



MONASH University

**The BAFF/APRIL system: emerging new and
unappreciated biomarkers in autoimmunity**

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MD, MSc.

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Abstract

Systemic lupus erythematosus (SLE) is a multifaceted autoimmune disease. Some evidence supports serum B cell-activating factor from the tumour necrosis factor family (BAFF), a key B cell survival factor, as a biomarker for SLE, with some specificity for some organ domains. However, inconsistent reports on the reliability of serum BAFF quantification across studies suggest the need to dissect the BAFF/a proliferation-inducing ligand (APRIL) system further. I will test the hypothesis that serum and urine biomarkers can be used to stratify subsets of SLE patients in relation to clinical phenotype. Primary Sjögren's syndrome (pSS) will be studied in parallel to assess specificity of findings for SLE disease. My thesis is focused on elements of the BAFF/APRIL system that are of emerging interest, such as soluble BAFF receptors in serum and urinary BAFF and APRIL levels, as well as on other systems known to have an interplay with this pathway.

My findings reveal distinct immunological profiles in SLE and pSS, which involve soluble components of the BAFF/APRIL, Fas/Fas ligand (FasL) and Type II interferon (IFN) systems. Serum sFas, BAFF and sTACI levels were related to SLE disease activity. At the organ level, serum BAFF and sFas, and urine (u)BAFF and uAPRIL were related to renal manifestations, while serum BAFF was related to musculoskeletal SLE. Serum sTACI was also negatively related to pSS disease activity, consistent with the decoy activity of sTACI. However, the absence of a relationship between serum BAFF and pSS disease activity raises questions about the exact contribution of this pathway in pSS pathogenesis. Since uBAFF was detectable in SLE but not in pSS, this biomarker may be specific to SLE. A potential relationship was found between sFasL and pSS lymphadenopathy.

Analysis of combinations of biomarkers from one or multiple pathways may offer benefits in SLE. BAFF/sTACI and BAFF/sBAFF-R ratios emerged as possible biomarkers for musculoskeletal disease. A relationship between sFasL/sFas ratio and mucocutaneous

SLE was identified in subset of patients with non-elevated BAFF. Also of interest was the relationship between serum BAFF combined with sFas, sFasL/sFas ratio or IFN- γ , with renal SLE. In contrast, combination of these biomarkers was not clinically relevant in pSS, suggesting specificity for SLE.

Serum sFasL and sBCMA, and uBAFF were increased in Asian compared to Caucasian patients. The relationships between serum sFas and sFasL/sFas ratio and renal SLE appeared specific to Asians.

Among the studies limitations, some patient subsets and healthy control groups were small; hence, caution should be taken when interpreting this data, while awaiting for future validation studies in larger cohorts. Data validation in an independent cohort would be of benefit; this is required prior to being able to develop a model.

Previously unrecognised immunological profiles reflecting distinct SLE phenotypes emerged from my work, that differed between clinical subsets. Combinations of biomarkers may better define pathway-dependent SLE manifestations. Variation in biomarker levels and their relationships with SLE in relation to ethnicity suggest disease mechanisms depending on genetic and/or environmental differences. Identification of non-invasive biomarkers specific for subgroups of patients with defined clinical manifestations may offer advantages in predicting which therapeutic approach is more likely to be effective, and improve clinical trials design with the recruitment of better stratified groups of patients. This work describes some unexpected observations, providing new insight into the mechanisms driving autoimmunity in subgroups of patients and may open the possibility of new strategies for therapeutic intervention. New methods for determining the most optimal target for treatment may improve outcomes while reducing health cost. My studies represent multiple contributions to this continuously evolving literature, and should serve as the foundation for future research.

Declaration

This thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

Publications during enrolment

Publications from PhD

Figgett WA, **Vincent FB**, Saulep-Easton D and Mackay F. Roles of ligands from the TNF superfamily in B cell development, function, and regulation. *Semin Immunol* 2014; 26:191-202.

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Publications during PhD (other enrolment)

Vincent FB[†], Pavy S[†], Krzysiek R, Lequerré T, Sellam J, Taoufik Y, Mariette X[†] and Miceli-Richard C[†]. Effect of serum anti-tumour necrosis factor (TNF) drug trough concentrations and antidrug antibodies (ADAb) to further anti-TNF short-term effectiveness after switching in rheumatoid arthritis and axial spondyloarthritis. *Joint Bone Spine*. 2016; [Epub ahead of print]

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Thesis including published works General Declaration

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes one original papers published in peer reviewed journals and zero unpublished publications. The core theme of the thesis is the BAFF/APRIL system in systemic lupus erythematosus. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the candidate, working within the Department of Immunology Central Clinical School, Faculty Of Medicine, Nursing And Health Sciences under the supervision of Professor Fabienne Mackay and co-supervisor Prof Eric Morand.

(The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research.)

In the case of Chapter I my contribution to the work involved the following:

(If this is a laboratory-based discipline, a paragraph outlining the assistance given during the experiments, the nature of the experiments and an attribution to the contributors could follow.)

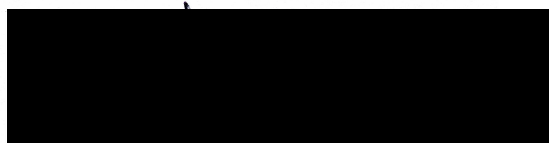
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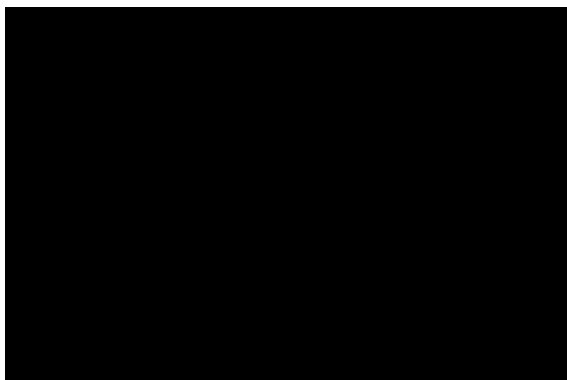
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TABLE OF CONTENTS

ABSTRACT	2
KEYWORDS	20
ABBREVIATIONS	21
CHAPTER I: LITERATURE REVIEW	23
1. INTRODUCTION	23
2. THE B CELL ACTIVATING FACTOR FROM THE TUMOUR NECROSIS FACTOR FAMILY (BAFF)/A PROLIFERATION-INDUCING LIGAND (APRIL) SYSTEM IN AID	26
<i>Published Review I have authored</i>	26
<i>Latest insights into BAFF as a biomarker in AID</i>	54
<i>Soluble BAFF receptors in AID</i>	55
<i>Strategies for the combination of biomarkers in AID</i>	56
3. A NEW LOOK AT THE FAS/FASL SYSTEM IN AID	58
<i>Interplay between the BAFF/APRIL and Fas/FasL systems in AID</i>	61
<i>Soluble forms of Fas and FasL in AID</i>	62
<i>The Fas/FasL system and organ involvement in SLE</i>	64
4. THE TYPE II IFN SYSTEM IN AID	67
<i>Interplay between the BAFF/APRIL and the Type II IFN systems in AID</i>	67
<i>Type II IFN as AID biomarker</i>	68
5. CONCLUSION	70
CHAPTER II: MATERIALS & METHODS	72
1. PATIENTS AND CLINICAL ASSESSMENTS	72
2. OVERALL AND SPECIFIC ORGAN DOMAIN DISEASE ACTIVITY ASSESSMENT	75
<i>SLE</i>	75
<i>pSS</i>	78
3. COLLECTION OF HUMAN BIOLOGICAL SAMPLES	79

<i>Serum</i>	79
<i>Urine</i>	79
4. ELISA	80
<i>For Chapters III to VII</i>	80
<i>For Chapter VIII</i>	81
5. COBAS ANALYSIS	81
6. INTERFERENCE BY sBAFF-R IN SERUM BAFF QUANTIFICATION	82
7. LUMINEX	82
8. ELISA AND LUMINEX COMMERCIAL KIT SELECTION	83
9. STATISTICAL ANALYSIS	84

CHAPTER III: ASSOCIATIONS OF SFAS, SFASL AND BAFF IN SYSTEMIC LUPUS

ERYTHEMATOSUS	86
1. KEY FINDINGS	86
2. INTRODUCTION	88
3. RESULTS.....	89
<i>Patient characteristics</i>	89
<i>Associations of serum sFas with SLE</i>	94
<i>Associations of serum sFasL with SLE</i>	97
<i>Associations of sFasL/sFas ratio with SLE</i>	100
<i>Relationship between serum BAFF and sFas and sFasL in SLE</i>	104
<i>Influence of ethnicity on serum sFas and sFasL and their relationships with SLE disease activity</i>	116
<i>Impact of anti-BAFF and anti-CD20 therapies on serum sFas and sFasL concentrations in SLE</i>	125
4. DISCUSSION.....	126

CHAPTER IV: ASSOCIATIONS OF SFAS, SFASL AND BAFF IN PRIMARY SJÖGREN'S SYNDROME	134
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1.	KEY FINDINGS	134
2.	INTRODUCTION	135
3.	RESULTS.....	135
	<i>Patient characteristics</i>	135
	<i>Associations of serum sFas with pSS.</i>	140
	<i>Associations of serum sFasL with pSS.</i>	141
	<i>Associations of sFasL/sFas ratio with pSS.</i>	143
	<i>Relationship between serum BAFF and sFas in pSS</i>	145
	<i>Relationship between serum BAFF and sFasL in pSS</i>	148
	<i>Relationship between serum BAFF and sFasL/sFas ratio in pSS</i>	149
4.	DISCUSSION.....	150
 CHAPTER V: COMBINING MEASUREMENT OF SERUM BAFF AND IFN-GAMMA IS ASSOCIATED WITH A RENAL-CENTRIC DISEASE ACTIVITY IN SYSTEMIC LUPUS ERYTHEMATOSUS..... 153		
1.	KEY FINDINGS	153
2.	INTRODUCTION	154
3.	RESULTS.....	155
	<i>Patient characteristics</i>	155
	<i>Associations of serum BAFF with SLE</i>	155
	<i>Associations of serum IFN-γ with SLE</i>	161
	<i>Relationship between serum IFN-γ and BAFF in SLE</i>	165
4.	DISCUSSION.....	169
 CHAPTER VI: SOLUBLE FORM OF BAFF RECEPTORS IN SYSTEMIC LUPUS ERYTHEMATOSUS..... 175		
1.	KEY FINDINGS	175
2.	INTRODUCTION	177
3.	RESULTS.....	178

<i>Patient characteristics</i>	178
<i>Difference in quantifying serum BAFF: ELISA vs. multiplex assays</i>	178
<i>Interference of sBAFF-R with serum BAFF detection by ELISA</i>	180
<i>Effect of anti-BAFF and anti-CD20 therapies on serum BAFF and soluble BAFF receptor concentrations</i>	182
<i>Associations of serum sTACI with SLE</i>	184
<i>Associations of serum sBCMA with SLE</i>	186
<i>Associations of serum sBAFF-R with SLE</i>	189
<i>Relationship between serum BAFF and soluble BAFF receptors in SLE</i>	191
<i>Associations of BAFF/sTACI ratio with SLE</i>	193
<i>Associations of BAFF/sBCMA ratio with SLE</i>	197
<i>Associations of BAFF/sBAFF-R ratio with SLE</i>	198
<i>Influence of ethnicity on serum soluble BAFF receptors in SLE</i>	200
4. DISCUSSION.....	212

CHAPTER VII: SOLUBLE TACI AS A BIOMARKER IN PRIMARY SJÖGREN'S

SYNDROME	222
1. KEY FINDINGS	222
2. INTRODUCTION	224
3. RESULTS.....	224
<i>Patient characteristics</i>	224
<i>Associations of serum sTACI with pSS</i>	224
<i>Associations of serum sBCMA with pSS</i>	226
<i>Associations of serum BAFF with pSS</i>	227
<i>Relationship between serum BAFF and soluble BAFF receptors in pSS</i>	229
<i>Associations of BAFF/sTACI ratio and pSS</i>	231
<i>Associations of BAFF/sBCMA ratio with pSS</i>	234
4. DISCUSSION.....	235

CHAPTER VIII: URINARY BAFF AND APRIL IN LUPUS NEPHRITIS	240
1. KEY FINDINGS	240
2. INTRODUCTION	242
3. RESULTS.....	243
<i>Patient characteristics</i>	<i>243</i>
<i>Associations of uBAFF with SLE</i>	<i>246</i>
<i>Associations between serum and urinary BAFF.....</i>	<i>250</i>
<i>Associations of uBAFF with SLE disease activity.....</i>	<i>251</i>
<i>Associations of uBAFF with renal SLE.....</i>	<i>253</i>
<i>Associations of baseline uBAFF with subsequent SLE disease activity</i>	<i>257</i>
<i>Associations of baseline uBAFF with subsequent renal SLE disease activity.....</i>	<i>260</i>
<i>Associations of uAPRIL in SLE.....</i>	<i>262</i>
<i>Associations between serum and urinary APRIL.....</i>	<i>264</i>
<i>Associations of uAPRIL with SLE disease activity</i>	<i>265</i>
<i>Associations of uAPRIL with renal SLE.....</i>	<i>267</i>
<i>Associations of baseline uAPRIL with subsequent SLE disease activity</i>	<i>271</i>
<i>Associations of baseline uAPRIL with subsequent renal SLE disease activity.....</i>	<i>273</i>
<i>Associations of uMCP-1 in SLE.....</i>	<i>274</i>
<i>Associations between serum and urinary MCP-1</i>	<i>275</i>
<i>Associations of uMCP-1 with SLE disease activity.....</i>	<i>276</i>
<i>Associations of uMCP-1 with renal SLE.....</i>	<i>277</i>
<i>Associations of baseline uMCP-1 with subsequent SLE disease activity</i>	<i>280</i>
<i>Associations of baseline uMCP-1 with subsequent renal SLE disease activity.....</i>	<i>282</i>
4. DISCUSSION.....	283
CHAPTER IX: CONCLUDING DISCUSSION.....	292
REFERENCES.....	311
APPENDIX 1.....	322

APPENDIX 2	325
APPENDIX 3	329
APPENDIX 4	334
APPENDIX 5	338
APPENDIX 6	340
APPENDIX 7	342
APPENDIX 8	344
APPENDIX 9	345
APPENDIX 10	348
APPENDIX 11	350
APPENDIX 12	354
APPENDIX 13	359

Keywords

A proliferation-inducing ligand (APRIL); Autoimmunity; B cell-activating factor from the tumour necrosis factor family (BAFF); BAFF receptor (BAFF-R); B cell maturation antigen (BCMA); Fas; Fas ligand (FasL); Interferon-gamma (IFN- γ); Lupus nephritis (LN); Primary Sjögren's syndrome (pSS); Systemic Lupus Erythematosus (SLE); Transmembrane activator and cyclophilin ligand interactor (TACI).

Abbreviations

Ab: antibodies; ACR: American College of Rheumatology; AICD: innate activation-induced cell death; AID: autoimmune diseases; ALPS: autoimmune lymphoproliferative syndrome; ALRB: Australian Lupus Registry and Biobank; AMS, Adjusted mean SLEDAI-2k; ANA: antinuclear antibody; APRIL: a proliferation-inducing ligand; AZA: azathioprine; BAFF: B cell-activating factor from the tumour necrosis factor family; BAFF-R: BAFF receptor; BAFF-Tg: BAFF-transgenic; BBB: blood-brain barrier; BCMA: B cell maturation antigen; BLyS: B lymphocyte stimulator; C3: complement component 3; C4: complement component 4; CCP: cyclic citrullinated peptide; CI: confidence interval; CLE: cutaneous lupus erythematosus; CNS: central nervous system; CRP: C-reactive protein; CSF: cerebrospinal fluid; CYA: cyclosporine A; CYM: cyclophosphamide; DAS28: disease activity score for 28 joints; DLE: discoid lupus erythematosus; dsDNA, double-stranded deoxyribonucleic acid; eGFR, estimated glomerular filtration rate; ELISA, Enzyme-Linked Immunosorbent Assay; ENA: extractable nuclear antigen; ESR: erythrocyte sedimentation rate; ESSDAI: European League Against Rheumatism Sjögren's syndrome disease activity index; EULAR: European League Against Rheumatism; FasL: Fas ligand; FS: focus score; gld: generalized lymphoproliferative disease; GN: glomerulonephritis; Hb: haemoglobin; HCQ: hydroxychloroquine; HD: healthy donor; IA: Indigenous Australian; IFN: interferon; Ig: immunoglobulin; IQR: inter-quartiles ranges; ISN/RPS: International Society of Nephrology/Renal Pathological Society; LE: lupus erythematosus; LN: lupus nephritis; LOCF: last observation carried forward; LPS: lipopolysaccharides; m: membrane-bound; mAb: monoclonal antibodies; MCP-1: monocyte chemoattractant protein 1; MDD: minimum detectable dose; MS: multiple sclerosis; MTX: methotrexate; MZ: marginal zone; PGA: physician's global assessment; PNS: peripheral nervous system; pSS: primary Sjögren's syndrome; r: recombinant; RA: rheumatoid arthritis; RBC, red blood cells; RF: rheumatoid

factors; RNP, ribonucleoprotein; s: soluble; SD: standard deviation; SDI, SLE Damage Index; SELENA-SLEDAI, Safety of Estrogens in Lupus Erythematosus National Assessment SLE Disease Activity Index; SEM: standard error of mean; SLE: Systemic lupus erythematosus; SLEDAI: SLE disease activity index; SLEDAI-2K: SLEDAI 2000; SLICC: systemic lupus international collaborating clinics; Sm: Smith; SS: Sjögren's syndrome; SSA: SS antigen A; SSB: SS antigen B; SSZ: sulfasalazine; TACI: transmembrane activator and cyclophilin ligand interactor; T_{FH}: follicular helper T cells; Tg: transgenic; TLR: Toll-like receptor; TNF: tumour necrosis factor; TRAIL: TNF-related apoptosis-inducing ligand; UPCR: urine protein/creatinine ratio; VAS: visual analogue scale; WBC, white blood cells; WCC: total white cell count; WT, wild-type.

CHAPTER I: Literature Review¹

1. Introduction

Systemic lupus erythematosus (SLE) and primary Sjögren's syndrome (pSS) are heterogeneous chronic systemic autoimmune diseases (AID), sharing common aspects of pathogenesis, including a multifactorial aetiology (reviewed in[93, 111, 190]). In both SLE and pSS, many organs/systems can be affected, such as the skin, joints, kidney, and the central nervous system (CNS). At the clinical level, pSS is characterized by sicca syndrome caused by immune-mediated damage to exocrine glands, with systemic complications being less common. In contrast, in SLE, any organ can be affected, particularly the skin, joints, and kidney. While SLE and pSS can present differently, they are both AID with a heterogeneous clinical presentation. Patients with SLE and pSS are also at higher risk of developing lymphoma (reviewed in[111]). Focusing on their pathophysiology, these diseases also share some striking similarities as underlined by Nocturne *et al.*,[111] particularly with involvement of the B cell-activating factor from the tumour necrosis factor family (BAFF)/ a proliferation-inducing ligand (APRIL), and type I and II interferon (IFN) systems (reviewed in[111, 184]). The partially overlapping clinical presentation and the common involvement of biological systems suggests the potential for insights from studying both diseases in parallel.

In daily clinical practice, diagnosing patients suffering from AID can be extremely challenging. The same is true for assessing the activity of the disease, especially when some internal organs are affected, such as seen in neurological and renal SLE. Clinical measurements that enable physicians to stratify patients better and make optimal

¹ In this chapter, after a short introduction, I have provided a review of the BAFF/APRIL system using a published review article arising from this thesis. Thereafter, I have reviewed other aspects of AID relevant to the studies presented in this thesis.

therapeutical decisions for individual patients are lacking and urgently needed. This is particularly important since the approval of the first targeted biological therapy in SLE, belimumab, an expensive treatment to which only a proportion of patients respond, and for which there are currently no highly reliable biomarkers to guide patient selection.[54, 109]

Biomarkers are tools that can be used by physicians to improve diagnosis, assess and/or predict disease activity and severity, guide therapeutical decisions, and assess response to therapy. In the fast-approaching era of personalized medicine, one of the key questions is to define whether a biomarker, to assess renal SLE disease for instance, could be used across patients, or if it would rather be more beneficial in a pre-defined patient subset. Since SLE is a multifaceted AID, we and others have suggested the potential benefit of not only searching for biomarkers for overall SLE disease activity, but also markers discriminating disease activity in specific organ domains affected in patients (reviewed in[184]). Overall SLE disease activity reflects the activity of multiple organ domains, in turn potentially reflecting various immunological pathways; the same is true for pSS. Therefore, stratifying patients into more homogeneous subsets according to their clinical phenotype may be relevant for the discovery of new subset-specific biomarkers. For example, a biomarker of interest may fail to correlate with overall disease activity, yet it may associate strongly with particular organ domain(s), thus potentially demonstrating a relationship between an immunologic pathway and a phenotypical manifestation of the condition. In other words, patient stratification for the identification of new biomarkers may uncover a biological pathway particularly relevant for a specific manifestation of SLE, that have hitherto been elusive. These may help physicians better assess organ-specific disease activity, and decide which treatment may be more appropriate for individual patients, as well as follow subsets of patients' clinical response to therapies and predict disease outcomes. If validated, this biomarker approach would represent a step towards a new era for personalized medicine. In this thesis, I present a series of studies,

which reveal previously undetected associations between various cytokine pathways and elements of disease activity in clinically and/or biologically stratified patients.

2. The B cell activating factor from the tumour necrosis factor family (BAFF)/a proliferation-inducing ligand (APRIL) system in AID

Published Review I have authored

Please find below the published review focused on SLE and the BAFF/APRIL system I have authored (Vincent FB, Morand EF, Schneider P, Mackay F. The BAFF/APRIL system in SLE pathogenesis. Nat. Rev. Rheumatol. 2014;10:365-73), as the author's own version, namely unedited before publication, as per Springer Nature request. I have also attached all three figures from the review article in order to better illustrate the accompanying review. Thereafter, I will present the latest insights into BAFF as a biomarker in AID.

The BAFF/APRIL system in SLE pathogenesis

Fabien B. Vincent, Eric F. Morand, Pascal Schneider and Fabienne Mackay

Abstract | Systemic lupus erythematosus (SLE) is characterized by multisystem immune-mediated injury in the setting of autoimmunity to nuclear antigens. The clinical heterogeneity of SLE, the absence of universally agreed trial end points, and the paucity of validated therapeutic targets have, historically, contributed to a lack of novel treatments for SLE. However, in 2011, a therapeutic monoclonal antibody that neutralizes the cytokine TNF ligand superfamily member 13B (also known as B cell-activating factor of the TNF family, BAFF), belimumab, became the first targeted therapy for SLE to have efficacy in a randomized clinical trial. Because of its specificity, the efficacy of belimumab provides an opportunity to increase understanding of SLE pathophysiology. Although belimumab depletes B cells, this effect is not as powerful as that of other B cell-directed therapies that have not been proven efficacious in randomized clinical trials. In this article, therefore, we review results suggesting that neutralizing BAFF can have effects on the immune system other than depletion of B cells. We also identify aspects of the BAFF system for which data in relation to SLE are still missing, and we suggest studies to investigate the pathogenesis of SLE and ways to refine anti-BAFF therapies. The role of a related cytokine, TNF ligand superfamily member 13 (also known as a proliferation-inducing ligand [APRIL]) in SLE is much less well understood, and hence this review focuses on BAFF.

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Competing interests

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Key points

TNF ligand superfamily member 13B (also known as B cell-activating factor of the TNF family [BAFF]) and TNF ligand superfamily member 13 (also known as a proliferation-inducing ligand [APRIL]) are important modulators of autoimmunity

Data indicate that alteration of the BAFF/APRIL system affects the capacity of the innate immune system to regulate B-cell activation

BAFF and type I interferons function together in systemic lupus erythematosus (SLE) pathogenesis in both a TLR-dependent and independent manner

Defining the clinical manifestations of disease related to alterations of the BAFF/APRIL system might help to stratify patients with SLE into subgroups that are more likely to benefit from anti-BAFF treatment

Differences in the molecular forms of BAFF might affect the efficacy of BAFF-specific therapies

Introduction

Systemic lupus erythematosus (SLE) is an idiopathic, systemic autoimmune disease.¹ Although the cause of this disease is unclear, research over the past two decades has provided new insights into its pathogenesis. One breakthrough was the discovery, in 1999, of a crucial B-cell survival factor, TNF ligand superfamily member 13B (also known as B cell-activating factor of the TNF family [BAFF], B lymphocyte stimulator [BLyS], TNFSF13B, zTNF4, THANK and TALL-1),² which has an important role in autoimmunity, and in particular in SLE pathogenesis.^{3, 4} In 2011, belimumab, a monoclonal antibody targeting human BAFF, was shown in randomized clinical trials to be efficacious in a subset of patients with SLE and has now become the first approved targeted therapy for SLE.^{5, 6} As a consequence, clinical inhibition of BAFF has generated substantial interest and similar biologic agents are being tested in clinical trials. The efficacy of a specific anti-BAFF treatment demonstrated that SLE is not exclusively a T-cell-mediated disease and has renewed the scientific community's interest in the pathogenesis of human SLE.

Clinical trials and *post hoc* analysis of data obtained from belimumab-treated patients with SLE have provided useful information about BAFF and a related cytokine, TNF ligand superfamily member 13 (also known as a proliferation-inducing ligand [APRIL]). Data suggest that mechanisms other than B-cell depletion are affected during responses to treatment with belimumab. In addition, the deregulation of BAFF and APRIL is found in specific subsets of patients with SLE.⁷ The BAFF/APRIL system and the innate immune system coordinate in regulating the innate activation of B cells, and seem to be defective in a mouse model of SLE in which BAFF is overexpressed.⁸ Several receptors and various biochemical forms of the ligands exist in the BAFF/APRIL system,⁹⁻¹¹ but further work is required to elucidate their respective contributions to the pathogenesis and phenotype of SLE. In this article, we review the BAFF/APRIL system and highlight new discoveries that are relevant to the improvement of SLE management and might lead to therapeutic innovation. In

light of an ongoing debate about the role of APRIL in SLE, we have predominantly focused this Review on BAFF.

The BAFF/APRIL system

Two ligands, BAFF and APRIL, and three receptors, TNF receptor superfamily member 13C (also known as BAFF receptor [BAFF-R] or BLyS receptor 3 [BR3]), TNF receptor superfamily member 17 (also known as B-cell maturation antigen [BCMA]) and TNF receptor superfamily member 13B (also known as transmembrane activator and cyclophilin ligand interactor [TACI]) form the backbone of the BAFF/APRIL system. BAFF and APRIL can both interact with BCMA and TACI, whereas BAFF is the sole ligand for BAFF-R (reviewed previously¹³; Figure 1). BAFF-R is essential for both survival and maturation of immature B cells, whereas TACI is critical for T cell-independent responses of B cells to type I and type II antigens, negative regulation of the B cell compartment and class-switch recombination of B cells (reviewed previously¹⁴). BCMA is expressed by plasmablasts and plasma cells, and promotes plasma cell survival.

BAFF and APRIL are produced by myeloid cells, predominantly by macrophages, neutrophils and dendritic cells (DCs), and also by radiation-resistant stromal cells.¹² Lymphoid cells, including B cells and activated T cells, can also produce BAFF and APRIL. Finally, Toll-like receptor 9 (TLR9)-activated plasmacytoid DC and IL-2-activated natural killer cells also produce BAFF (reviewed previously¹³).

BAFF and APRIL are type II transmembrane proteins.^{3, 15, 16} BAFF can also be processed or not into a soluble cytokine after cleavage at a furin protease site,^{3, 15} whereas APRIL is mainly produced in a soluble form.^{16, 17} Two exceptions are the APRIL- δ isoform, first identified in malignant B-cell tumours,¹⁸ and TWE-PRIL, a hybrid protein of APRIL and TNF ligand superfamily member 12 (also known as TNF-related weak inducer of apoptosis [TWEAK]),¹⁹ that are membrane-bound. The receptor-binding domain of BAFF is trimeric;²⁰ however, 20 BAFF trimers can assemble into a BAFF 60-mer.^{10, 11} Furthermore, BAFF and APRIL can assemble as BAFF–APRIL heterotrimers of undefined stoichiometries, which have been detected in the serum of patients with rheumatic diseases (Figure 2). Recombinant BAFF–APRIL heterotrimers are biologically active, albeit less than BAFF or APRIL homotrimers.^{21, 22} Heterotrimers of various compositions might be differentially susceptible to BAFF-targeting therapies.

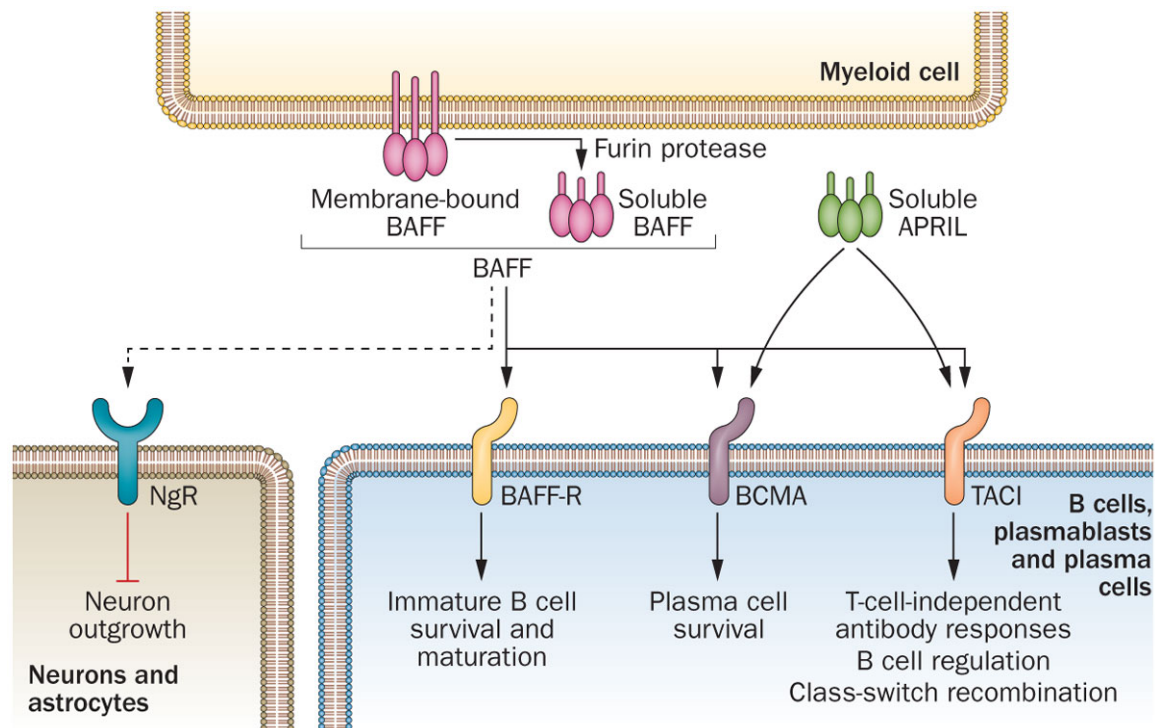


Figure 1. Soluble BAFF and APRIL signalling.

BAFF and APRIL are type II transmembrane proteins, but BAFF can be processed into a soluble cytokine after cleavage at a furin protease site. APRIL is soluble, having been cleaved intracellularly. BAFF only has weak affinity for BCMA. BAFF-R is essential for survival and maturation of immature B cells. TACI is critical for T-cell-independent responses of B cells to type I and type II antigens, negative regulation of the size of the B-cell compartment and class-switch recombination. BCMA promotes plasma-cell survival. Dashed line indicates that BAFF possibly binds NgR, which is expressed on neurons and astrocytes, and has been shown to mediate negative effects on neuron outgrowth. Abbreviations: APRIL, a proliferation-inducing ligand (also known as TNF ligand superfamily member 13); BAFF, B-cell-activating factor of the TNF family (also known as TNF ligand superfamily member 13B); BAFF-R, BAFF receptor (also known as TNF receptor superfamily member 13C); BCMA, B-cell maturation antigen (also known as TNF receptor superfamily member 17); NgR, Nogo-66 receptor (also known as reticulon 4 receptor); TACI, transmembrane activator and cyclophilin ligand interactor (also known as TNF receptor superfamily member 13B). (Figure 1 has been reproduced from a review I have authored[184]).

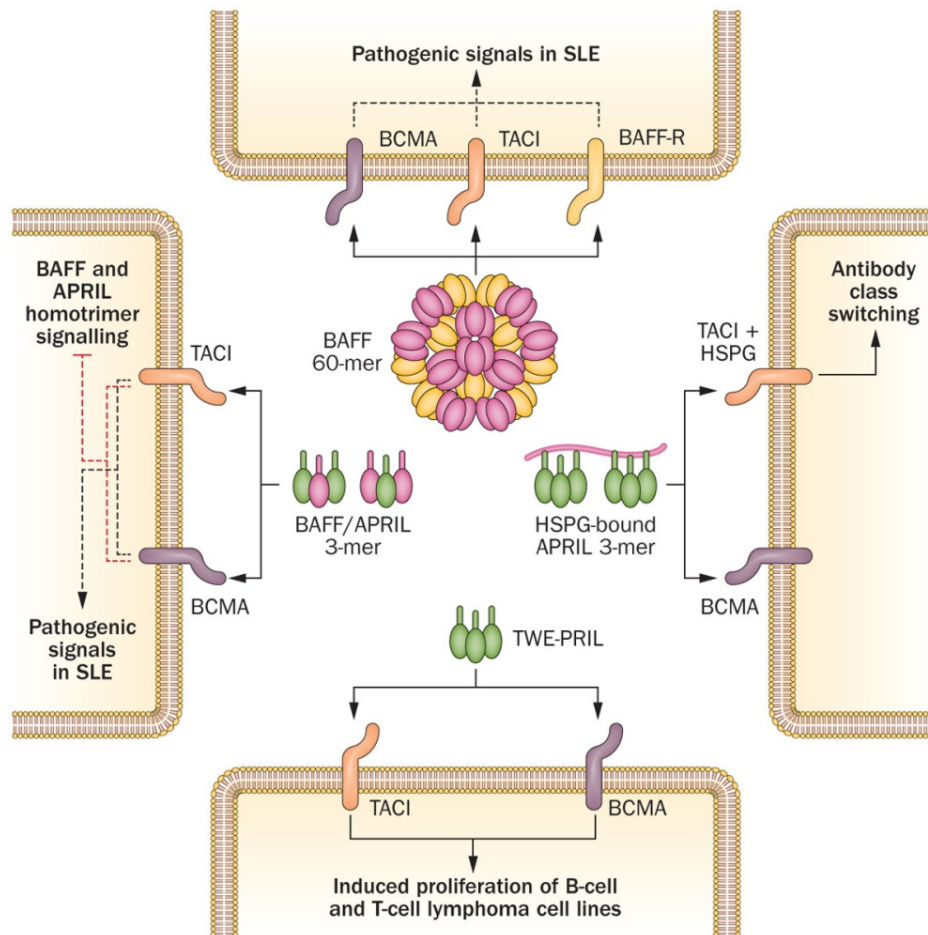


Figure 2. Structural variants of BAFF and APRIL.

Different BAFF–APRIL structures might be involved in the pathogenesis of SLE, or might counteract the effects of BAFF and APRIL homotrimers by sequestering monomeric components required for their formation, competing for receptor binding or inducing suboptimal signals. APRIL interacts with polysaccharide side chains of HSPG, and interactions of TACI and HSPG regulate antibody class-switching. Solid lines indicate known interactions. Dashed lines indicate hypothetical interactions. Abbreviations: APRIL, a proliferation-inducing ligand (also known as TNF ligand superfamily member 13); BAFF, B-cell-activating factor of the TNF family (also known as TNF ligand superfamily member 13B); BAFF-R, BAFF receptor (also known as TNF receptor superfamily member 13C); BCMA, B-cell maturation antigen (also known as TNF receptor superfamily member 17); HSPG, heparan sulphate proteoglycans; SLE, systemic lupus erythematosus; TACI, transmembrane activator and cyclophilin ligand interactor (also known as TNF receptor superfamily member 13B); TWEAK, TNF-related weak inducer of apoptosis (also known as TNF ligand superfamily member 12); TWE–PRIL; a hybrid of TWEAK and APRIL. (Figure 2 has been reproduced from a review I have authored[184]).

Mice expressing BAFF with a mutated furin cleavage-site have a phenotype similar to BAFF^{-/-} mice, suggesting that membrane-bound BAFF cannot substitute for soluble BAFF.²³ However, when treated with soluble BAFF, B cells from the BAFF furin mutant mice became more similar to B cells from wild-type mice than to B cells from BAFF^{-/-} mice that were also treated with soluble BAFF.²³ These results suggest a two-step process in BAFF-mediated B-cell maturation. First, soluble BAFF promotes B-cell survival by activating BAFF-R, and then the B-cell phenotype is modulated by membrane-bound BAFF signals, possibly by activating TACI.²³

Human BAFF is a 285 amino acids-long protein with two potential *N*-glycosylation sites.²⁴ Because some ELISAs detect only unglycosylated BAFF, whereas other ELISAs detect both unglycosylated and glycosylated BAFF, post-translational *N*-glycosylation status could explain the failure to consistently associate disease activity with serum BAFF concentrations.²⁴ Alternative splice variants of BAFF, particularly ΔBAFF, might have an important role in SLE pathogenesis.²⁵⁻²⁸ Mouse ΔBAFF decreases the bioactivity and release of full-length BAFF by associating with BAFF and forming inactive heterotrimers.²⁶ In mice, BAFF and ΔBAFF can both modulate B1 B cells.^{25, 26} BAFF-ϕ is a nonfunctional isoform identified in human cell lines.²⁶ Although currently unknown, it is possible that anti-BAFF biologic agents might differ in their ability to bind and neutralize the various human BAFF isoforms or glycosylation variants.

The BAFF/APRIL system in SLE

BAFF is necessary for B-cell maturation and survival. Mice genetically deficient in BAFF lack mature B cells and are immunodeficient,²⁹ whereas mice that overproduce BAFF have high numbers of mature B cells and antibodies, including autoantibodies, and develop an autoimmune disease similar to SLE in humans.² Together with the observation that BAFF blockade decreased symptoms of SLE in mouse models,³⁰ these findings promoted BAFF as a therapeutic target for the treatment of patients with SLE.

APRIL is important for antibody class-switching and plasma-cell survival (reviewed previously¹⁴). APRIL-overexpressing transgenic mice develop B1 B-cell neoplasia, but do not develop SLE-like pathology.³¹ Selective APRIL blockade can delay the development of disease in a lupus-prone mouse model (NZB/W F1 mice).³² Using NZM.*April*^{-/-} (NZM.*Tnfrsf13*^{-/-}) mice, Jacob *et al.*³³ showed that, in this model, APRIL was not necessary for

SLE pathogenesis. Although NZM.*Baff*^{-/-}*April*^{-/-} (NZM.*Tnfsf13b*^{-/-}*Tnfsf13*^{-/-}) mice had fewer bone-marrow plasma cells and autoantibodies than NZM.*Baff*^{-/-} (NZM.*Tnfsf13b*^{-/-}) mice, both strains had mild kidney immunopathology,³³ suggesting that blocking both BAFF and APRIL in patients with SLE might not be beneficial and could even increase the risk of toxicity. Further supporting this idea, a phase II/III clinical trial of the chimeric recombinant fusion protein atacicept (combining TACI with an immunoglobulin constant region), which neutralizes BAFF, APRIL and BAFF–APRIL heterotrimers in patients with active lupus nephritis, was stopped because of serum IgG depletion and an increased rate of infections.³⁴

Belimumab is a fully human recombinant monoclonal IgG_{1λ} antibody that impairs B-cell survival by targeting soluble, not membrane-bound, human BAFF.³⁵ The addition of belimumab to standard therapy has been studied in two multicenter, double-blind, placebo-controlled, randomized phase III clinical trials in patients with SLE, cumulatively examining 1,684 patients for up to 76 weeks.^{5, 6} Various scoring systems are used to evaluate disease activity in human SLE clinical trials, such as SLE disease activity index (SLEDAI), British isles lupus assessment group (BILAG) or SLE responder index (SRI).³⁶⁻³⁸ These composite scores of clinical and laboratory features enable scaled assessment of disease activity at a single time point. Patients with active SLE who tested positive for SLE-associated antibodies benefited from belimumab compared with placebo with respect to disease activity (SRI) and health-related quality of life, with a satisfactory drug safety profile.^{5, 6, 39, 40} Belimumab therapy was associated with reduced autoantibody levels and normalized, low, complement levels,⁴¹ and in *post hoc* analysis, patients with high disease activity (Safety Of Estrogens In Lupus Erythematosus National Assessment (SELENA)-SLEDAI), high anti-double stranded DNA antibody and low complement levels at baseline had superior treatment responses to belimumab.⁴² Belimumab therapy also reduced the number of circulating naive B cells, activated B cells and plasma cells, but did not reduce the number of circulating memory B cells or T cells.⁴¹ No effects on antibody responses to previous pneumococcal, tetanus or influenza immunizations were detected after one year of treatment, consistent with a preserved memory B-cell compartment.⁴³

BAFF and APRIL as biomarkers of SLE

Relative to healthy individuals, serum concentrations of BAFF and APRIL are increased in patients with numerous autoimmune diseases, such as SLE, primary Sjögren's syndrome and rheumatoid arthritis (reviewed previously¹⁴). Whether serum BAFF or APRIL can be used as

a biomarker in SLE has been a matter of debate over the past decade, with some studies reporting correlations between serum BAFF and APRIL concentrations and overall disease activity,⁴⁴⁻⁴⁷ whereas others found no such correlations.^{41, 47-50} Petri *et al.*⁵¹ showed, using multivariate analysis, that elevated baseline serum BAFF concentration (≥ 2 ng/ml) was predictive of moderate-to-severe SLE flares in patients receiving the standard therapy, prednisone with antimalarial (hydroxychloroquine) or immunosuppressive drugs (methotrexate, mycophenolate mofetil and azathioprine). This association is supported by another study, in which a high serum BAFF concentration was associated with flares of SLE disease activity (BILAG) after rituximab treatment.⁵² However, in our study,⁷ among others, no significant association between serum BAFF concentration and disease activity (SLEDAI-2k) was found. Moreover, in the belimumab phase III clinical trials, baseline serum BAFF concentration was not found to be predictive of outcome in either anti-BAFF or control-treated patients.⁴¹ These discrepancies might be caused by differences in assay sensitivity, disease activity scores or study populations. However, serum BAFF and APRIL concentrations might not accurately reflect total BAFF production or, in the case of APRIL (which binds proteoglycans), the concentration of the active cytokine in tissues. Urinary excretion of cytokines, for example, might reduce serum concentration in patients with renal disease, suggesting that the measurement of BAFF and APRIL in urine should be investigated by SLE researchers.⁵³

Perhaps the failure to reliably show an association between disease activity and the concentration of a cytokine known to be involved in the pathogenesis of SLE means that the measurement of disease activity requires re-examination. Moreover, given that *post hoc* analysis of clinical trial data revealed that BAFF inhibition was most effective in patients with active disease and high serum autoantibody titres,⁴² this subset of SLE patients might be best suited for analysis of clinical associations of BAFF and APRIL expression. We could, therefore, study SLE patient subsets, defined according to specific clinical or immunological phenotypes.

SLE clinical subsets

Some studies, measuring cytokines in patients with SLE, have focused on SLE subsets rather than overall disease activity measured with composite indices of disease activity.⁵⁴⁻⁶⁰ We showed that serum BAFF and APRIL concentrations, although not correlating with a composite measure of disease activity (SLEDAI-2k), were increased in subsets of patients

with SLE who also had renal or central nervous system (CNS) pathology.⁷ These data are supported by mouse studies, in which combinations of separate pathways that regulate autoimmunity, inflammation and renal damage led to differentiated phenotypes of disease.^{61, 62} Combining different genetic loci such as *Sle3* and *Sle5*, with or without *Sle1*, leads to differentiated phenotypes in lupus-prone mice.⁶² If distinct immunological pathways are activated in humans with different disease manifestations, then general biomarkers for SLE might not exist. Some subsets of patients with SLE might, however, benefit from specific therapies targeting these pathways. The identification of reliable biomarkers for these subsets would be useful as a tool for the selection of therapy. Serum BAFF, for example, is increased in patients with CNS or renal pathology, therefore, these patients might benefit from anti-BAFF therapy.⁷ Although patients with severe CNS manifestations or severe lupus nephritis were excluded from two phase III clinical trials of belimumab,^{5, 6} *post hoc* pooled analysis suggested that belimumab might be an effective treatment for patients with SLE and renal involvement.⁶³ Also, in the subset of SLE patients without renal disease activity at baseline, those treated with belimumab had less renal involvement than patients given placebo.⁶⁴ Further research is needed to investigate the accuracy of using serum BAFF and APRIL measurements to predict SLE phenotype, and therapeutic responses in patients within the different subgroups (Box 1).

Ethnicity and polymorphisms

Substantial ethnic variation in the prevalence and characteristics of SLE exists.^{66, 67} Asian, Indigenous Australian and African patients with SLE suffer from more severe disease compared with white people.^{7, 66-69} In one report, serum BAFF or APRIL concentration was not associated with Asian ethnicity;⁷ however, African American patients with SLE had higher serum BAFF concentrations than white American patients with SLE, although not significantly after multivariate analysis.⁷⁰ By contrast, increased serum BAFF concentration was associated with an increase in the SLEDAI in white American, but not in African American, patients.⁷⁰ Further research is needed to understand how particular ethnic subgroups respond to anti-BAFF therapies (Box 1).

Future research could focus on BAFF and APRIL single nucleotide polymorphisms (SNPs). Several SNPs have been identified in the promoter, coding and untranslated regions of *TNFSF13B* (the human BAFF gene), but in most studies no significant association with SLE susceptibility has been found.^{71, 72} Only one study reported an association (in Egyptian

patients with SLE) between disease susceptibility and 871C>T and 2701T>A SNPs in the BAFF promoter region.⁷³ *TNFSF13B* promoter SNPs were not significantly associated with symptoms of SLE. Monocytes from healthy individuals with the 871T allele had a significant increase in BAFF mRNA compared with monocytes from healthy individuals without the 871T allele.⁷¹

SNPs have also been found in *TNFSF13* (the human APRIL gene), including Gly67Arg, which was significantly associated with SLE susceptibility in Japanese patients.⁷⁴ Another study showed a possible association between Gly67Arg and SLE susceptibility in African American and Hispanic people, but not in white American people.⁷⁵ Additionally, the c.199A-c.287G (67Arg-96Ser) SNP was reported to be a protective haplotype whereas the c.199G-c.287A (67Gly-96Asn) haplotype was associated with disease susceptibility in Japanese patients with SLE.⁷⁶ In line with its protective effect, the protective haplotype 67Arg-96Ser decreased soluble APRIL secretion in transfection experiments.⁷⁷

BAFF and APRIL receptors in SLE

Exacerbation of disease in lupus-prone BCMA-deficient mice (MRL-*Fas^{lpr}/J.Tnfrsf17^{-/-}* and Nba2.*Tnfrsf17^{-/-}* mice), suggests that BCMA has a direct or indirect negative regulatory role.⁷⁸ In these mouse models, exacerbation of disease might occur by abnormal signalling of BAFF or APRIL through TACI or BAFF-R in the absence of BCMA. Compared with wild-type and single-mutant control strains, BCMA-deficient lupus-prone mice have more mature-B cells and plasma cells in secondary lymphoid organs, more serum autoantibodies and BAFF, and more immune complex deposition in the kidneys.⁷⁸ An increase in the number of BAFF-producing cells, such as DCs and macrophages, was also detected in these mice.⁷⁸ BCMA signalling might have an indirect regulatory role in the homeostasis of BAFF-producing cells, because BCMA was not expressed by splenic DCs in all mouse strains in this study (MRL-*Fas^{lpr}/J, Tnfrsf17^{-/-}*, MRL-*Fas^{lpr}/J.Tnfrsf17^{-/-}* and wild-type mice).⁷⁸ Jacob *et al.*⁷⁹ showed that, in NZM lupus-prone mice, the deletion of BAFF-R alone, which leads to profound B-cell depletion, or of BCMA or TACI alone, did not protect against SLE. These data suggest that, unlike BAFF-overexpressing transgenic mice⁸⁰, T cells might have an important role in the pathogenesis of NZM lupus-prone mice.^{79, 80}

The reticulon-4 receptor (also known as the Nogo-66 receptor [NgR]), a novel BAFF receptor that has not been confirmed by other laboratories, is expressed on neurons and astrocytes, and

has been shown to mediate negative effects on neuron outgrowth via transducing BAFF signalling.⁹ Whether BAFF signalling through NgR is relevant to the pathogenesis of SLE in the CNS is not known. We found that subsets of patients with SLE and CNS pathology had substantially more serum BAFF and less serum APRIL than patients with SLE and no CNS pathology.⁷ BAFF and APRIL production by astrocytes has also been reported in patients with multiple sclerosis, another autoimmune disease affecting the CNS.^{27, 81} In the cerebrospinal fluid of patients with SLE, BAFF and APRIL were significantly higher than in cerebrospinal fluid from healthy individuals.⁸² In patients with SLE, BAFF and APRIL found in the CNS might be locally produced by astrocytes, or immigrant immune cells, or it might derive from systemic cytokine production that might cross the blood–brain barrier. NgR expression in the CNS might explain clinical associations between BAFF expression and CNS pathology in patients with SLE, but this remains to be confirmed. Whether APRIL can also interact with NgR is not known.

BAFF, TLR cross-talk and IFN signatures

Type I interferons, and particularly IFN- α , are thought to have a major role in SLE pathogenesis. SLE is characterized by increased expression of IFN- α -induced genes, referred to as the IFN- α signature, in peripheral blood mononuclear cells and in target organs, such as the kidneys.^{54, 83, 84} In some studies, serum IFN- α concentrations and IFN-induced genes positively correlate with SLE disease activity (SLEDAI).^{83, 85} *IFNG* expression is also increased in SLE,⁶⁰ and a higher serum IFN- γ level has been reported in patients with SLE compared with healthy individuals.⁸⁶ Plasmacytoid DCs are the major source of IFN- α produced in response to viruses and bacteria (reviewed previously⁸⁷). Activation of endosomal TLR7 and TLR9 by their respective agonists, single-stranded RNA and unmethylated CpG-rich DNA, transduces signals through myeloid differentiation primary response protein MyD88 (MyD88) and upregulates IFN- α expression. In SLE, antibody–nucleic acid immune complexes, for example RNA or DNA from necrotic or apoptotic cells,⁸⁸ are suggested to be bound by immunoglobulin γ Fc region receptor IIa (Fc γ RIIa) and internalized by plasmacytoid DCs in the form of autoimmune complexes, subsequently activating endosomal TLRs and upregulating IFN- α production (Figure 3).^{87, 89}

Several studies indicate that interferons induce BAFF expression: IFN- α upregulates BAFF expression by mouse macrophages;⁹⁰ IFN- α and IFN- γ both upregulate BAFF and APRIL expression by human DCs;⁹¹ and IFN- α and IFN- γ can upregulate BAFF expression

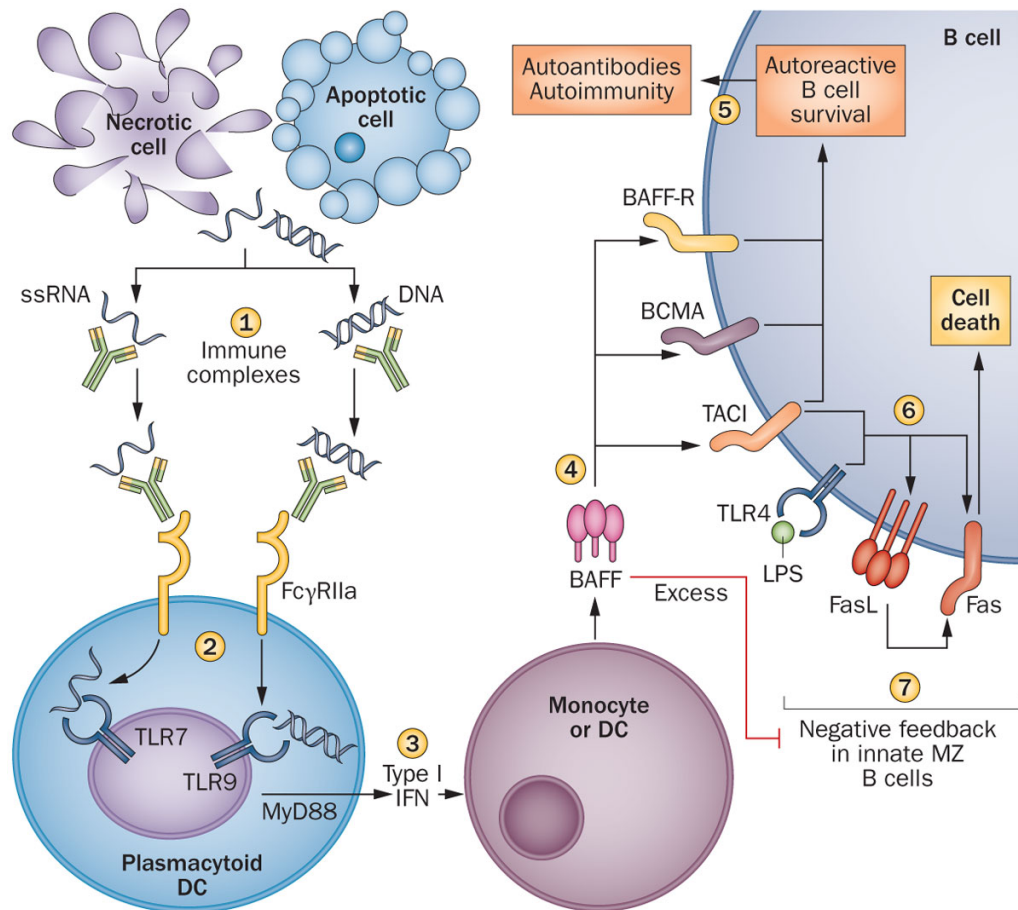


Figure 3. Role of BAFF in the pathogenesis of SLE.

In SLE, it is believed that antibody–nucleic acid immune complexes (1), for example ssRNA or DNA from dead cells, are bound by FcγRIIa, activating TLRs and IFN-α production (2). IFN-α increases BAFF production (3). BAFF interacts with receptors on B cells (4). Excess BAFF can increase autoreactive B-cell survival, driving autoimmunity (5). TLR4 and TACI signalling cooperate to commit MZ B cells to apoptosis via induction of Fas and FasL (6), possibly contributing to the mechanism that terminates the short-lived antibody response of activated innate B cells. This mechanism is defective in BAFF-overexpressing transgenic mice (7; Box 1). Abbreviations: BAFF, B-cell-activating factor of the TNF family (also known as TNF ligand superfamily member 13B); BAFF-R, BAFF receptor (also known as TNF receptor superfamily member 13C); BCMA, B-cell maturation antigen (also known as TNF receptor superfamily member 17); DC, dendritic cell; FasL, Fas ligand; FcγRIIa, immunoglobulin γ Fc region receptor IIa; LPS, lipopolysaccharide; MyD88, myeloid differentiation primary response protein MyD88; MZ, marginal zone; ssRNA, single-stranded RNA; TACI, transmembrane activator and cyclophilin ligand interactor (also known as TNF receptor superfamily member 13B); TLR4, Toll-like receptor 4. (Figure 3 has been reproduced from a review I have authored[184]).

by human monocytes.⁹¹ In response to IFN- γ , more BAFF was produced by monocytes from patients with SLE than by monocytes from healthy donors.⁹² In a phase Ia clinical trial of patients with SLE treated with an anti-IFN- α monoclonal antibody, BAFF mRNA expression in whole blood was suppressed.⁹³ Strikingly, in NZM lupus-prone mice, BAFF deficiency prevented glomerulonephritis and clinical disease in response to IFN- α treatment.⁹⁴ The activity of IFN- α in serum from patients with SLE positively correlated with serum BAFF concentrations.⁷⁰ Moreover, we demonstrated a regulatory feedback loop between TLR7 or TLR9 and TACI,⁸⁰ and in lupus-prone mice demonstrated cooperation between TACI and TLR signalling in Fas-induced apoptosis, a defective mechanism in BAFF-overexpressing transgenic mice (Box 2).⁸ Collectively, these mouse and human studies of SLE pathogenesis suggest the roles of BAFF and IFN- α are closely linked (Figure 3). One study showed that, through an IFN- α -dependent pathway, monocytes from healthy individuals can differentiate into DCs when cultured with serum from patients with SLE (SLE-DCs), and this induction of DC differentiation also correlated with SLE disease activity (SLEDAI).⁹⁵ SLE-DCs can induce B-cell proliferation and plasmablast differentiation.⁹⁶ Interestingly, DCs differentiated from monocytes, from healthy individuals, and cultured with IFN- α plus granulocyte macrophage colony-stimulating factor (IFN-DCs), phenotypically differ from SLE-DCs. One of the main differences is that IgG secretion was enhanced (dependent on BAFF) by SLE-DCs but not IFN-DCs.⁹⁶

Therapeutic innovation

Although known anti-BAFF biologic agents bind to soluble or membrane-bound BAFF, the specificity for homotrimer, heterotrimer and 60-mer forms is unclear. Belimumab targets only soluble human BAFF.³⁵ Tabalumab (also known as LY2127399), a fully-human IgG₄ monoclonal antibody, neutralizes both soluble and membrane-bound human BAFF.⁹⁷ Blisibimod (also known as A-623) is a fusion polypeptide protein that targets human BAFF and binds to both soluble and membrane-bound BAFF in mouse models of SLE and rheumatoid arthritis.⁹⁸

Major differences between BAFF trimer and 60-mer forms have been investigated using mouse models; however, in humans, these forms of BAFF have not been studied in detail and the 60-mer has not been described. In primary B cells isolated from mice, TACI is activated by the BAFF 60-mer, but not the BAFF trimer.²⁰ TACI interacts with MyD88, a signalling adaptor critical for the development of BAFF-mediated autoimmunity in mice,⁸⁰

suggesting that TACI, and hence the BAFF 60-mer, might be important in regulating autoimmune disease. One of the major unanswered questions is whether human BAFF exists in both trimer and 60-mer forms, and what functions the different forms might have in disease. Indeed, if the BAFF 60-mer is the active form in humans, active-form-specific anti-BAFF therapies might be developed by neutralizing only the active BAFF 60-mer, or by therapeutically disrupting the active 60-mer into the less active trimer (Figure 2). The BAFF 60-mer is not an established molecular form of BAFF *in vivo* and has yet to be characterized in mice and humans.

If findings on the reduced bioactivity of recombinant BAFF–APRIL heterotrimers apply to the native heterotrimer, heterotrimer formation could have a role in determining BAFF or APRIL activity *in vivo*. Analysis performed on a small cohort of 36 patients with SLE showed a non-significant trend towards a positive correlation between serum BAFF–APRIL heterotrimer concentration and disease activity (SLEDAI).²¹ If supported by larger longitudinal, prospective studies, these data could be interpreted to mean either that heterotrimer formation drives specific pathogenic signals or that it reflects the action of a negative feedback loop without which the pathology is more severe (Figure 2). Whether heterotrimer formation occurs through *de novo* assembly of monomers in a cell producing both BAFF and APRIL, or whether homotrimers equilibrate into heterotrimers after synthesis and release is not known. However, heterotrimers can be predicted not to form 60-mers. BAFF contains a loop sequence (the ‘flap’) that protrudes out of the monomer and hooks to the flap of BAFF in adjacent trimers.¹⁰ Size analyses of recombinant BAFF 60-mers, showed some BAFF trimers in addition to the 60-mer, but no 6-mer or 9-mer (*etc.*) intermediates,¹¹ suggesting that flap–flap interactions are weak and are probably only important when they act cooperatively in the 60-mer. APRIL has no flap sequence⁹⁹ and, therefore, theoretically cannot be incorporated into a BAFF 60-mer; if APRIL were incorporated it would most likely lead to 60-mer dissociation. A better understanding of the relative proportions and activity of BAFF–APRIL homotrimers and heterotrimers might redirect therapeutic targeting of the BAFF/APRIL system.

Conclusions

The approval of BAFF inhibition as the first targeted therapy for SLE is a major advance in the treatment of this disease, and confirms a role for BAFF in the pathogenesis of SLE. However, the diversity of SLE manifestations and the monetary cost of biologic therapy may

restrict the generalized use of anti-BAFF biologic agents in the treatment of SLE. The role of the BAFF/APRIL system in the pathogenesis of SLE needs to be understood in order to improve stratification of patients with SLE for effective anti-BAFF therapy. Studies of clinical subsets of patients who overexpress BAFF, but who were previously excluded from clinical trials, are particularly needed. Novel data suggest the potential for various approaches to target the BAFF/APRIL system in patients with SLE, such as targeting BAFF 60-mers, heterotrimers or novel receptors. Further work on the biology of the BAFF/APRIL system, and its connection to type I interferons and innate immunity (Box 3), together with rapid translation to clinical validation, is required. The role of current and future BAFF/APRIL system-targeting therapies in SLE will be determined by careful application of experimental and clinical approaches.

Review criteria

We performed a PubMed search for relevant articles from 1999–2013, using key words, including “systemic lupus erythematosus”, “BAFF”, “BLyS”, “THANK”, “TALL-1”, “zTNF4”, “TNFSF13B”, “APRIL”, “TNFSF13”, “heterotrimer”, “TACI”, “BAFF-R”, “BCMA”, “Nogo-66 receptor”, “B cell”, “T cell”, “natural killer”, “dendritic cell”, “neutrophils”, “interferon”, “toll-like receptor”, “Belimumab”, “GSK1550188”, “HGS1006”, “Blisibimod”, “AMG623”, “A-623”, “Tabalumab”, and “LY2127399”. Articles in English were reviewed by title and abstract and selected if deemed important and relevant.

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Author contributions

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Figure 1 | Soluble BAFF and APRIL signalling. BAFF and APRIL are type II transmembrane proteins, but BAFF can be processed into a soluble cytokine after cleavage at a furin protease site. APRIL is soluble, having been cleaved intracellularly. BAFF only has weak affinity for BCMA. BAFF-R is essential for survival and maturation of immature B cells. TACI is critical for T-cell-independent responses of B cells to type I and type II antigens, negative regulation of the size of the B-cell compartment and class-switch recombination. BCMA promotes plasma-cell survival. Dashed line indicates that BAFF possibly binds NgR, which is expressed on neurons and astrocytes, and has been shown to mediate negative effects on neuron outgrowth. Abbreviations: APRIL, a proliferation-inducing ligand (also known as TNF ligand superfamily member 13); BAFF, B-cell-activating factor of the TNF family (also known as TNF ligand superfamily member 13B); BAFF-R, BAFF receptor (also known as TNF receptor superfamily member 13C); BCMA, B-cell maturation antigen (also known as TNF receptor superfamily member 17); NgR, Nogo-66 receptor (also known as reticulon 4 receptor); TACI, transmembrane activator and cyclophilin ligand interactor (also known as TNF receptor superfamily member 13B).

Figure 2 | Other molecular structures of BAFF and APRIL. Different BAFF–APRIL structures might be involved in the pathogenesis of SLE, or might counteract the effects of BAFF and APRIL homotrimers by sequestering monomeric components required for their formation, competing for receptor binding or inducing suboptimal signals. APRIL interacts with polysaccharide side chains of HSPG, and interactions of TACI and HSPG regulate antibody class-switching. Solid lines indicate known interactions. Dashed lines indicate hypothetical interactions. Abbreviations: APRIL, a proliferation-inducing ligand (also known

as TNF ligand superfamily member 13); BAFF, B-cell-activating factor of the TNF family (also known as TNF ligand superfamily member 13B); BAFF-R, BAFF receptor (also known as TNF receptor superfamily member 13C); BCMA, B-cell maturation antigen (also known as TNF receptor superfamily member 17); HSPG, heparan sulphate proteoglycans; SLE, systemic lupus erythematosus; TACI, transmembrane activator and cyclophilin ligand interactor (also known as TNF receptor superfamily member 13B); TWEAK, TNF-related weak inducer of apoptosis (also known as TNF ligand superfamily member 12); TWE-PRIL is a hybrid of TWEAK and APRIL.

Figure 3 | Role of BAFF in the pathogenesis of SLE. In SLE, it is believed that antibody–nucleic acid immune complexes (1), for example ssRNA or DNA from dead cells, are bound by FcγRIIa, activating TLRs and IFN-α production (2). IFN-α increases BAFF production (3). BAFF interacts with receptors on B cells (4). Excess BAFF can increase autoreactive B-cell survival, driving autoimmunity (5). TLR4 and TACI signalling cooperate to commit MZ B cells to apoptosis via induction of Fas and FasL (6), possibly contributing to the mechanism that terminates the short-lived antibody response of activated innate B cells. This mechanism is defective in BAFF-overexpressing transgenic mice (7; Box 2). Abbreviations: Abs, Antibodies; BAFF, B-cell-activating factor of the TNF family (also known as TNF ligand superfamily member 13B); BAFF-R, BAFF receptor (also known as TNF receptor superfamily member 13C); BCMA, B-cell maturation antigen (also known as TNF receptor superfamily member 17); DC, dendritic cell; Fas, TNF receptor superfamily member 6; FcγRIIa, immunoglobulin γ Fc region receptor IIa; LPS, lipopolysaccharide; MyD88, myeloid differentiation primary response protein MyD88; MZ, marginal zone; ssRNA, single-stranded RNA; TACI, transmembrane activator and cyclophilin ligand interactor (also known as TNF receptor superfamily member 13B); TLR4, Toll-like receptor 4.

Box 1 | Therapeutic blockade of BAFF/APRIL system in SLE

The BAFF/APRIL system has a potential role in defined subsets of patients with SLE, for example in patients with renal or CNS pathology. Understanding associations between clinical manifestations and alteration of the BAFF/APRIL system might help to classify patients with SLE into subsets of potential responders to anti-BAFF treatment. Studies of subgroups previously excluded from clinical trials^{5,6} are of interest. Ethnicity might help to classify patients who could benefit from anti-BAFF therapy, because increased serum BAFF concentrations were reported to be associated with increased disease activity in some ethnic groups.⁷⁰ Collectively, these data could make SLE treatment with BAFF-targeting therapies more personalized and cost-effective.

Abbreviations: APRIL, a proliferation-inducing ligand (also known as TNF ligand superfamily member 13); BAFF, B-cell-activating factor of the TNF family (also known as TNF ligand superfamily member 13B); SLE, systemic lupus erythematosus.

Box 2 | Innate activation-induced cell death of marginal zone B cells

The BAFF/APRIL system is important in the regulation of innate B-cell activation. Nonspecific activation of innate-like marginal zone B cells by TLR4 causes an efficient and rapid antibody response that is usually short-lived. The mechanism of nonspecific activation of marginal zone B cells by TLR4 leads to increased expression of TACI. Simultaneous TLR4 activation and BAFF or APRIL signals promote expression of Fas and FasL by marginal zone B cells, and repress expression of antiapoptotic proteins. These events prepare marginal zone B cells for apoptosis, ultimately terminate the response to TLR4 activation and possibly also maintain tolerance. Defective FasL upregulation on TLR4-activated marginal zone B cells has been observed in lupus-prone BAFF-overexpressing transgenic mice.⁸ Abbreviations: BAFF, B-cell-activating factor of the TNF family (also known as TNF ligand superfamily member 13B; APRIL, a proliferation-inducing ligand (also known as TNF ligand superfamily member 13); TLR4, Toll-like receptor 4; SLE, systemic lupus erythematosus.

Box 3 | BAFF and immunity

- Studies showed that BAFF (and APRIL) is involved in splenic neutrophil activation of marginal zone B cells, promotion of plasma cell differentiation and increased immunoglobulin production¹⁰⁰
- Cytoplasmic anti-neutrophil antibodies stimulate BAFF production by neutrophils leading to enhanced B-cell survival¹⁰¹
- Neutrophils infiltrating the joints of patients with rheumatoid arthritis release BAFF after TNF activation¹⁰²
- Human NK cells produce BAFF when stimulated with IL-2¹⁰³
- An *in vitro* study of mouse splenocytes showed that soluble BAFF can indirectly enhance NK-cell activity through upregulation of IL-2 and IFN- γ production by CD4⁺ T cells¹⁰⁴

Abbreviations: BAFF, B-cell-activating factor of the TNF family (also known as TNF ligand superfamily member 13B; APRIL, a proliferation-inducing ligand (also known as TNF ligand superfamily member 13); NK, natural killer.

Table of Contents blurb

In this Review the authors enlighten our understanding of the cytokines BAFF and APRIL, their receptors and signalling pathways. The efficacy of belimumab, an anti-BAFF biologic agent, used in clinical trials for SLE, is used to develop an argument that this 'BAFF/APRIL system' is an important regulator of autoimmunity. Future therapies for SLE could fine tune the complex BAFF/APRIL system to regulate autoreactive B-cell survival and autoimmunity. [OK]

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Latest insights into BAFF as a biomarker in AID

As reported in the above review article I have authored,[184] the utility of serum BAFF as a SLE biomarker in clinical practice is unresolved, with conflicting results regarding its relationship with overall SLE disease activity. When particularly focused on specific organs involved in SLE, data from the literature also appear conflicting. Positive relationships have been reported between serum BAFF levels with the presence and/or activity of some individual organ manifestations in SLE, namely renal,[101, 127, 185] neurological/neuropsychiatric,[73, 185] musculoskeletal,[101] mucocutaneous,[101, 127] and serosal disease,[127, 135] as well as immunological manifestations.[101] Conversely, other studies reported no relationship between serum BAFF levels and specific organ disease.[15, 44, 135, 150, 173]

The type of assay used to quantify serum BAFF and its sensitivity, potential assay interference with the presence of rheumatoid factors (RF), the potential presence of BAFF in different conformations, such as BAFF-APRIL heterotrimers, BAFF isoforms and/or glycosylation variants, and even the potential presence of BAFF in a 60-mer conformation, which may or not be recognised by the assays used, is of interest when comparing these studies (**Figure 2**; reviewed in[184]). Finally, the potential presence of other proteins that could interfere with BAFF detection, for instance by hiding the epitope recognised in ELISA assays,[88] has to be taken into account.

Soluble BAFF receptors in AID

Hoffmann *et al.* reported the existence of a soluble form of TACI (sTACI) as the ectodomain of trans-membrane TACI (mTACI), produced following cleavage of mTACI by the metalloproteinase ADAM10 from the cell surface of activated B cells. When further cleaved by γ -secretase, sTACI can oligomerize to act as a decoy receptor for both BAFF and APRIL.[70] The authors therefore suggested that sTACI may play a role in the negative regulation of BAFF/APRIL signalling in B cells. The same group also reported the presence of soluble form of BCMA (sBCMA) in human sera, produced from cleavage of mBCMA from plasma cells by γ -secretase, which *in vitro* acts as a decoy receptor specific for APRIL.[90] Indeed, sBCMA, as opposed to mBCMA, fail to bind BAFF but bound APRIL. Moreover, production of sBCMA led to the modulation of bone marrow plasma cell populations. Only one study has reported the existence of sBAFF-R.[41] This soluble receptor form was released by human decidual stromal cells *ex vivo*, and an inhibitory role for sBAFF-R decoy in the regulation of monocyte cytokine production (IL-6 and TNF) has been suggested.[41] Some published studies report the presence of soluble forms of BAFF receptors in human serum. sTACI and sBCMA have been described in multiple sclerosis (MS), multiple myeloma and patients with chronic lymphocytic leukaemia.[70, 88, 90, 136, 166] Only one published study has described the presence of sTACI, and, a separate report described the presence of sBCMA, in SLE.[70, 90] In both cases the SLE cohort was modest ($N < 50$), and the articles only reported relationships with overall disease activity, and whether patients were untreated vs. treated. The relationship between sTACI, sBAFF-R or sBCMA and disease activity at the organ level in SLE and pSS has never been published. However, it is worth to note that serum sTACI has also been reported higher in a cohort of 56 SLE patients, and particularly those with renal manifestations, in an abstract presented at the

ACR 2001.[39] The work presented in this thesis shows that these markers can be used to stratify patients with various manifestation of the disease.

Strategies for the combination of biomarkers in AID

Since BAFF is a validated pathogenic driver in SLE, one of the main questions is whether there is a way to use serum BAFF levels as a reliable biomarker for the assessment of phenotypically heterogeneous patients encountered in clinical practice. Stratification of SLE patients into subsets according to their clinical and/or immunological phenotype may refine the use of BAFF as a biomarker for SLE and also as a therapeutic target (reviewed in[184]). I hypothesize that a strategy combining quantification of serum BAFF levels and components of other immunological systems involved in SLE pathogenesis may be a more powerful and reliable approach for the identification of biologically similar patients. For example, would other components of the immune system emerge as useful clinical biomarkers, when combined with measurements of BAFF?

Decades ago, al-Janadi *et al.* suggested that differences in cytokine levels could help distinguish SLE subsets, highlighting differences in the immunological pathways involved in SLE.[3] As previously discussed in a review, I have authored,[184] differences in clinical manifestations are likely related to differences in the immunological pathways involved in disease progression, therefore finding a single biomarker that best correlate with all forms of SLE is not realistic (reviewed in[184]). Rather, selected components of some immunological pathways may be involved in some pathogenic manifestation but not all, and may provide biomarkers specific for particular phenotypic manifestations. Refined biomarkers are likely to guide therapeutical intervention, or lead to the development of novel therapeutic agents specifically targeting the immunological pathway(s) involved in driving specific clinical manifestation(s). In fact, avoiding the use of expensive biologic therapies, like belimumab, in

patients unlikely to respond to them will spare costs and unnecessary exposure to the known side effects of this treatment. Given the many factors potentially contributing to the progression of AID, one approach is to use as a starting point a biological system with a demonstrated association with disease in mice and humans– such as the BAFF/APRIL system. Among the different immunological systems that are characterized by an interplay with the BAFF/APRIL system, the Fas/Fas ligand (FasL), and Type I and II interferon (IFN) systems stand out for investigation (**Figure 3**).

3. A new look at the Fas/FasL system in AID

The Fas/FasL system comprises FasL (TNFSF6, CD178; a type II membrane protein), which is the ligand for the receptor Fas (TNFRSF6, CD95/Apo-1; a type I membrane protein). Another receptor, DcR3 (TNFRSF6b) acts as a decoy for FasL. Signalling by the TNF superfamily ligand FasL through Fas leads to apoptosis (reviewed in[50]). Mutations in mouse genes encoding for Fas [*lpr* (lymphoproliferation)] and FasL [*gld* (generalized lymphoproliferative disease)] lead to spontaneous mouse models of systemic AID, particularly reminiscent of human SLE.[33, 157, 192] This phenotype is due to impaired activation-induced cell death, controlled by the Fas/FasL axis. This underlines a potential role for both Fas and FasL in human AID pathogenesis.

Defects in Fas-mediated apoptosis pathway are the cause of a rare autoimmune lymphoproliferative disorder, autoimmune lymphoproliferative syndrome (ALPS). While the genetic defect is unknown in one third of ALPS patients (ALPS-U), two-thirds have a mutation in FAS, most commonly a heterozygous germline mutation (ALPS-FAS), being more frequent than the somatic mutation (ALPS-sFAS). Of note, less than 1% of ALPS patients have a germline mutation in FASLG (ALPS-FASLG) or in CASP10 (ALPS-CASP10) (reviewed in[92, 97]). ALPS patients typically present with lymphoproliferative symptoms (most frequently lymphadenopathy and splenomegaly), autoimmunity features (most frequently autoimmune hemolytic anemia and immune-mediated thrombocytopenia), and are at increased risk of malignancy, especially lymphoma (reviewed in[92, 97]). While other autoimmune manifestations are less frequent, they appear reminiscent of SLE manifestations, such as renal, skin and joint involvement (reviewed in[163]).

FasL is expressed as a membrane-bound form (mFasL), which can be released in a soluble trimeric form after proteolytic cleavage by metalloprotease,[82] resulting in soluble FasL (sFasL), which can also bind its receptor, Fas.[139, 140] mFasL appears to induce

apoptosis via signalling through Fas, while sFasL may mediate non-apoptotic effects, also transduced through Fas.[115] As opposed to mFasL, sFasL cannot induce Fas oligomerisation on the cell surface, without which no Fas-mediated apoptosis occurs.[80] This difference in function between mFasL and sFasL has been recently reported in mouse photoreceptor cells,[100] and in rheumatoid arthritis (RA) fibroblast-like synoviocytes (FLS),[9] showing that sFasL harbours anti-apoptotic and proliferative, but not proapoptotic, functions. However, there is evidence that sFasL inhibits mFasL interaction with Fas, inhibiting Fas-mediated apoptosis.[71, 152] Fas also exists as a soluble form (sFas). sFas is translated from an alternatively spliced variant of *Fas* mRNA, and can block Fas-mediated apoptosis *in vitro*, and impede regulation of lymphocyte development and proliferation *in vivo*. [24, 29] Thus, the soluble forms of Fas and FasL both act as decoys for the Fas-mediated apoptosis pathway, potentially leading to increased survival of Fas-bearing target cells, which are of primary relevance in AID pathogenesis. Whether sFas can neutralise the proinflammatory effects of endogenous sFasL has not been established, but would be expected. Interestingly, the presence of autoAbs against FasL[155] and Fas[158] in human SLE has been described. Thus, the Fas/FasL system appears complex, with mediation of apoptotic pathways through interaction between membrane-bound receptor and ligand, modulated by their respective soluble forms, and in addition, a proinflammatory role for sFasL, which might also be modulated by the presence of sFas. Measurement of the ratio between sFasL and sFas could be informative, but has not been studied in details in human AID.

There is, however, another layer of complexity in this system. The presence of endogenous sFas oligomers has been reported in the sera of RA[125, 164] and SLE patients,[164] while only the monomeric form is found in healthy individuals.[125, 164] Cytotoxic activity of this oligomeric form of sFas has been demonstrated on HeLa cells and

primary lymphocyte in cultures.[125, 164] This supports a potential “reverse signalling” function of mFasL, whereby sFas could act as a proapoptotic ligand for mFasL acting, in turn, as a membrane-bound receptor.[42, 125, 154] This mFasL-mediated apoptosis through reverse signalling has been demonstrated in CD4⁺ T cells, but not in CD8⁺ T cells.[42, 154] Conversely, Suzuki *et al.* demonstrated the existence of a positive reverse signalling in CD8⁺ T cells, enhancing their proliferation.[154] This suggests that CD4⁺ and CD8⁺ T cells may potentially receive two different signals through Fas and mFasL, and their response may be modulated by relative sensitivity to one of the two receptors or by which signal occurs first.[154]

sFas-mediated cytotoxicity was reported as being dependent on its oligomerisation, since oligomeric but not monomeric sFas induced apoptosis by reverse signalling.[125] The authors suggested sFas oligomerisation/depolymerisation as the mechanism modulating its dual anti-/pro-apoptotic functions. Oligomeric forms of sFas were detected in sera from patients with AID harbouring high abundance of serum sFas, but not in sera from healthy subjects,[125, 164] with lower serum sFas levels in some studies. Other studies also suggested a proapoptotic effect of sFas in SLE.[147] In summary, sFas and sFasL both have mainly anti-apoptotic functions, with the exception of the proapoptotic function of oligomeric, but not monomeric, sFas, presumably active when sFas is in high abundance in patients with AID (**Figure 4**).

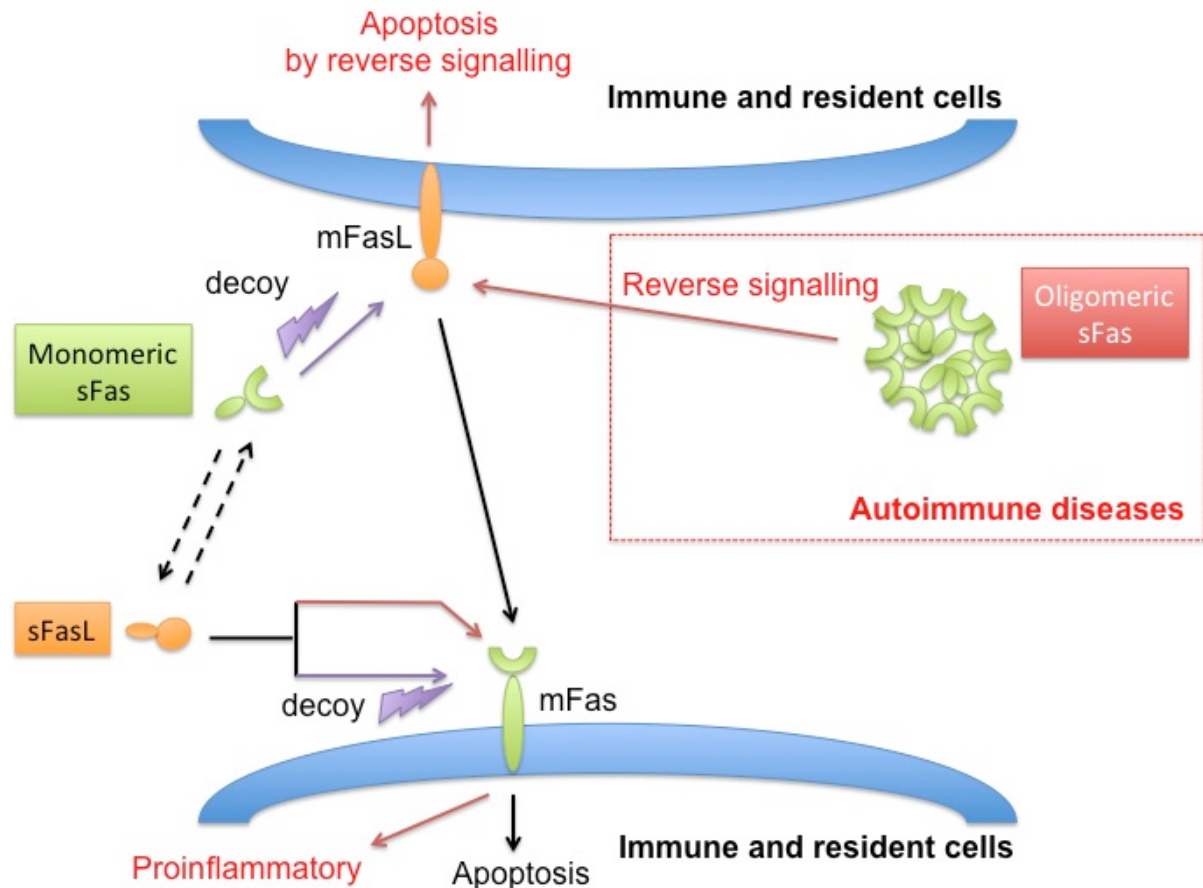


Figure 4. The Fas/FasL system in AID.

mFasL interacts with Fas on the cell surface to induce apoptosis. Both sFas and sFasL act as decoy blocking Fas-mediated apoptosis. An oligomeric form of sFas is only detected in AID. Oligomeric sFas can induce apoptosis on mFasL-bearing cells by reverse signalling. sFasL also triggers proinflammatory function when signalling through Fas. Dashed arrows indicate hypothetical decoy function of sFas for sFasL in relation to the proinflammatory function of the latter.

Interplay between the BAFF/APRIL and Fas/FasL systems in AID

With a homozygous *lpr* gene mutation, the MRL-*lpr/lpr* SLE-prone mouse is characterized by up-regulation of IFN-gamma (IFN- γ) and high serum BAFF levels.[66, 91] Published work from our laboratory recently described an interaction between the BAFF/APRIL system and Fas/FasL system-mediated marginal zone (MZ) B cells apoptosis in mice.[49] Concomitant Toll-like receptor (TLR) 4 and TACI signalling following lipopolysaccharide (LPS) activation, induce expression of both mFasL and Fas on MZ B cells, while repressing

inhibitors of this apoptotic pathway, leading to apoptosis of these cells [innate activation-induced cell death (AICD)]. Interestingly, in BAFF-Tg mice, BAFF overexpression appeared to disturb this regulatory mechanism, through impaired mFasL, but not Fas, up-regulation.[49] This emphasizes the potential link between the BAFF/APRIL and Fas/FasL systems, in the pathogenesis of AID. The interplay between the BAFF/APRIL and the Fas/FasL systems is depicted in Figure 3. It could be speculated that the defect observed by flow cytometry of mFasL up-regulation in MZ B cells from BAFF-Tg mice might be explained by the presence of sFas bound to mFasL, which may have hidden the epitope recognized by the Ab used for flow cytometry.[49] Hence, increased levels of serum sFas in the setting of BAFF overexpression may reflect Fas/FasL immune dysregulation, for example in human SLE and pSS. To further characterize the relationship between BAFF and sFas, it would also be of interest to determine whether BAFF plays a role in sFas oligomerisation.

Soluble forms of Fas and FasL in AID

Compared to healthy individuals, serum sFas has been reported elevated in SLE patients in many studies,[4, 7, 17, 19, 29, 30, 37, 68, 81, 104, 114, 118, 130, 132, 133, 170-172, 176, 179, 180] but not in all,[52, 59, 67, 84] and has even been reported to be lower in SLE in a few studies.[134, 168] Reported findings are inconsistent with regard to the relationship between serum sFas and overall SLE disease activity, where a positive relationship has been shown in some studies,[17, 68, 81, 132, 133, 168, 179] but not in others.[4, 37, 130, 134, 164] Only a very few studies investigated serum sFas as a biomarker for specific organ activity. Serum sFas has been variously reported to be associated with the presence of renal and CNS SLE,[132] and with the presence of oral ulcers,[164] arthritis,[164] serositis,[164] LN,[19, 164] haematological,[164] immunological disorders,[164] and cutaneous lupus.[5, 55] A positive relationship between serum sFas and SLE organ damage has also been

shown,[4, 179] with serum sFas reported to particularly associate with organ damage involving CNS,[68, 179] renal[68, 179] and serosal domains.[68] Plasma sFas concentrations have also been reported to predict SLE relapse.[180] Thus, while studies show conflicting results regarding the relationship between serum sFas with overall SLE disease activity, some studies suggest its potential role as an organ-specific SLE biomarker, particularly in renal, neurological and mucocutaneous SLE.

Fewer studies have focused on sFasL in SLE. sFasL has been reported to be elevated in SLE compared to healthy controls in most studies,[7, 162, 168, 172, 176] but not in all.[114] Only a very few studies investigated the relationship between serum sFasL and SLE disease activity, and were rarely focused on phenotypic manifestations. A positive relationship between sFasL and SLE disease activity has been reported in one study,[168] but not in another.[74] Thus, while an increase in serum sFasL levels is observed across most studies, there is no clear picture as its potential as an overall and/or organ-specific disease activity biomarker with SLE.

While the use of a ratio between serum concentrations of sFas and sFasL has been investigated in other conditions,[2, 106, 195] only one study has reported the use of this ratio in SLE. Using a small cohort of 15 SLE patients, Turi *et al.* reported that the ratio sFas/sFasL was slightly higher in SLE patients compared to healthy individuals (no *p* value provided), while no relationship with either overall disease activity or organ-specific involvement was observed.[176] As noted earlier, there are potential benefits from using this ratio in light of the complex Fas/FasL system, where measuring only one component of this system may not perfectly reflect the whole system activity.[176]

The Fas/FasL system and organ involvement in SLE

The role of the Fas/FasL system in renal SLE has been well documented. Renal expression of both Fas and FasL has been previously reported in mice,[63, 95] with increased expression of mFasL and sFasL in LN-prone mice.[95] Interestingly, FasL is constitutively expressed by renal tubular cells in healthy rodents, but glomerular (mesangial cells and/or infiltrated immune cells) FasL expression can be observed in models of LN.[95] *In vivo* stimulation of Fas-expressing murine renal glomerular mesangial cells with anti-Fas Ab induced their apoptosis.[63] Another study showed mFas-mediated apoptosis in mFas-bearing human glomerular mesangial cells.[62] In human LN, glomerular cells express mFas,[47, 159, 177] and both glomerular cells and tubular epithelium have increased mFasL expression[10, 38, 175] and greater numbers of apoptotic renal cell.[38] Fathi *et al.* reported the increased expression of both glomerular mFas and serum sFas levels in LN, compared to normal controls.[47]

The role of the Fas/FasL system in mucocutaneous SLE has also been studied. In human normal skin, Fas expression has been described, at the levels of the epidermis (including keratinocytes and epidermal dendritic cells), and dermis (including infiltrated mononuclear lymphoid cells), as well as in endothelial cells, secretory glands and hair follicles.[117] Conversely, epidermal keratinocytes[11, 108] and hair follicles[11] did not express FasL, while some dermal mononuclear cells,[11] and dermal histiocytes (macrophage, not Langerhans cells),[108] which are likely to be the same mononuclear cell type, did. Fas-mediated apoptosis has been demonstrated *in vitro* in the Fas-expressing human epidermal keratinocyte cell line KJD.[117] Fas and FasL expression has been reported up-regulated in the skin of patients suffering from cutaneous lupus[131, 146], but no correlation between apoptosis and Fas expression was observed.[146] Higher apoptosis in epidermal, keratinocytes and inflammatory infiltrate has been shown in patients with

cutaneous lupus,[11, 169] compared to normal controls. In cutaneous lupus, Fas expression was detected in the epidermis,[169] keratinocytes,[11, 108, 117] hair follicles,[108] and in infiltrating lymphocytes and mononuclear cells,[11, 108] while FasL was expressed mainly by dermal infiltrated mononuclear cells and histiocytes,[11, 108] and by CD4⁺ T cells,[108] but not by epidermal keratinocytes.[11].

In the brain, both Fas and mFasL are expressed on neurons and astrocytes, and oligodendrocytes only express Fas (reviewed in[16]). *In situ* brain production of sFasL has been described in a mouse model of cerebellar degeneration, but the specific sFasL-producing cell(s) still remains to be identified.[181] Whilst the proapoptotic role of Fas in the brain is acknowledged, recent studies revealed crucial non-apoptotic functions in the CNS (reviewed in[16]). Indeed, Corsini *et al.*, showed that Fas/FasL interaction was important for adult neurogenesis.[36] Interestingly, Vernet-der Garabedian *et al.* showed that a non-apoptotic Fas/FasL pathway signalling is involved in chronic CNS inflammation occurring in a mouse model of cerebellar degeneration triggered by neuron loss, particularly following up-regulation of Fas and sFasL expression.[181] Authors also underscored the integrity of the blood-brain barrier (BBB) in this model, ruling out potential interaction with infiltrated immune cells.[181] A recent *in vitro* mouse study showed that Fas signalling could lead to the proliferation of brain endothelial cells when stimulated with low dose of recombinant sFasL, and, conversely, induce apoptosis when stimulated by high doses.[197] Hence, the role of the Fas/FasL system appears very complex in the brain, with FasL interaction with Fas leading to different signalling pathways, such as apoptosis, neurogenesis (including brain repair), neuronal branching, and even tumorigenesis (reviewed in[16]). When taking CNS infiltrated Fas- and/or mFasL-bearing cells, the situation appears even more complex. As mentioned earlier, only few studies investigate serum sFas in CNS lupus.[68, 132, 179]

Similarly, very few studies looked at sFas and/or sFasL in pSS/SS. Serum sFas concentrations have been reported to be significantly higher in pSS/SS, but not in secondary SS, compared to healthy subjects in some studies.[52, 114, 174] Interestingly, significantly higher serum sFas levels have been found in pSS patients with extra-glandular disease compared to those without.[52] Serum sFasL concentrations are also significantly higher in SS compared to healthy subjects in one study,[114] but not in another.[34] No association between serum sFas with disease activity has been reported in SS.[134] Thus, serum sFas appears to be higher in pSS, while conflicting reports with serum sFasL in pSS have not allowed drawing any conclusion on the significance of this biomarker. This is a limited number of studies and further studies with larger cohorts are required to investigate these aspects in more details. Moreover, only one study has reported the relationship between sFas and pSS/SS disease activity,[134] while no published study has reported sFasL or the ratio of sFasL/sFas as a potential pSS biomarker. None of these studies used the European League Against Rheumatism (EULAR) SS disease activity index (ESSDAI) composite scoring system,[143, 145] or investigated organ-specific pSS disease activity. As already mentioned for studies investigating the role of serum BAFF as a biomarker for AID, and suggested by authors of these studies, discrepancies between these studies may come from differences in the assays used to quantify soluble forms of Fas and/or FasL, potentially detecting oligomeric and/or monomeric forms of sFas, as well as differences in assay sensitivity, study population, and the disease activity score used.

In light of these discrepancies, using a ratio between sFas and sFasL may be of interest, since both proteins belong to the same immunological pathway and are able to modulate the Fas/FasL system.[176] The use of this ratio may better reflect the Fas/FasL biological activity in SLE,[176] and in other AID, hence lead to more consistent study outcomes regarding the relationship between serum sFas and sFasL with AID.

4. The Type II IFN system in AID

Interplay between the BAFF/APRIL and the Type II IFN systems in AID

In the published review I have authored (please see above section 2), I have described the interplay between the BAFF/APRIL system and both type I and II IFN systems in humans and mice in the context of SLE pathogenesis.[184] Interestingly, both IFN- α and IFN- γ up-regulate BAFF expression by human dendritic cells (DCs) and monocytes,[184] and BAFF is increased in response to stimulation with IFN- γ in human neutrophils.[138] Regulation of BAFF production from resident cells by IFN- α and/or IFN- γ in target organs of human with AID has also been reported. BAFF up-regulation has been demonstrated following IFN- α and IFN- γ stimulation in both pSS and healthy subject salivary gland epithelial cells (SGEC).[78] A recent study showed that BAFF-R-mediated BAFF signalling in mouse follicular helper T cells (T_{FH}) cells led to up-regulation of IFN- γ along with proliferation, while BCMA appeared to have the opposite effect. Moreover, T_{FH} -produced IFN- γ enhanced BAFF expression by mouse DCs.[35] This potentially created a positive feedback loop between IFN- γ and BAFF production. A positive association between serum BAFF and IFN- γ levels in human SLE has been suggested,[35] but no direct correlation or any statistical test was presented. Szodoray *et al.* reported the potential of both BAFF and IFN- γ in discriminating between pSS patients with or without ectopic salivary gland germinal center formation, but did not report a correlation between BAFF and IFN- γ .[156]

Type II IFN as AID biomarker

Type I IFN, and particularly IFN- α (IFN- α), have been extensively studied in SLE, where an IFN- α signature has been extensively detailed (reviewed in[128, 184]). I did not focus on the Type I IFN pathway in the studies presented here, therefore, I will not review the literature extensively other than to state that serum IFN- α levels have not emerged as a useful clinical predictive biomarker in SLE. SLE patients were also reported to have elevated serum levels of IFN- γ , a prototypical Th1 cytokine and sole representative of the Type II IFN system (reviewed in[184]). In fact, higher levels of serum IFN- γ have been reported in SLE,[3, 18, 23, 31, 35, 46, 48, 61, 83, 103, 196] including in active SLE.[53] However, conflicting reports showed that levels of serum IFN- γ are lower than,[160] or not different to healthy individuals in other studies.[6, 12, 94] Most studies reported no relationship between serum IFN- γ and overall SLE disease activity,[6, 12, 31, 61, 83, 160] with only a few reporting a positive,[3, 46] or a negative relationship.[103] Several studies also showed a potential use of serum IFN- γ as an organ-specific SLE biomarker, despite the absence of a relationship between IFN- γ levels and overall disease activity.[83] A relationship has been reported between serum IFN- γ levels and the presence and/or activity of mucocutaneous,[46, 53, 83] fever,[53] musculoskeletal, [46] and renal SLE manifestations.[22, 23, 46, 53, 103, 196] Conversely, photosensitivity,[83] alopecia,[83] Raynaud's phenomenon,[83] musculoskeletal,[53, 83] neuropsychiatric [83] and renal manifestations,[31, 83] were not shown to be related to IFN- γ levels. Al-Janadi *et al.* also reported significantly higher serum levels of IFN- γ in SLE patients with lymphadenopathy, nephrotic syndrome, CNS and thrombocytopenia compared to healthy individuals, however, the authors did not compare these levels to SLE patients without these clinical manifestations.[3] Collectively, most studies suggest serum IFN- γ as a potential renal-centric SLE biomarker. Of course, showing

an association for IFN- γ as a biomarker also suggests the possibility of IFN- γ being a therapeutic target in AID.

Serum levels of IFN- γ are higher in LN compared to healthy individuals,[22, 196, 199] and to SLE without LN.[22, 23] Of note, levels of serum IFN- γ are higher in LN with or without active lesions compared to healthy individuals.[196] Interestingly, the same authors reported levels of serum IFN- γ higher in focal proliferative, diffuse proliferative and rapidly progressive, but not in membranous LN compared to class I LN (WHO classification) and healthy subjects.[196] Conversely, one study reported lower levels of serum IFN- γ in LN compared to SLE without LN,[103] while others reported no difference between LN and either healthy individuals[85] or SLE without LN.[31] Serum IFN- γ levels were also related to the histological activity index, but not chronicity index, and with leukocyte infiltration in LN.[196] Not all studies have found histopathological associations with IFN- γ . [83, 199] Finally, serum levels of IFN- γ have been reported associated with clinical response in LN,[196] although another study did not find this association.[199] Urinary IFN- γ levels were also reported higher in LN compared to SLE without LN.[22] Interestingly, urinary expression of IFN- γ is higher in SLE than in healthy individuals, and positively correlated with both overall and renal SLEDAI as well as with proteinuria levels, but not with the kidney biopsy histological activity index. Interestingly, urinary IFN- γ expression is higher in active LN compared to LN in remission and non-renal SLE, and with LN in remission higher than non-renal SLE.[25] Collectively, while these data show a potential for serum IFN- γ as a renal-centric SLE biomarker, divergence among studies suggest that further studies are required with larger cohorts of patients.

5. Conclusion

As mentioned earlier, several studies have investigated the presence of serum BAFF-pathway members, sFas and sFasL, as well as their ratio, in human SLE. Most studies focused on BAFF and sFas, only a few on sFasL, and only one investigated the ratio between serum sFas and sFasL in SLE.[176] Despite the emergence of anti-BAFF therapies, the use of the BAFF family of proteins as biomarkers to identify potentially responsive subsets of patients has not been fully or successfully explored. Since BAFF appears to play a role in the regulation of the Fas/FasL apoptotic pathway,[49] measuring key components of both the BAFF and Fas systems may be of interest in human SLE. To the best of our knowledge, only one study looked at both BAFF and components of the Fas/FasL system in their soluble forms, within a larger pool of 52 soluble factors.[105] A number of questions remain unanswered in relation to the BAFF/APRIL system and its relationship with Fas/FasL and type II IFN systems in AID. In particular, it is unknown whether combinations of these measurements affords greater specificity as a composite biomarker for organ involvement in SLE. Of course, biomarkers are potentially of great value in identifying targetable immunological pathways for specific subsets of patients with AID. Membrane expression, but also biological activity of soluble forms of receptors of the Fas/FasL and the BAFF/APRIL systems, have already been studied, as reviewed earlier in this Chapter. Hence, the focus of the studies presented in this thesis is on measuring expression of the soluble forms of these receptors, as well as their cognate ligands, in human autoimmune diseases, where a significant knowledge gap remains. In this thesis, studies have been designed to address the gaps in knowledge in this area, as outlined by specific aims in each Chapter.

Anti-BAFF therapies are of particular interest in the presented studies. Belimumab, which targets BAFF, is the sole biologic therapy approved in SLE, but patient selection for use of this therapy is not currently guided by any biomarker approach and much remains

unknown about which patient subgroups in which to apply it. I propose that one possible outcome of my studies could be refinement of patient selection for this drug, but acknowledge that further studies including studies of treatment response to belimumab in patients stratified according to these markers are required in the future.

In this PhD thesis, I will test the overarching hypothesis that serum and urine biomarkers can be used to identify subsets of SLE patients that relate in turn to clinical phenotype. To do this, I will test the sub-hypotheses (i) that analysis of associations of serum biomarkers, in concert with patient stratification by clinical phenotype, may characterize subsets of patients with pathway-dependent SLE manifestations; (ii) that SLE patients can be stratified using combinations of these biomarkers; (iii) that biomarkers measured in combination may better reflect the immunological pathways involved in SLE, compared to individual markers on their own; (iv) that measurement of BAFF and APRIL in the urine of SLE patients may be clinically relevant, particularly in lupus nephritis; (v) that ethnicity may influence serum biomarkers and their relationships with clinical manifestations in SLE; and (vi) that biomarker-identified pathway-dependent clinical phenotypes, composite biomarker relationships, and the clinical relevance of urinary measurement of BAFF, may be specific to SLE, by studying pSS in parallel.

CHAPTER II: Materials & Methods

1. Patients and clinical assessments

Adult SLE patients fulfilling the 1982 ACR revised criteria for SLE diagnosis[161] were enrolled from the Lupus clinic at Monash Medical Centre (MMC) (Clayton, Victoria, Australia) between December 2009 and July 2014, and The Queen Elizabeth Hospital (TQEH) (Woodville, South Australia, Australia) in December 2005, for the studies presented in Chapters III, V and VI. One SLE patient was enrolled in these studies at the time of diagnosis and the remainder had established disease, summarized in the disease duration data provided in Table 2. A total of 130 SLE patients (MMC: 129, TQEH: 1) were enrolled and had blood samples collected. Six SLE patients were followed with subsequent clinical assessment and blood collection. Five SLE patients were on clinical trials receiving either tabalumab (anti-BAFF) (n=4) or epratuzumab (anti-CD22) (n=1) at the time of blood and data collection. One, three and two SLE patients were receiving belimumab, rituximab or abatacept, respectively, at the time of or within 12 months of blood sample collection. For all statistical analysis involving SLE patients, unless otherwise specified, patients receiving anti-BAFF therapies (tabalumab and belimumab), and anti-CD20 and anti-CD22 drugs (rituximab and epratuzumab, respectively) at the time of or within 12 months of blood sample collection, were excluded. Thus, 122 patients (MMC: 121, TQEH: 1) were included in the SLE cohort at baseline, and five in the follow-up cohort.

In the study presented in Chapter VIII, consecutive patients fulfilling the 1982 ACR criteria for the classification of SLE[161] who were aged over 18 and prepared to provide a urine sample for study were recruited from the Lupus Clinic of MMC, Melbourne, Australia between March 2010 and April 2013. Forty SLE patients were enrolled in both serum biomarker studies (Chapters III, V and VI) and in the urine biomarker study presented in Chapter VIII. All SLE patients enrolled in these studies had established disease as

summarized by the disease duration data in Table 7. All patients received standard-of-care therapy; patient who had received treatment with rituximab in the preceding 12 months were excluded and no patient had received treatment with belimumab in the preceding 12 months. These SLE studies were retrospective analyses on serum samples already prospectively collected and stored.

Adult patients fulfilling the 2002 revised American-European Consensus Criteria for Sjögren's syndrome classification for pSS diagnosis[188] were prospectively enrolled at TQEH between April 2013 and August 2014. All pSS patients enrolled in these studies had established disease as summarized by the disease duration data in Table 4.

Consecutive adult patients with IgA nephropathy confirmed by biopsy were recruited from the Nephrology departments of MMC, St Vincent's and Royal Melbourne Hospitals, Melbourne, Australia, as a non-SLE nephropathy control group for the study presented in Chapter VIII. Patients suffering from IgA nephropathy were chosen as control subjects in the study presented in Chapter VIII, because, like LN, this disease is characterised by glomerulonephritis with immune complex deposits.[89] IgAN was also used as the disease control for LN in a previous published study.[28] Hence, this disease control group represented a non-SLE nephropathy.

Healthy individuals were enrolled at Monash Health Department of Immunology, as a healthy control group for studies presented in Chapters III to VII. This cohort encompassed 17 healthy volunteers, recruited by a call for volunteers at Monash University. The exclusion criteria were having an inflammatory disease or being on glucocorticoid. The same healthy control group samples were used in Chapters III to VII. Healthy subjects aged over 18 were enrolled as another healthy control group for the study presented in Chapter VIII. This cohort comprised of 36 healthy individuals recruited by a call for volunteers at Monash University,

and the exclusion criteria were any personal or familial history of autoimmune disease, any current infection, and being under medication, particularly on anti-inflammatory drugs.

In the MMC Lupus clinic, consenting patients' clinical data matched to serum samples were collected prospectively at each visit, as described.[60, 185, 186] Baseline demographical and clinical data were recorded, including date of birth, gender, ethnicity, disease duration, disease activity, and treatment. Ethnicity in SLE and pSS patients enrolled in the studies presented in this thesis was self-reported as previously described in studies of ethnicity associations in SLE.[60] In the pSS cohort, while ethnicity status was not known for some patients, ethnicity status of Indigenous Australian (IA) or non-IA was recorded. In SLE, flare was defined according to the SELENA-SLEDAI definition.[121] Damage was recorded at baseline using the Systemic Lupus International Collaborating Clinics (SLICC) SLE Damage Index (SDI),[151] as previously described.[185, 186] Histological classification of LN was performed according to the International Society of Nephrology/Renal Pathological Society (ISN/RPS) 2003 criteria.[193]

Routine laboratory tests were performed at accredited Australian laboratories for patients from MMC (Southern Cross Pathology), and at SA Pathology for patients from TQEH. In most of SLE patients enrolled at the MMC center, ANA was measured by direct immunofluorescence, and anti-ENA and anti-dsDNA by Luminex assay. To deal with discrepancies arising from different upper limit of normal for anti-dsDNA autoantibody levels, this variable was also categorized as positive or negative based on the respective normal range. In SLE patients enrolled at the MMC, ENA, ANA, anti-CCP and RF were measured at enrolment rather than at the time of sampling for my studies. This means that these laboratory tests were performed when SLE patients first enrolled at the Lupus clinic at

MMC [now named Australian Lupus Registry and Biobank (ALRB)], and not at the time of serum and/or urine sampling for the studies presented in the thesis.

When there was some missing demographical, clinical or biological data, analysis have been performed in patients with available data. This means that subjects lacking complete data in a given domain were excluded from the relevant analysis. All patients gave written informed consent. This study was approved by the Human Research Ethics Committee, Southern Health, the Human Research Ethics Committee (TQEH/LMH/MH), the Monash University Human Research Ethics Committee (MUHREC), and St Vincent's Hospital Ethics Committee, Melbourne, Victoria, Australia.

2. Overall and specific organ domain disease activity assessment

SLE

Overall SLE disease activity was assessed using both the SLE disease activity index 2000 (SLEDAI-2k)[58] and the physician's global assessment (PGA) scores.[120] The PGA score is based on a 0-3 point visual analogue scale (VAS). The 24 items of the SLEDAI-2k scoring system were individually recorded and, then, grouped into nine organ domains, in order to stratify patients into phenotypic SLE clinical subsets and to assess their individual organ domain disease activity (**Table 1**). Patients without complete SLEDAI-2k were excluded from clinical subset analysis. In some cases, last observation carried forward (LOCF) was used to complete incomplete SLEDAI-2k scores. Active and inactive overall SLE was defined as $\text{SLEDAI-2k} \geq 4$ and $\text{SLEDAI-2k} < 4$, respectively. Renal disease activity was measured by adding the renal components of the SLEDAI-2k (renal SLEDAI-2k).[123] The renal components of the SLEDAI-2k encompass the four following items, each of them scored "0" or "4": urinary casts, haematuria, proteinuria, and pyuria. Hence, renal SLEDAI-

2k score can be “0”, “4”, “8”, “12”, or “16”. Active renal disease was defined as having a positive renal SLEDAI-2k score, that is renal SLEDAI-2k >0. Similarly, neurological SLE was defined by the addition of the seven neurological SLEDAI-2k items, as presented in the **Table 1**, each of them scored “0” or “8”, namely: seizure, psychosis, organic brain syndrome, visual disturbance, cranial nerve disorder, lupus headache, cerebrovascular accident. Active neurological SLE disease was then defined as having a positive neurological SLEDAI-2k score, that is neurological SLEDAI-2k >0. SLEDAI-2k data were completed within one month of blood sample collection.

Regarding the study presented in Chapter VIII, subsequent assessments were made at routine visits over a period of 24 months after the baseline assessment. SLE disease activity was measured at baseline and at each subsequent visit during the period of observation, using the SLEDAI-2k and PGA, as above and as previously described.[120] The time-adjusted mean SLEDAI-2k (AMS) was calculated as the SLEDAI-2k area under the curve divided by the time observed.[75]

Table 1. SLEDAI-2k scoring system.

SLEDAI-2k organ domains	SLEDAI-2k items
Neurological	Seizure, Psychosis, Organic brain syndrome, Visual disturbance, Cranial nerve disorder, Lupus headache, Cerebrovascular accident (CVA)
Renal	Urinary casts, Haematuria, Proteinuria, Pyuria
Mucocutaneous	Rash, Alopecia, Mucosal ulcers
Musculoskeletal	Arthritis, Myositis
Serosal	Pleurisy, Pericarditis
Vascular	Vasculitis
Immunological	Low complement, Increased DNA binding
Haematological	Thrombocytopenia, Leukopenia
Fever	Fever

DNA: deoxyribonucleic acid; SLE: systemic lupus erythematosus; SLEDAI-2k: SLE disease activity index 2000.[58]

pSS

The ESSDAI score was used to assess overall pSS disease activity. An initiative project of the EULAR SS task force, the ESSDAI score was first developed in 2009,[143] then validated in a prospective international study which included 395 patients enrolled in more than 10 countries (reviewed in[144]). As noted earlier, disease activity states, but also score improvements representing for treatment response, have since been defined.[141] Amongst the pSS scores currently available for pSS studies, the use of the ESSDAI score was justified on the basis of this validation.

The 12 specific organ domains of the ESSDAI scoring system were recorded, i.e. constitutional, lymphadenopathy, glandular, articular, cutaneous, pulmonary, renal, muscular, peripheral nervous system (PNS), CNS, haematological and biological domains. Low and moderate to high pSS disease activity were defined as $\text{ESSDAI} < 5$ and $\text{ESSDAI} \geq 5$, respectively.[141] Some pSS patients had labial salivary gland biopsies reports available, all performed prior to blood sample collection. ESSDAI data were available in 35 pSS patients. Some clinical laboratory data were available and analysed in pSS patients with no available ESSDAI data. pSS patients without complete ESSDAI were analysed for other available demographical or biological data. ESSDAI data were completed within a maximum of 2.2 months (median [IQR] time interval between ESSDAI score assessment and blood sample collection 0.4 [0-0.7] months).

3. Collection of human biological samples

Serum

Blood samples were collected by venepuncture at the time of routine clinical testing. Serum was then isolated, using serum-separating blood collection tubes, and stored at -80°C until further use.

Urine

Urine samples were obtained at patients' routine clinic visits and healthy subject assessment visits. Samples were stored on ice for a maximum of three hours before processing. Urine samples from SLE patients were first centrifuged (1,500xg, 10 min.) to separate debris, and then distributed into 500µl aliquots to which was added 2.5µl of Protease Inhibitor Cocktail (PIC) (Sigma, Castle Hill, NSW, Australia). No PIC was added to urine samples from healthy subjects or patients with IgA nephropathy, or in a subset of SLE patients. The handling of urine samples for cytokine analysis in the setting of human disease is important. In this SLE cohort, PIC was added in 86 urine samples while not in 37 other. In order to evaluate the potential effect of the absence of PIC on urine cytokine concentrations, analyses were repeated in the subset comprising only urine samples with PIC. No change in the conclusions emerged when comparing outcomes generated in the whole cohort to those in the subset with only urine samples with PIC. Data analysis comparing the results in the whole cohort with results on the subset of SLE patients whom urine samples had PIC added are presented as an appendix (**Appendix 2-4**). The presented data in the Results section of Chapter VIII included urine samples with and without PIC. Samples were stored at -80°C until use.

4. ELISA

For Chapters III to VII

A commercial ELISA kit (Quantikine, Cat #SBLYS0B, R&D Systems, Minneapolis, MN, USA) was used to quantify human serum BAFF concentrations, following the manufacturer's protocol. Serum sBAFF-R concentrations were quantified using commercial ELISA kits (Cat #ABIN1371267, antibodies-online.com), following the manufacturer's protocol. sBAFF-R was not quantified in two serum samples from SLE patients and six serum samples from HD, so the number of SLE patients and HD analysed was 120 and 11 for this analyte, respectively. Samples were tested in duplicate when possible.

Serum BAFF levels are reported to be higher in SLE, and other autoimmune diseases including pSS and RA, when compared to healthy individuals, and to be associated with SLE disease activity in some studies (reviewed in[184]). However, only a small proportion of SLE patients appears to respond to anti-BAFF therapy in the two phase III clinical trials that have led to belimumab approval,[54, 109] suggesting the presence of anti-BAFF therapy resistant subsets and/or BAFF-independent disease. Of note, some SLE phenotypes were excluded from these trials, namely severe renal and neurological SLE. In the study presented in Chapter III, I stratified patients according to their serum levels of BAFF, namely being elevated or not, as potentially being an indication of the BAFF/APRIL system involvement in the patient disease pathogenesis. The threshold for elevated serum BAFF concentrations was defined as the mean + 2 standard deviations (SD) of serum BAFF concentrations in healthy individuals enrolled in the control group [mean (SD) 1039 (199.6) pg/ml].[65] This threshold was used to categorise AID patients as having high (≥ 1438.2 pg/ml) or non-elevated serum BAFF (< 1438.2 pg/ml).

For Chapter VIII

BAFF, APRIL and monocyte chemoattractant protein 1 [(MCP-1), also known as chemokine ligand 2 (CCL2)] concentrations in urine and serum were quantified by commercial ELISA (R&D Systems, Minneapolis, MN, USA; eBiosciences, San Diego, CA, USA; and R&D Systems, Minneapolis, MN, USA respectively), as per the manufacturers' protocols. Samples were tested in duplicate when possible. To correct for effects on urinary cytokine concentrations due to dilution, cytokine concentrations were normalised against creatinine concentration in the same sample. Urinary BAFF/creatinine ratio, APRIL/creatinine ratio and MCP-1/creatinine ratio were designated uBAFF, uAPRIL and uMCP-1, respectively.[21] BAFF was detectable in 2.4% (1/41) of urine samples from non-SLE IgA nephropathy control patients. However, no urine creatinine was quantified in this patient to calculate uBAFF. Hence, only 40 urine samples are displayed for uBAFF in this cohort, all being not detectable. Two SLE patients (follow-up cohort) and one HD had detectable urinary APRIL, however no urine creatinine levels quantified. Hence, these three subjects were not included in data analysis of uAPRIL. One SLE patient had detectable urinary MCP-1 in the follow-up cohort, however no matching urine creatinine levels were quantified. Hence, this SLE patient was not included in data analysis of uMCP-1.

5. Cobas analysis

In Chapter VIII, urine creatinine concentrations were quantified by Cobas Integra 400 plus (Roche Diagnostics, Rotkreuz, Switzerland).

6. Interference by sBAFF-R in serum BAFF quantification

Serum from a patient suffering from pSS was incubated with recombinant human (rh) BAFF-R/TNFRSF13C chimera (Cat #1162-BR, R&D Systems) at different concentrations (2, 4 and 8 µg/ml) for 15 minutes at room temperature before loading on the pre-coated BAFF Quantikine ELISA microplate. Each concentration used for rhBAFF-R was controlled by adding the same volume of reagent diluent. Serum BAFF concentrations quantification was then carried out, as described above. All samples were tested in duplicate.

7. Luminex

A commercial Human Luminex Screening Assay (polystyrene beads; Cat #LXSAH, R&D Systems) was used to simultaneously quantify serum sBCMA, sTACI, sFas, sFasL, and IFN- γ concentrations, using a Bio-Rad Bio Plex 200 system, following the manufacturer's protocol. One additional (lower) standard point dilution was added to extend the standard curve. Two serum samples from SLE patients, different from those where sBAFF-R was not quantified, were not quantified, because of sampling error or technical issue during the washing step, so the number of SLE patients analysed was 120 for these analytes. Serum samples with undetectable IFN- γ were assigned a value of "0" for IFN- γ concentrations. One outlier value in the control cohort for serum IFN- γ concentrations was excluded, as being > 95 confidence interval (CI) (24.21 pg/ml). Serum sTACI concentrations were not detectable in one SLE sample; the sample was excluded from analysis. Serum sBCMA concentrations were out of range (high) in one SLE sample, and this sample was excluded from analysis. Serum BAFF concentrations were also quantified using the same commercial Human Luminex Screening Assay, and which combined with the other five analytes made a 6-plex multiplex assay. Samples were tested in duplicate when possible.

8. ELISA and Luminex commercial kit selection

The choice of the commercial ELISA and Luminex kits in the present studies was mainly based on kit sensitivity. Unfortunately, no data from the commercial BAFF ELISA kit used in these studies was available regarding the potential detection of unglycosylated/glycosylated BAFF, alternative BAFF splice variants, as well as BAFF/APRIL heterotrimers, or BAFF 60-mer, the latter currently unknown if being potentially present in human sera (reviewed in[184]). As mentioned above, samples were tested in duplicate when possible for ELISA and Luminex assays. When serum and urine samples were tested in duplicate, the mean was used to generate final sample concentrations for analysis using the standard curve run in each plate. However, in some cases, biological sample volume was too low to allow testing in duplicate. This consideration applies to less than 10% of samples.

9. Statistical analysis

All statistical analysis were performed using Graphpad software (Prism, version 6.0h) or Stata version 14 (StataCorp, College Station, Texas, USA). Data are presented as median and inter-quartiles ranges [IQR], unless otherwise stated. Because data were not normally distributed, non-parametric tests were used. Spearman's test was used to test correlations between variables. Mann Whitney and Kruskal-Wallis tests (followed by Dunn's multiple comparisons test) were used to analyse differences between two and more than two variables, respectively. Mann Whitney tests were two-tailed, unless otherwise stated. Group of categorical data were analysed by Fisher's exact test or Chi-square test. A p value of < 0.05 was considered statistically significant. No sample size calculation has been performed for the studies presented in this thesis. Statistical advice provided to the candidate was that power calculations were not possible in the absence of data on the magnitude of variation in biomarker levels between groups.

Non-normally distributed variables were log₁₀-transformed before being used in a linear regression model. Serum cytokines and soluble receptors levels were tested as continuous and categorical variables (categorized into quartiles, then into a binary variable as 4th quartile vs. other quartiles) in a logistic regression model. For statistical analyses performed in clinical subsets, the following potential confounders were tested for inclusion in multivariable regression models: age, gender, ethnicity and disease duration. Before including SLEDAI-2k or ESSDAI organ domains in multivariable regression models, a preliminary collinearity test using polychoric analysis was performed, where r value greater than $|0.7|$ precluded using the variable in multivariable analysis models. Associations of potential confounders with both exposure and outcome variables was required prior to include potential confounders into multivariable regression models. A p value of <0.1 in

univariable analysis was used as threshold to include potential confounders into multivariable regression models.

When no multivariable analysis could be performed, to correct for multiple group testing, Bonferroni correction has been applied, generating a corrected p value of $0.05/(\text{number of tested groups})$, as the threshold for statistical significance. To correct for multiple groups testing shown in this thesis, where association between serum biomarkers and SLE organ domain activity has been tested in seven SLE subsets (amongst the 9 organ domains of the SLEDAI-2k score encompassing more than three patients), and where association between serum biomarkers and pSS organ domain activity has been tested in seven pSS subsets (amongst the 12 organ domains of the ESSDAI score encompassing more than three patients), a Bonferroni correction generated an adjusted p value threshold of <0.007 to be considered as statistically significant. To correct for multiple testing according to ethnicity or according to serum BAFF levels, a Bonferroni correction generated an adjusted p value of <0.025 as the threshold for statistical significance.

CHAPTER III: Associations of sFas, sFasL and BAFF in systemic lupus erythematosus

1. Key findings

- 1.1. The data presented confirm the observation of increased levels of serum sFas in patients with SLE compared to healthy individuals.
- 1.2. Serum sFas was related to overall SLE disease activity and to renal SLE disease activity.
- 1.3. Increased levels of serum sFasL were observed in patients with SLE compared to healthy individuals. Although a relationship between serum sFasL and anti-double-stranded deoxyribonucleic acid (dsDNA) and anti-Smith (Sm) Abs emerged, no relationship was noted with overall SLE disease activity.
- 1.4. The ratio of serum sFasL/sFas was related to overall SLE disease activity and renal SLE disease activity.
- 1.5. A significant relationship between serum BAFF and sFas was noted in SLE. This relationship was specific for patients with immunological and musculoskeletal manifestations of disease. The relationship between serum sFas and renal SLE was only seen in patients with high levels of serum BAFF, consistent with the known interaction between the BAFF/APRIL and Fas/FasL systems.
- 1.6. The relationship between the sFasL/sFas ratio and mucocutaneous SLE disease activity was seen only in patients with non-elevated serum BAFF. In contrast, the relationship between the sFasL/sFas ratio and renal SLE was restricted to patients with high serum BAFF levels.

- 1.7. The relationship between serum sFas and overall SLE disease activity appeared to be influenced by ethnicity, as it was specific to Caucasian patients. In contrast, the relationship between serum sFas and renal SLE was a specific feature of patients of Asian ethnicity.
- 1.8. Ethnicity also appears to influence the levels of serum sFasL in SLE, as these levels are higher in Asian compared to Caucasian patients.
- 1.9. No significant difference was noted regarding the sFasL/sFas ratio according to ethnicity. However, the relationship between the sFasL/sFas ratio and overall SLE disease activity was solely observed in Asian patients. The relationship between the sFasL/sFas ratio and renal SLE appeared to also be particularly dependent on Asian ethnicity.
- 1.10. The relationship between serum BAFF and sFas was also influenced by ethnicity.

2. Introduction

As outlined in the literature review (Chapter I), previously reported data suggest that serum sFas might be a potential pathogenic molecule and biomarker in SLE, particularly for some clinical phenotypes of the condition, including renal and neurological manifestations. However, conflicting results have been reported in these studies for relationships with overall disease activity, and only a few studies focused on organ-specific disease activity. Fewer studies have focused on serum sFasL and its potential relationship with SLE. Only one prior study assessed the ratio between sFas and sFasL to investigate the relationship between this ratio and SLE disease activity, but the cohort was very small with only 15 patients. Only one study has investigated BAFF in relation to soluble components of the Fas/FasL system in SLE.

Here, I have tested the sub-hypothesis that SLE patient stratification by clinical phenotype in analysis of associations of serum biomarkers from the Fas/FasL system may characterize subsets of patients with pathway-dependent SLE manifestations. I have also tested the sub-hypothesis that SLE patients can be characterised using combinations of these biomarkers. A particular aim of this study was to investigate clinical associations of the ratio of sFas and sFasL in a larger and better-defined SLE cohort than any previously described. This study also aimed to investigate whether the combination of sFas and sFasL (and/or a ratio thereof) with serum BAFF levels could be revealing of subsets in SLE. I have also tested the sub-hypothesis that biomarkers measured in combination may better reflect immunological pathways involved in SLE, compared to individual markers on their own. Finally, I have tested the sub-hypothesis that ethnicity may influence serum biomarker levels from the Fas/FasL system and their relationships with SLE.

3. Results

Patient characteristics

The cohort of SLE patients was nearly 85% female. Nearly half of the cohort was Asian, while about the other half was primarily Caucasian (**Table 2**). One patient was from Indigenous Australian (IA) background. Median age and disease duration were 43.6 and 7.7 years, respectively. SLE patients were significantly older than healthy subjects in the present study ($p=0.049$; data not shown; **Table 2**). Median SLEDAI-2k and PGA scores were 4 and 0.3, respectively. Approximately 36% of patients had two or more organ domains involved. Most patients were characterized by an active immunological domain, whilst 20.5% and 18% of them presented with active mucocutaneous and renal SLE manifestations, respectively (**Table 3**). All SLE patients were seropositive for antinuclear antibody (ANA) and/or anti-dsDNA autoAbs, with about 20% having detectable anti-Sm autoAbs (**Table 2**). Regarding laboratory data for the healthy control group, median (IQR) white cell count, lymphocyte count and monocyte count were 7.6 [6.8, 8.1], 1.8 [1.5, 2.7] and 0.5 [0.4, 0.5] $\times 10^9/L$, respectively. As previously mentioned in the Methods Chapter, sFas and sFasL were not quantified in two serum samples because of sampling error or technical issue during the washing step, so the number of patients analysed was 120 for these analytes.

Appendix 10 displays demographic, clinical and biological characteristics of SLE patients categorised by ethnicity. Most of the observed difference between Asian and Caucasian SLE patients in the present study, such as younger age, higher PGA, and higher anti-Sm and anti-Ro autoAb frequency in Asians, are in line with a previous published study from my co-Supervisor.[60]

Table 2. Demographical, clinical and biological characteristics of SLE patients and HD.

Characteristics	HD (N = 17)	SLE (N=122)	<i>p</i>
Age (years)	41 [27-45]	43.6 [33.2-55.6]	0.049
Female, n (%)	15 (88.2)	103 (84.4)	NS
<i>Ethnicity</i>			
Caucasian, n (%)	12 (70.6)	59 (48.4)	NS
Asian, n (%)	5 (29.4)	58 (47.5)	0.04
Hispanic, n (%)	0 (0)	1 (0.8)	N/A
IA, n (%)	0 (0)	1 (0.8)	N/A
Maori, n (%)	0 (0)	2 (1.6)	N/A
Samoan, n (%)	0 (0)	1 (0.8)	N/A
Unknown, n (%)	0 (0)	0 (0)	
Disease duration (years)	N/A	7.7 [4.5-15.2]	N/A
<i>Overall disease activity</i>			
SLEDAI-2k	N/A	4 [2-6]	N/A
PGA	N/A	0.3 [0.15-0.6]	N/A
<i>Treatment</i>			
Prednisone or equivalent, n (%)	N/A	69 (69.7)	N/A
Prednisone or equivalent (mg/day)	N/A	5 [0-8.5]	N/A
Hydroxychloroquine (HCQ), n (%)	N/A	106 (94.6)	N/A
HCQ (mg/day)	N/A	400 [200-400]	N/A
Methotrexate, n (%)	N/A	11 (9.02)	N/A
Azathioprine, n (%)	N/A	29 (23.8)	N/A
Mycophenolate mofetil, n (%)	N/A	16 (13.1)	N/A

Characteristics	HD (N = 17)	SLE (N=122)	<i>p</i>
Mycophenolic acid, n (%)	N/A	3 (2.5)	N/A
Cyclosporine A, n (%)	N/A	0 (0)	N/A
Cyclophosphamide, n (%)	N/A	0 (0)	N/A
<i>Clinical laboratory data</i>			
Hb (g/l)	N/A	131 [121.8-139.3]	N/A
Platelets (x 10⁹/l)	N/A	232.5 [189.8-285.8]	N/A
CRP (mg/l)	N/A	1.6 [0.7-3.4]	N/A
ESR (mm/h)	N/A	15 [7.8-28.8]	N/A
C3 (g/l)	N/A	0.89 [0.69-1.07]	N/A
C4 (g/l)	N/A	0.16 [0.11-0.22]	N/A
Anti-dsDNA Ab (IU/ml)	N/A	29 [7-145.5]	N/A
Anti-dsDNA Ab +, n (%)	N/A	64 (52.5)	N/A
ANA +, n (%)	N/A	118 (96.7)	N/A
Anti-Sm Ab +, n (%)	N/A	25 (20.5)	N/A
Anti-Ro (SSA) Ab +, n (%)	N/A	52 (42.6)	N/A
Anti-La (SSB) Ab +, n (%)	N/A	29 (23.8)	N/A
UPCR (g/mmol)	N/A	0.02 [0.01-0.03]	N/A
eGFR (ml/min)	N/A	90 [87.3-90]	N/A

N: number of individuals in each cohort. Data are expressed as median [IQR] or as number (percentage). Percentages (%) are calculated based on N. Mann Whitney test was used to analyse differences between two variables. Mann Whitney tests were two-tailed. Group of categorical data were analysed by Fisher's exact test.

Ab: antibody; ANA: anti-nuclear antibody; C3: complement component 3; C4: complement component 4; CRP: C-reactive protein; dsDNA: double-stranded deoxyribonucleic acid; eGFR: estimated glomerular filtration rate; ESR: erythrocyte sedimentation rate; Hb: haemoglobin; HCQ: hydroxychloroquine; HD: healthy donors; IA: Indigenous Australian; IU, international units; NA: non available; N/A: non applicable; ND: non determined; NS: not significant; PGA: Physician's Global Assessment; SLE: systemic lupus erythematosus; SLEDAI-2k: SLE Disease Activity Index 2000; Sm: Smith; SSA: Sjögren's syndrome antigen A; SSB: Sjögren's syndrome antigen B; UPCR: Urine protein/creatinine ratio.

Table 3. Disease activity by organ domains in the SLE cohort.

SLE patients with active organ domain	
<i>Organ domains</i>	SLE cohort (N = 122)
Fever, n (%)	0 (0)
Neurological, n (%)	4 (3.3)
Renal, n (%)	22 (18)
Mucocutaneous, n (%)	25 (20.5)
Musculoskeletal, n (%)	10 (8.2)
Serosal, n (%)	3 (2.5)
Vascular, n (%)	0 (0)
Immunological, n (%)	88 (72.1)
Haematological, n (%)	5 (4.1)

N: number of individuals in the SLE cohort. Data are expressed as number (percentage).

Percentages (%) are calculated based on N.

Individual organ domain disease activity was assessed by the SLEDAI-2k score.

SLE: Systemic lupus erythematosus; SLEDAI-2k: SLE disease activity index 2000.

Associations of serum sFas with SLE

Serum sFas was detectable in all tested SLE and HD samples. Serum sFas concentrations were significantly higher in SLE patients compared to HD ($p<0.0001$) (**Figure 5A**). Serum sFas concentrations were higher in SLE patients with active disease compared to those without ($p=0.02$; **Figure 5B**). With respect to laboratory markers of SLE, sFas concentrations were correlated with albumin ($r=-0.39$, $p<0.0001$) and the inflammation marker ESR ($r=0.21$, $p=0.023$) (data not shown). No significant relationship was observed with the presence of anti-dsDNA and anti-extractable nuclear antigen (ENA) Abs, or with complement component 3 (C3) and complement component 4 (C4) (data not shown).

I next tested whether sFas could be specifically related to clinical phenotypes of SLE. Serum sFas concentrations were higher in SLE patients with active renal disease compared to those without ($p=0.001$; **Figure 5C**), and were also correlated with renal SLE disease activity ($r=0.3$, $p=0.001$; **Figure 5D**). The association of sFas with active renal disease was also found in univariable analysis using logistic regression (OR 1.0002; 95% CI 1.0001, 1.0004; $p=0.001$). No confounder was identified. With respect to laboratory markers of renal function, sFas concentrations were weakly but significantly correlated with serum creatinine ($r=0.2$, $p=0.03$; **Figure 5E**) and urine protein/creatinine ratio (UPCR) ($r=0.2$, $p=0.029$; **Figure 5F**).

Serum sFas concentrations were significantly lower in SLE patients with active neurological disease compared to those without ($p=0.004$; **Figure 5G**). The association of sFas with active neurological disease was also found in univariable analysis using logistic regression (OR 0.9995; 95% CI 0.999, 0.9999; $p=0.02$), and confirmed in multivariable analysis after adjusting for gender (OR 0.9996; 95% CI 0.999, 0.9999; $p=0.037$). However, only four SLE patients had an active CNS disease.

Of note, no significant relationship emerged between serum sFas concentrations and mucocutaneous, serosal, musculoskeletal, immunological, or haematological SLE disease activity (data not shown).

Data presented in Figure 5 was reanalysed after excluding one outlier value for serum sFas in SLE. No major difference in the results or conclusions was observed after excluding this outlier, with all data analysis presented in Figure 5 remaining statistically significant at the same level (**Appendix 5**).”

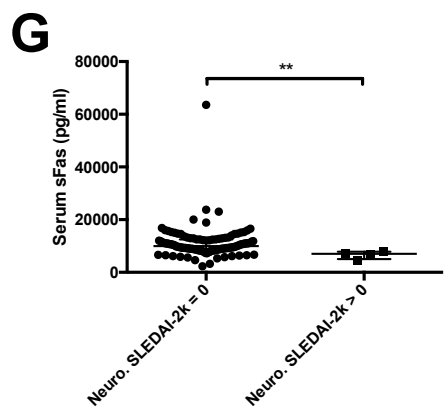
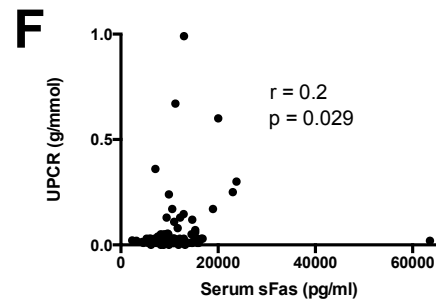
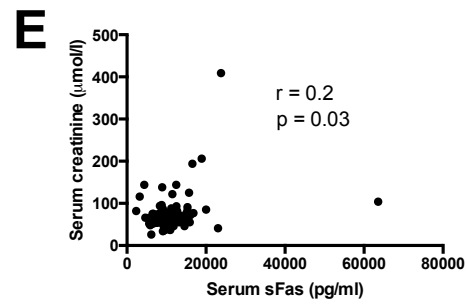
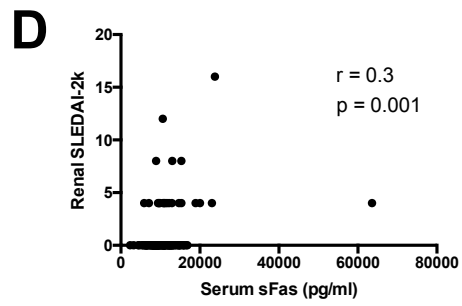
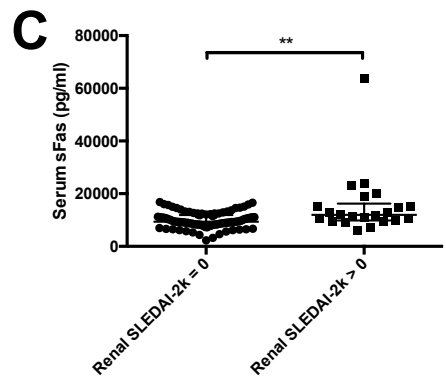
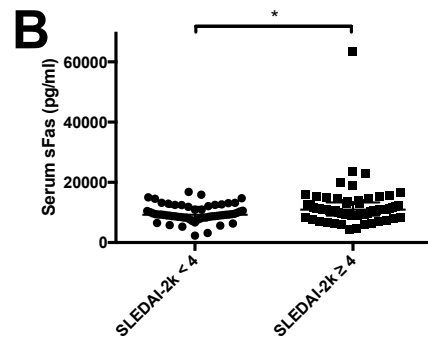
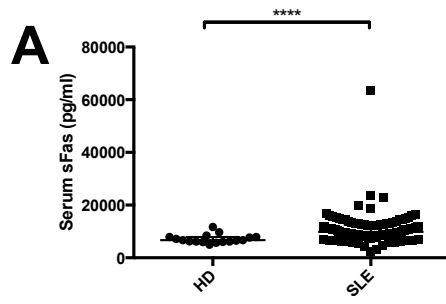


Figure 5. Associations between serum sFas with SLE disease activity.

Overall and renal SLE disease activity was assessed by the SLEDAI-2k score. (A) Serum sFas in SLE patients (n=120) compared to HD (n=17). (B) Serum sFas concentrations according to overall SLE disease activity (SLEDAI-2k < 4: n=59; SLEDAI-2k ≥ 4: n=61). (C) Serum sFas concentrations according to renal SLE disease activity (Renal SLEDAI-2k = 0: n=98; Renal SLEDAI-2k > 0: n=22). (D) Correlation between serum sFas and renal SLEDAI-2k (n=120). (E) Correlation between serum sFas and serum creatinine (n=118). (F) Correlation between serum sFas and UPCR (n=115). (G) Serum sFas concentrations according to neurological SLE disease activity (Neuro. SLEDAI-2k = 0: n=116; Neuro. SLEDAI-2k > 0: n=4).

Serum sFas concentrations are expressed in pg/ml. Serum creatinine and UPCR are expressed in μmol/l and g/mmol, respectively. Neuro. SLEDAI-2k stands for neurological SLEDAI-2k.

In panels A-C and G, horizontal bars indicate the median with [IQR].

**** $p < 0.0001$; ** $p < 0.01$; * $p < 0.05$

Associations of serum sFasL with SLE

Serum sFasL was detectable in all tested SLE and HD samples. Serum sFasL was significantly higher in SLE compared to HD ($p=0.047$; one-tailed; **Figure 6A**). Serum sFasL concentrations were not significantly related to overall SLE disease activity in this cohort (**Figure 6B-C**). However, serum sFasL concentrations were correlated with anti-dsDNA levels ($r=0.2$, $p=0.03$; **Figure 6D**), and were significantly higher in SLE patients with anti-Sm Abs compared to those without ($p=0.043$; **Figure 6E**). No significant relationship emerged regarding the presence of other anti-ENA Abs, or with complement components C3 and C4 (data not shown).

Serum sFasL concentrations were significantly lower in SLE patients with active neurological disease compared to those without ($p=0.004$; **Figure 6F**). The association of sFasL with active neurological disease was also found in univariable analysis using logistic regression (OR 0.9996; 95% CI 0.999, 0.9999; $p=0.012$). No confounder was identified. However, as for sFas analysis, only four SLE patients had an active CNS disease.

No significant relationship emerged between serum sFasL concentrations with renal, mucocutaneous, serosal, musculoskeletal, immunological, or haematological SLE disease activity (data not shown).

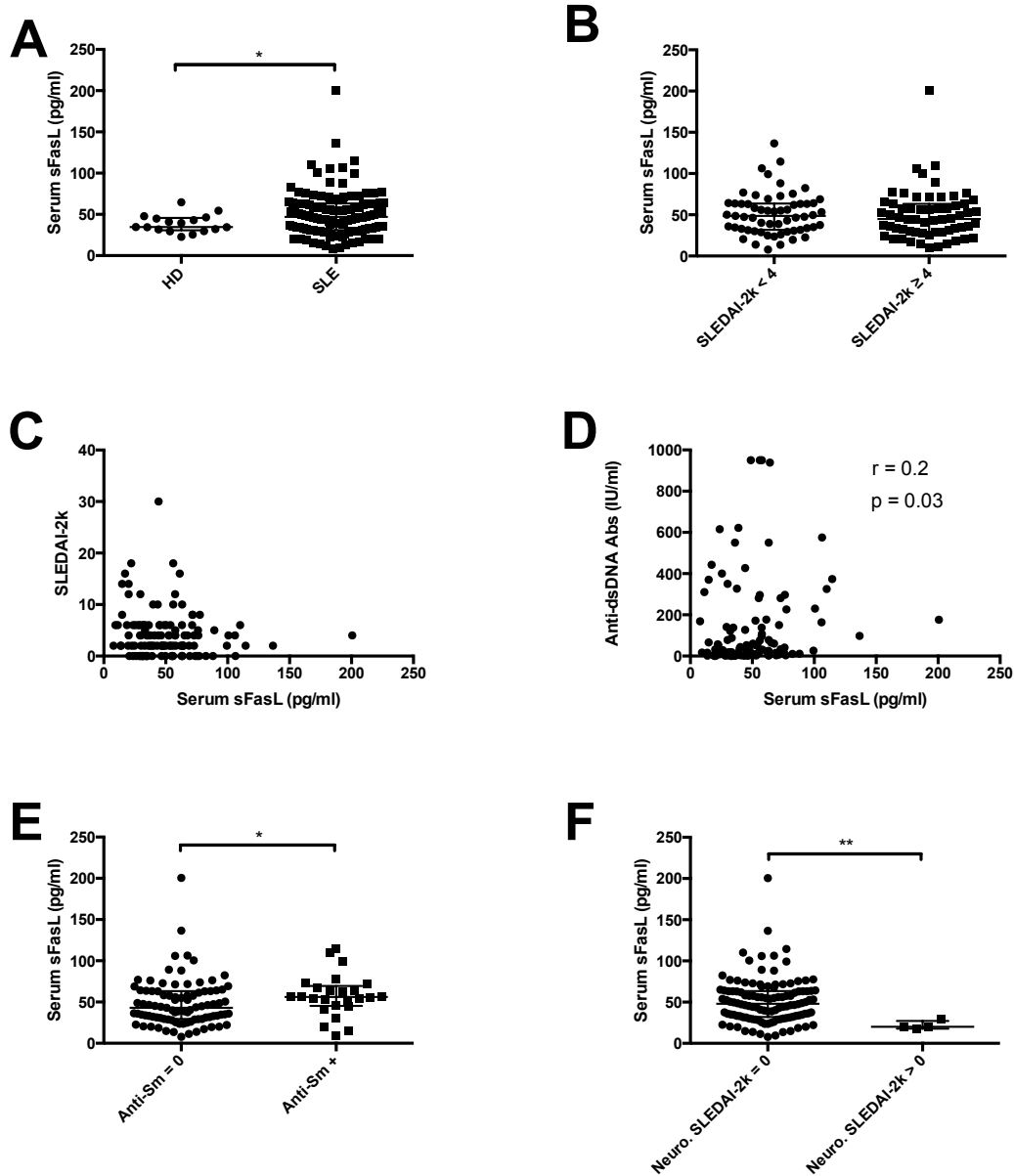


Figure 6. Associations between serum sFasL with SLE disease activity.

Overall SLE disease activity was assessed by the SLEDAI-2k score. (A) Serum sFasL concentrations in SLE patients (n=120) compared to HD (n=17). (B) Serum sFasL concentrations according to overall SLE disease activity (SLEDAI-2k < 4; n=59; SLEDAI-2k ≥ 4; n=61). (C) Correlation between serum sFasL concentrations and SLEDAI-2k (n=120). (D) Correlation between serum sFasL concentrations and anti-dsDNA Abs levels (n=119). (E) Serum sFasL concentrations according to the presence of anti-Sm Abs (Anti-Sm = 0; n=91; Anti-Sm +; n=25). (F) Serum sFasL concentrations according to neurological SLE disease activity (Neuro. SLEDAI-2k = 0; n=116; Neuro. SLEDAI-2k > 0; n=4).

Serum sFasL concentrations are expressed in pg/ml. Anti-dsDNA Abs levels are expressed in IU/ml. Neuro. SLEDAI-2k stands for neurological SLEDAI-2k.

In panels A-B, and E-F, horizontal bars indicate the median with [IQR].

One-tailed p value is shown in panel A.

** p<0.01; * p<0.05

Associations of sFasL/sFas ratio with SLE

There was no significant difference in the ratio of sFasL over sFas (sFasL/sFas) between SLE and HD (**Figure 7A**). Of note, no significant relationship was observed between concentrations of sFas and sFasL in the same sample, either in SLE or in HD (data not shown). The sFasL/sFas ratio was significantly lower in SLE patients with active disease compared to those without ($p=0.03$; **Figure 7B**), and was significantly negatively correlated with SLEDAI-2k ($r=-0.22$, $p=0.016$; **Figure 7C**). With respect to laboratory markers of SLE, sFasL/sFas ratio was significantly correlated with albumin ($r=0.32$, $p=0.0005$) and the inflammation marker ESR ($r=-0.23$, $p=0.011$) (data not shown). No significant relationship emerged regarding the presence of anti-dsDNA and anti-ENA Abs, or with complement components C3 and C4 (data not shown).

When examining sFasL/sFas ratio in relation to a particular clinical SLE phenotype, I observed that sFasL/sFas ratio was significantly lower in patients with active renal disease compared to those without ($p=0.021$; **Figure 7D**), and was also significantly correlated with renal SLE disease activity ($r=-0.21$, $p=0.019$; **Figure 7E**). The sFasL/sFas ratio was not significantly correlated with SLEDAI-2k when renal SLEDAI-2k was subtracted. This suggests that the renal component of the SLEDAI-2k drove the association observed between sFasL/sFas ratio and overall SLEDAI-2k. The association of sFasL/sFas ratio with active renal disease was also found in univariable analysis using logistic regression (OR 7.4E-100; 95% CI 5.0E-190, 1.09E-09; $p=0.031$). No confounder was identified. With respect to laboratory SLE renal function markers, sFasL/sFas ratio was also significantly correlated with UPCR ($r=-0.22$, $p=0.017$; **Figure 7F**). There was also a non-significant trend toward a correlation between sFasL/sFas ratio and serum creatinine ($r=-0.18$, $p=0.053$; data not shown).

There was a non-significant trend toward a lower sFasL/sFas ratio in SLE patients with active mucocutaneous disease compared to those without ($p=0.055$; **Figure 7G**). Accordingly, no significant association of sFasL/sFas ratio with active mucocutaneous disease was found in univariable analysis using logistic regression. No significant relationship emerged between sFasL/sFas ratio with serosal, musculoskeletal, immunological, or haematological SLE disease activity (data not shown).

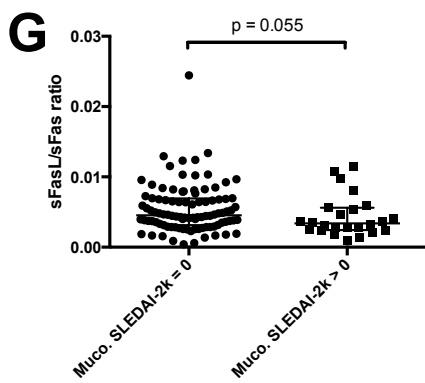
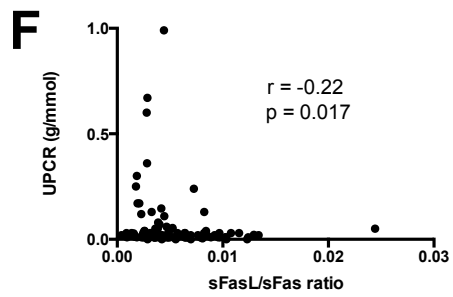
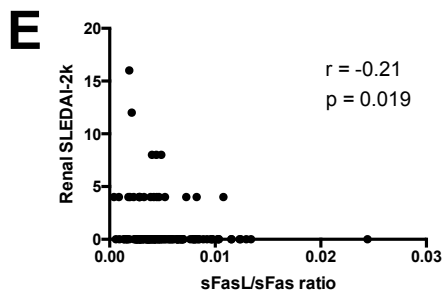
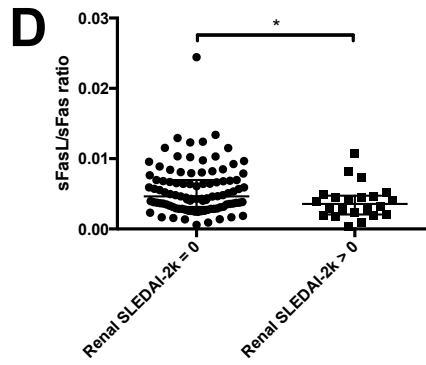
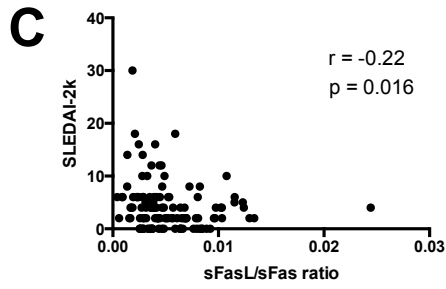
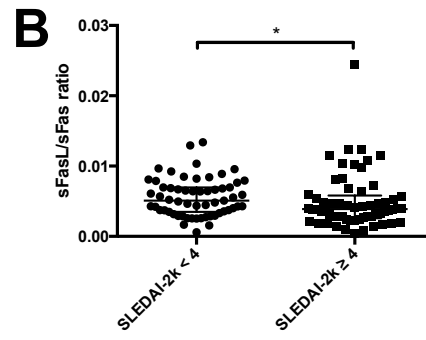
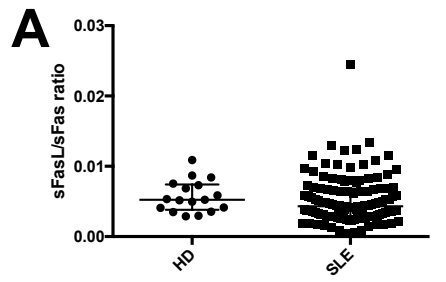


Figure 7. Associations between sFasL/sFas ratio with SLE disease activity.

Overall, renal and mucocutaneous SLE disease activity was assessed by the SLEDAI-2k score. (A) sFasL/sFas ratio in SLE patients (n=120) compared to HD (n=17). (B) sFasL/sFas ratio according to overall SLE disease activity (SLEDAI-2k < 4: n=59; SLEDAI-2k ≥ 4: n=61). (C) Correlation between sFasL/sFas ratio and SLEDAI-2k (n=120). (D) sFasL/sFas ratio according to renal SLE disease activity (Renal SLEDAI-2k = 0: n=98; Renal SLEDAI-2k > 0: n=22). (E) Correlation between sFasL/sFas ratio and renal SLEDAI-2k (n=120). (F) Correlation between sFasL/sFas ratio and UPCR (n=115). (G) sFasL/sFas ratio according to mucocutaneous SLE activity (Muco. SLEDAI-2k = 0: n=96; Muco. SLEDAI-2k > 0: n=24).

Serum sFas and sFasL concentrations are expressed in pg/ml. UPCR is expressed in g/mmol. Muco. SLEDAI-2k stands for mucocutaneous SLEDAI-2k.

In panels A-B, D and G, horizontal bars indicate the median with [IQR].

* $p < 0.05$

Collectively, the above data indicate that serum sFasL/sFas ratio, and serum sFas to a lesser extent, were related to overall SLE disease activity. Both serum sFas and sFasL/sFas ratio were not only associated with the presence of renal SLE disease, but also correlated with renal disease activity as assessed by the SLEDAI-2k score, and with the key renal disease activity marker UPCR. However, the association between sFasL/sFas ratio and renal SLE disease appeared weaker compared to sFas alone. Hence, both serum sFas and sFasL/sFas ratio may be renal-centric SLE disease activity biomarkers. The present data also suggest a potential association between serum sFasL/sFas ratio and mucocutaneous SLE.

Relationship between serum BAFF and sFas and sFasL in SLE

Analyses of associations between serum BAFF with SLE disease activity are presented in Chapter V.

I next investigated the relationship between serum sFas and sFasL with serum BAFF in SLE. A stratification of SLE patients based on the presence of high vs. non-elevated serum BAFF levels was applied, potentially defining subsets of patients with BAFF-mediated or non-BAFF-mediated SLE, respectively. Serum BAFF concentrations were defined as non-elevated (< 1438.2 pg/ml) or high (≥ 1438.2 pg/ml), as described in the Methods Chapter. I then investigated the relationships between serum sFas, sFasL and sFasL/sFas ratio, with overall and individual organ domains of disease according to this stratification. Of note, in the patient subset with high serum levels of BAFF, only 2.6% (1/39) of patients had active neurological disease, meaning it was not possible to compare the relationship between sFas and sFasL with neurological disease according to serum BAFF levels.

Of note, there was no significant relationship between serum sFas and sFasL concentrations, or sFasL/sFas ratio, with serum BAFF levels in the cohort of HD (data not shown).

Relationship between serum BAFF and sFas in SLE

Serum sFas levels significantly positively correlated with serum BAFF concentrations ($r=0.22$, $p=0.017$; **Figure 8A**). The next analysis aimed to determine whether the observed relationship between BAFF and sFas was specific to a particular SLE phenotype. A positive correlation was observed in the subset of patients with immunological manifestations ($r=0.25$, $p=0.023$; **Figure 8B**), but not in the subset without (data not shown). A strong and significant positive correlation between sFas and BAFF was also observed in the subset of patients with musculoskeletal manifestations ($r=0.77$, $p=0.013$; **Figure 8C**), while only a

non-significant trend was noted in the subset of patients without ($r=0.17$, $p=0.07$) (data not shown). No significant relationship was observed in other studied phenotypic subsets, including renal and mucocutaneous, as well as when comparing active and inactive disease SLE subsets (data not shown). A significant association was found between BAFF and sFas in univariable analysis using linear regression (coef. 0.17, 95% CI 0.04, 0.29, $p=0.008$). Immunological and musculoskeletal manifestations were not found as potential confounders. Of note, after adjusting for neurological and renal active domain in multivariable analysis, only a non-significant trend toward an association was observed (coef. 0.11, 95% CI -0.01, 0.22, $p=0.073$).

Serum sFas was significantly higher in SLE patients with high serum BAFF concentrations compared to those with non-elevated serum BAFF concentrations ($p=0.044$; **Figure 8D**). The relationships between sFas with renal SLE disease noted earlier in this Chapter were only observed in the patient subset with high serum BAFF levels. 35.9% (14/39) of “high BAFF” patients had active renal disease, as opposed to 9.9% (8/81) in the non-elevated BAFF SLE subset ($p=0.001$). Serum sFas levels were significantly higher in patients with active renal disease in the subset of patients with high BAFF levels ($p=0.019$; **Figure 8E**), but not in the subset of patients with non-elevated BAFF levels (**Figure 8F**). Serum sFas levels were also correlated with renal SLEDAI-2k in the subset of patients with high BAFF levels ($r=0.38$, $p=0.017$; **Figure 8G**). Of note, there were only two possible values for renal SLEDAI-2k in the subset of patients with non-elevated BAFF levels, precluding correlation statistical tests. As noted earlier, a significant association was found between sFas with renal SLE disease in univariable analysis using logistic regression. In multivariable analysis, the association remained significant after adjusting for BAFF (OR 1.0002; 95% CI 1.0001, 1.0004; $p=0.003$). This suggests an independent association between sFas with renal SLE. With respect to laboratory markers of renal function, serum sFas

concentrations were significantly correlated with UPCR in the subset with high BAFF levels ($r=0.34$, $p=0.036$; **Figure 8H**), but not in the subset of patients with non-elevated BAFF levels (**Figure 8I**).

In summary, previously noted associations between sFas and active renal SLE were significant only in patients with elevated BAFF, and absent in patients without elevated BAFF, suggesting a previously unsuspected relationship between BAFF and sFas in renal SLE.

Data presented in Figure 8 was reanalysed after excluding one outlier value for serum sFas in SLE. No major difference was observed after excluding this outlier, with all but one analysis presented in Figure 8 remaining statistically significant at the same level (**Appendix 6**). In fact, the exclusion of this outlier led to a non-significant trend toward higher serum sFas levels in SLE patients with high serum BAFF levels compared to those without ($p=0.0695$; **Appendix 6D**), while this difference was initially significant when including this outlier ($p=0.044$; **Figure 8D**). Ultimately, while the exclusion of this outlier moderately affected the relationship shown between serum sFas and BAFF when using BAFF as a categorical variable, the positive correlation between these two analytes still remained statistically significant when analysed as continuous variables (**Appendix 6A**).

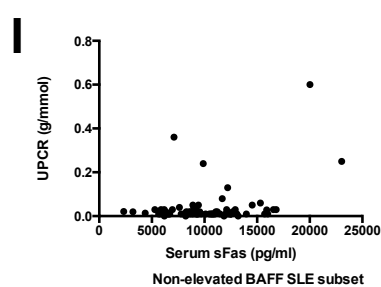
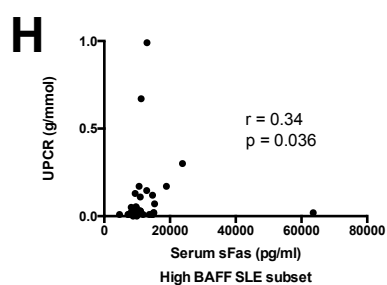
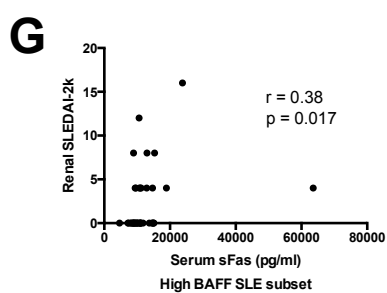
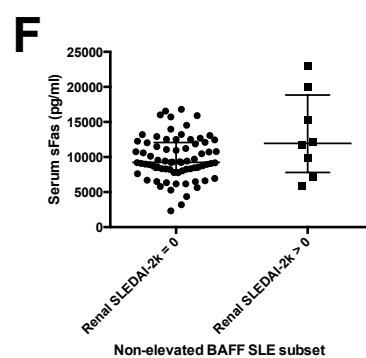
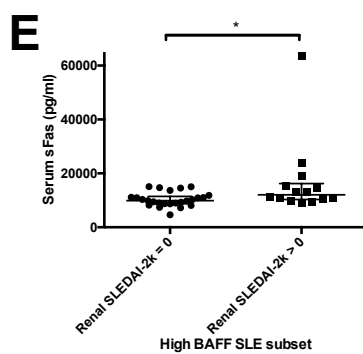
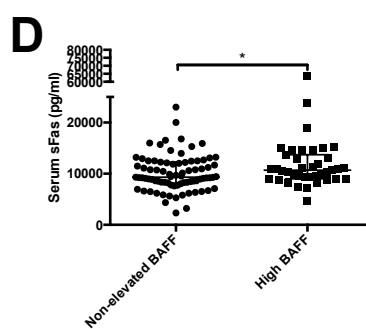
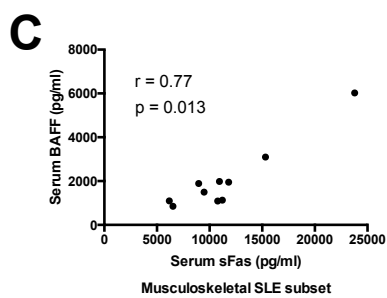
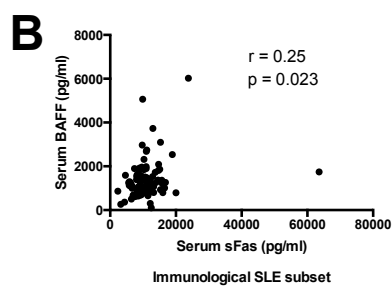
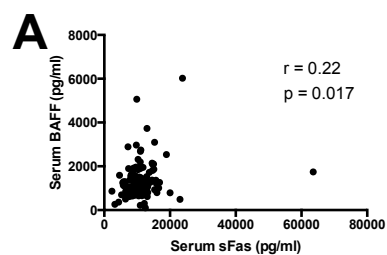


Figure 8. Relationship between serum sFas and BAFF in SLE.

Serum BAFF concentrations were defined as non-elevated (< 1438.2 pg/ml) or high (≥ 1438.2 pg/ml), as per the Methods Chapter. Renal SLE disease activity was assessed by the SLEDAI-2k score. (A) Correlation between serum sFas and BAFF concentrations in SLE ($n=120$). Correlation between serum sFas and BAFF concentrations in (B) immunological ($n=86$) and (C) musculoskeletal SLE subsets ($n=10$). (D) Serum sFas concentrations according to serum BAFF concentrations in SLE (Non-elevated BAFF: $n=81$; High BAFF: $n=39$). Serum sFas concentrations according to renal SLE disease activity in the (E) high (Renal SLEDAI-2k = 0: $n=25$; Renal SLEDAI-2k > 0 : $n=14$) and (F) non-elevated serum BAFF SLE subsets (Renal SLEDAI-2k = 0: $n=73$; Renal SLEDAI-2k > 0 : $n=8$). (G) Correlation between serum sFas concentrations and renal SLEDAI-2k in the high serum BAFF SLE subset ($n=39$). Correlation between serum sFas concentrations and UPCR in the (H) high ($n=38$) and (I) non-elevated serum BAFF SLE subsets ($n=77$). Serum sFas and BAFF concentrations are expressed in pg/ml. UPCR is expressed in g/mmol. In panels D-F, horizontal bars indicate the median with [IQR].

* $p<0.05$

Relationship between serum BAFF and sFasL in SLE

In contrast to my findings in relation to serum sFas, no significant relationship emerged between serum BAFF and sFasL in SLE (**Figure 9A-B**). No significant relationship of sFasL emerged with either overall or phenotypic clinical manifestations when stratifying patients according to serum BAFF levels (data not shown).

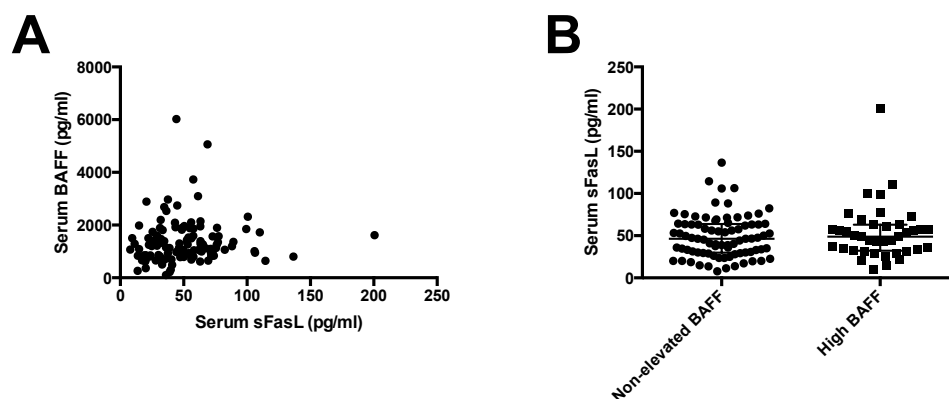


Figure 9. Relationship between serum sFasL and BAFF in SLE.

Serum BAFF concentrations were defined as non-elevated (< 1438.2 pg/ml) or high (≥ 1438.2 pg/ml), as per the Methods Chapter. (A) Correlation between serum sFasL and BAFF concentrations in SLE ($n=120$). (B) Serum sFasL concentrations according to serum BAFF concentrations in SLE (Non-elevated BAFF: $n=81$; High BAFF: $n=39$).

Serum sFasL and BAFF concentrations are expressed in pg/ml.

In panel B, horizontal bars indicate the median with [IQR].

Relationship between serum BAFF and sFasL/sFas ratio in SLE

Similarly to serum sFasL, no significant relationship was observed between serum BAFF and sFasL/sFas ratio (**Figure 10A-B**). In contrast to my findings in relation to sFas, the relationship between sFasL/sFas ratio and overall SLE disease activity observed in the overall cohort was limited to the SLE subset with non-elevated serum BAFF levels. sFasL/sFas ratio was significantly lower in SLE patients with active disease compared to those with inactive disease in the non-elevated BAFF subset ($p=0.044$; **Figure 10C**), but not in the subset with high BAFF levels (**Figure 10D**). Moreover, sFasL/sFas ratio was significantly correlated with SLEDAI-2k in the subset with non-elevated serum BAFF levels ($r=-0.23$, $p=0.044$; **Figure 10E**) but not in the subset with high BAFF levels (**Figure 10F**). The potential for BAFF being a confounder for the association between sFasL/sFas ratio and overall SLE disease activity could not be tested in multivariable analysis, as no significant association was observed in univariable analysis using logistic regression between sFasL/sFas ratio and overall SLE disease activity. In order to correct for multiple testing, a Bonferroni correction has been applied, generating an adjusted p value of 0.025 as the threshold for statistical significance for this presented association. The relationships between sFasL/sFas ratio with overall SLE disease activity observed in the patient subset with non-elevated BAFF was not significant after Bonferroni correction.”

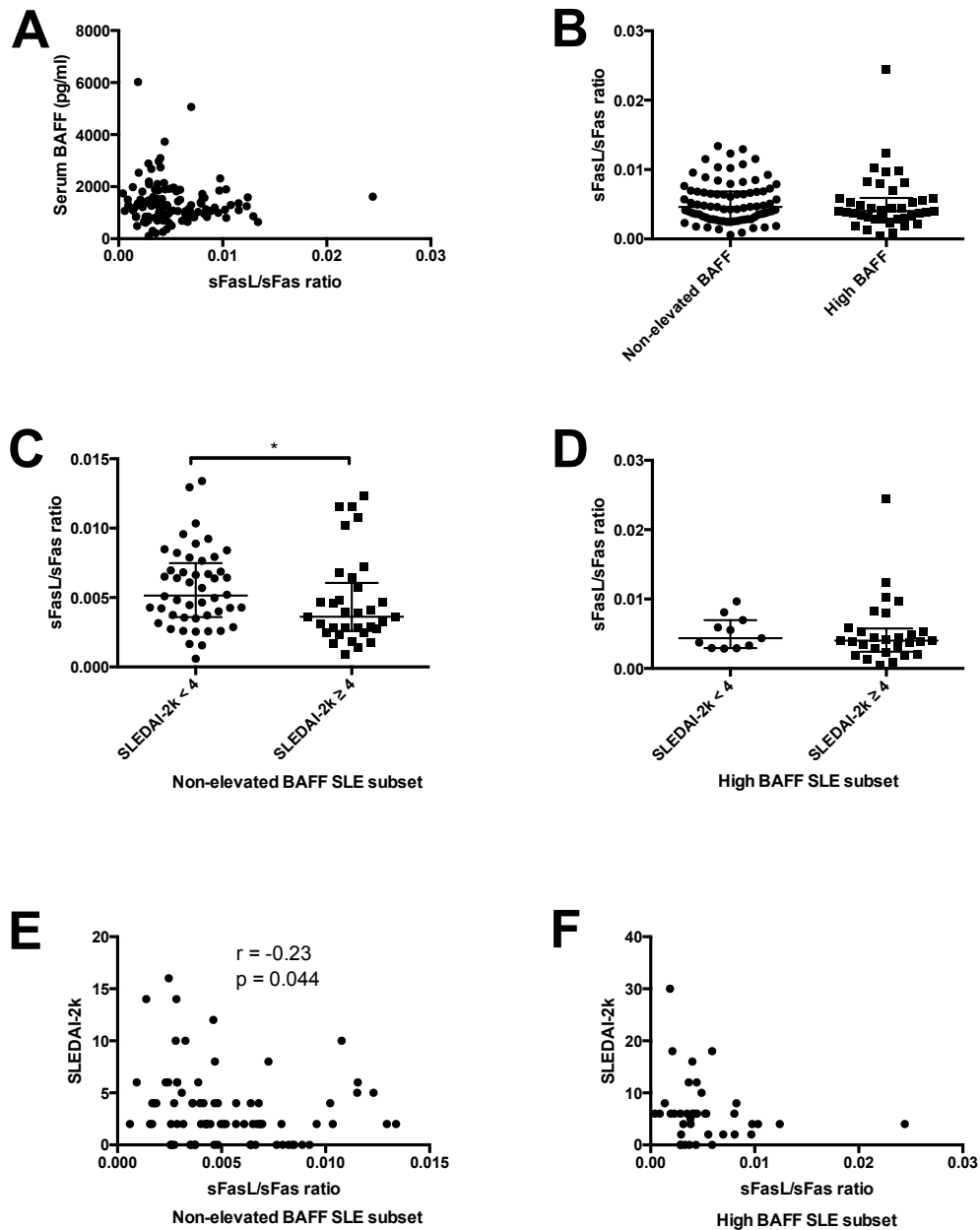


Figure 10. Relationship between sFasL/sFas ratio and BAFF in SLE.

Serum BAFF concentrations were defined as non-elevated (< 1438.2 pg/ml) or high (≥ 1438.2 pg/ml), as per the Methods Chapter. Overall SLE disease activity was assessed by the SLEDAI-2k score. (A) Correlation between sFasL/sFas ratio and BAFF concentrations in SLE ($n=120$). (B) sFasL/sFas ratio according to serum BAFF concentrations (Non-elevated BAFF: $n=81$; High BAFF: $n=39$). sFasL/sFas ratio according to SLE disease activity in the (C) non-elevated (SLEDAI-2k < 4 : $n=48$; SLEDAI-2k ≥ 4 : $n=33$) and (D) high serum BAFF SLE subsets (SLEDAI-2k < 4 : $n=11$; SLEDAI-2k ≥ 4 : $n=28$). Correlation between sFasL/sFas ratio and SLEDAI-2k in the (E) non-elevated ($n=81$) and (F) high serum BAFF subsets ($n=39$).

Serum sFas and sFasL concentrations are expressed in pg/ml.

In panels B-D, horizontal bars indicate the median with [IQR].

* $p < 0.05$

Of particular interest was the emergence of a significant relationship between sFasL/sFas ratio with mucocutaneous SLE disease in patients with non-elevated serum BAFF levels, whereas only a non-significant trend was observed in the whole SLE cohort. 18.5% (15/81) of patients in the non-elevated BAFF subset had active mucocutaneous disease as opposed to 23.1% (9/39) in the high BAFF subset. In the subset of patients with non-elevated serum BAFF, sFasL/sFas ratio was significantly lower in SLE patients with active mucocutaneous disease compared to those without ($p=0.016$; **Figure 11A**). The potential for BAFF being a confounder for the association presented between sFasL/sFas ratio and mucocutaneous SLE disease could not be tested in multivariable analysis, as no significant association was observed in univariable analysis using logistic regression between sFasL/sFas ratio and mucocutaneous SLE disease. In order to correct for multiple testing, a Bonferroni correction has been applied, generating an adjusted p value of 0.025 as the threshold for statistical significance for these presented associations. The relationships between sFasL/sFas ratio with mucocutaneous disease observed in the patient subset with non-elevated BAFF remained significant after Bonferroni correction. Of note, in the non-elevated BAFF subset, sFasL/sFas ratio was not correlated with the SLEDAI-2k after subtraction of mucocutaneous SLEDAI-2k, suggesting that the mucocutaneous component of the SLEDAI-2k drove the observed association between sFasL/sFas ratio and SLEDAI-2k. Thus, sFasL/sFas ratio appears to be primarily related to mucocutaneous disease in the SLE subset characterized by non-elevated BAFF levels. No significant association between sFasL/sFas ratio and mucocutaneous SLE manifestations was observed in the high BAFF SLE subset (**Figure 11B**).

As was observed for serum sFas, the relationship between sFasL/sFas ratio and renal SLE was only observed in the subset with high serum BAFF. sFasL/sFas ratio was significantly lower in SLE patients with active renal disease compared to those without, in

the subset with high serum BAFF ($p=0.03$; **Figure 11C**), but not in the non-elevated BAFF subset (**Figure 11D**). sFasL/sFas ratio was also correlated with renal SLEDAI-2k in the subset with high serum BAFF ($r=-0.35$, $p=0.029$; **Figure 11E**). Of note, as for sFas, there were only two possible values for renal SLEDAI-2k in the non-elevated BAFF SLE subset, precluding correlation statistical tests. As reported earlier, a significant association was also found between sFasL/sFas ratio and renal disease activity in univariable analysis using logistic regression. BAFF was not found to be a potential confounder for this association. sFasL/sFas ratio was also significantly correlated with UPCR in SLE patients subset with high serum BAFF ($r=-0.36$, $p=0.029$; **Figure 11F**), but not in the non-elevated BAFF subset (**Figure 11G**).

In conclusion, as was the case for serum sFas, significant relationships of sFas/sFasL ratio with active renal SLE were observed only in patients stratified according to elevated serum BAFF.

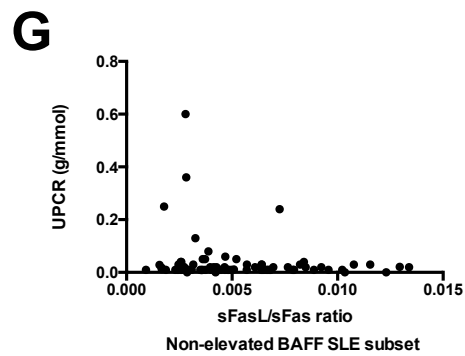
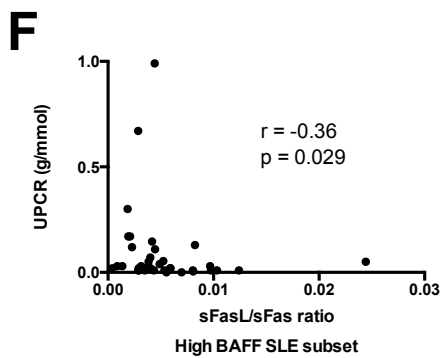
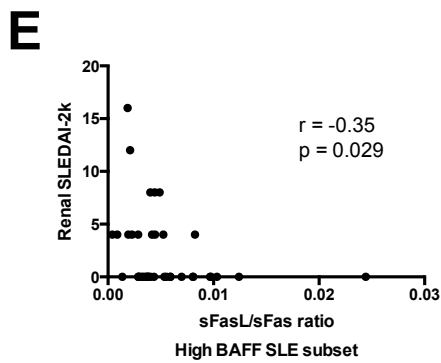
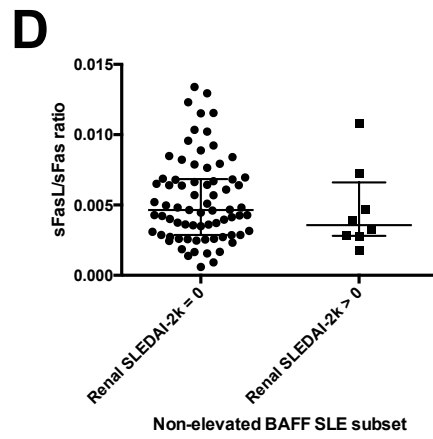
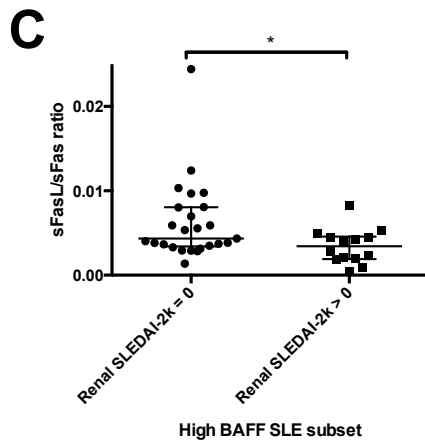
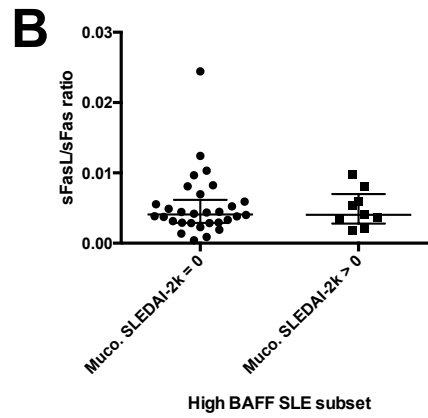
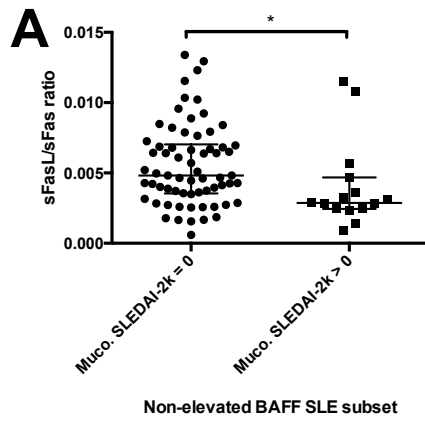


Figure 11. Relationship between sFasL/sFas ratio and BAFF in SLE.

Serum BAFF concentrations were defined as non-elevated (< 1438.2 pg/ml) or high (≥ 1438.2 pg/ml), as per the Methods Chapter. Mucocutaneous and renal SLE disease activity was assessed by the SLEDAI-2k score. sFasL/sFas ratio according to mucocutaneous SLEDAI-2k in the (A) non-elevated (Muco. SLEDAI-2k = 0: n=66; Muco. SLEDAI-2k > 0 : n=15) and (B) high serum BAFF SLE subsets (Muco. SLEDAI-2k = 0: n=30; Muco. SLEDAI-2k > 0 : n=9). sFasL/sFas ratio according to renal SLEDAI-2k in the (C) high (Renal SLEDAI-2k = 0: n=25; Renal SLEDAI-2k > 0 : n=14) and (D) non-elevated serum BAFF SLE subsets (Renal SLEDAI-2k = 0: n=73; Renal SLEDAI-2k > 0 : n=8). (E) Correlation between sFasL/sFas ratio with renal SLEDAI-2k in the high serum BAFF SLE subset (n=39). Correlation between sFasL/sFas ratio with UPCR in the (F) high (n=38) and (G) non-elevated serum BAFF SLE subset (n=77).

Serum sFas and sFasL concentrations are expressed in pg/ml. UPCR is expressed in g/mmol. Muco. SLEDAI-2k stands for mucocutaneous SLEDAI-2k.

In panels A-D, horizontal bars indicate the median with [IQR].

* $p < 0.05$

Collectively, these data showed a relationship between serum BAFF and sFas in SLE, which was selective to patients with immunological and musculoskeletal manifestations. These data also showed that sFasL/sFas ratio was related to mucocutaneous SLE disease phenotype, potentially in the subset characterized by a non-elevated BAFF. Potentially most importantly, the relationships between both serum sFas and sFasL/sFas ratio with renal SLE disease activity appeared to be specific to potentially BAFF-mediated SLE disease.

Influence of ethnicity on serum sFas and sFasL and their relationships with SLE disease activity

Influence of ethnicity on sFas in SLE

No significant difference was noted regarding serum sFas concentrations according to ethnicity in both SLE and HD (**Figure 12A-B**). The relationship between serum sFas and overall SLE disease activity appeared influenced by ethnicity. A significant difference in serum sFas according to disease activity was seen in Caucasian ($p=0.049$; **Figure 12C**) but not in Asian (**Figure 12D**) patients. A significant association was found between sFas and overall SLE disease activity in univariable analysis using logistic regression (OR 1.0001; 95% CI 1.00002, 1.0003; $p=0.02$) and multivariable models adjusted for age (OR 1.0002; 95% CI 1.00004, 1.0003; $p=0.012$). Of note, ethnicity was not a confounder for this association.

The relationship between sFas and renal SLE disease activity observed in the overall cohort was not observed in Caucasian subjects ($p=0.079$; **Figure 12E**), but was significant in Asians ($p=0.003$; **Figure 12F**). Similarly, only a non-significant trend towards a correlation between sFas and renal SLEDAI-2k was observed in Caucasian patients ($r=0.24$, $p=0.07$; **Figure 12G**), but a significant trend was observed in the Asian subset ($r=0.38$, $p=0.003$; **Figure 12H**). As noted earlier, a significant association was found between sFas with renal disease activity in univariable analysis using logistic regression. Ethnicity was not a confounder for this association. However, due to the limited number of SLE patients with active renal disease in the Caucasian subset compared to Asians, a potential underestimation of such relationship in Caucasians cannot be excluded.

Data presented in Figure 12 was reanalysed after excluding one outlier value for serum sFas in SLE. No major difference in the results or conclusions was observed after

excluding this outlier, with all data analysis presented in Figure 12 remaining statistically significant at the same level (**Appendix 7**).

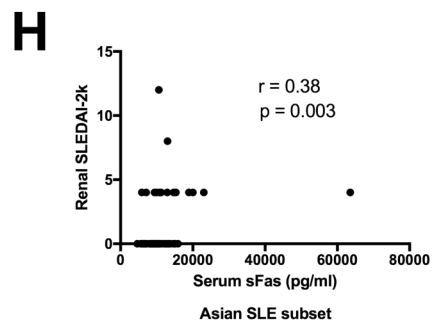
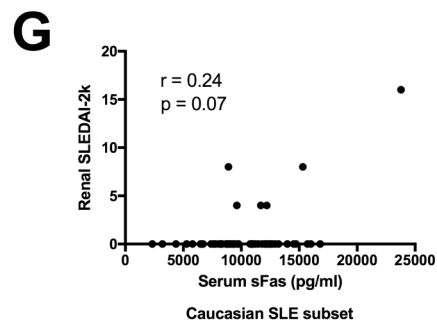
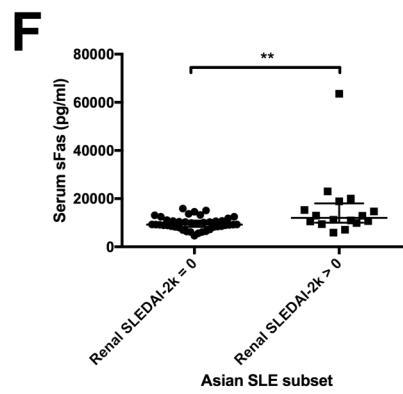
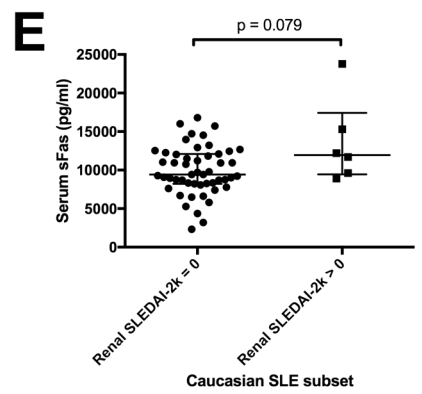
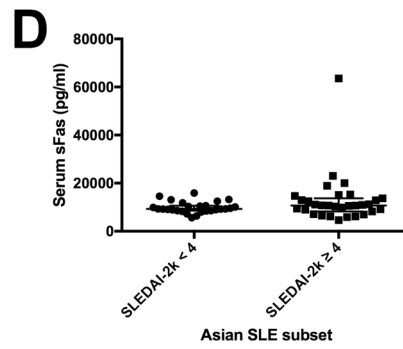
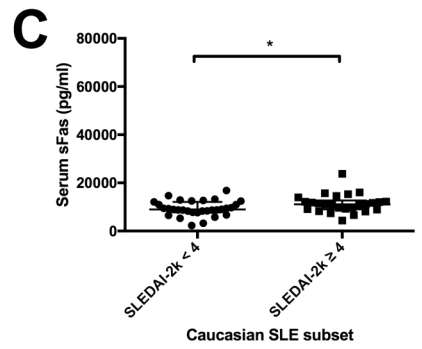
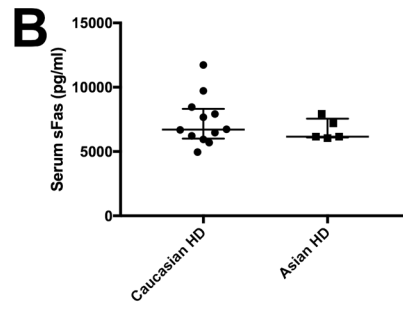
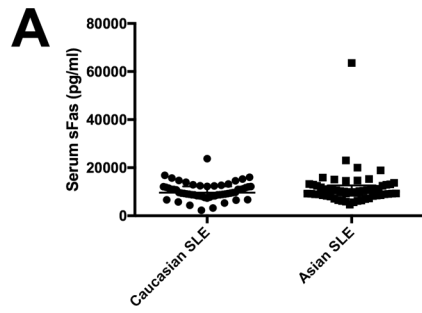


Figure 12. Influence of ethnicity on the association between serum sFas with SLE.

Overall, renal and musculoskeletal SLE disease activity was assessed by the SLEDAI-2k score. (A) Serum sFas according to ethnicity [Caucasian (n=57) vs. Asian (n=58)] in SLE. (B) Serum sFas according to ethnicity [Caucasian (n=12) vs. Asian (n=5)] in HD. Serum sFas concentrations according to overall disease activity in (C) Caucasian (SLEDAI-2k < 4: n=31; SLEDAI-2k ≥ 4: n=26) and (D) Asian SLE subsets (SLEDAI-2k < 4: n=27; SLEDAI-2k ≥ 4: n=31). Serum sFas concentrations between patients with inactive and active renal SLE disease in (E) Caucasian (Renal SLEDAI-2k = 0: n=51; Renal SLEDAI-2k > 0: n=6) and (F) Asian SLE subsets (Renal SLEDAI-2k = 0: n=42; Renal SLEDAI-2k > 0: n=16). Correlation between serum sFas concentrations and renal SLEDAI-2k in (G) Caucasian (n=57) and (H) Asian SLE subsets (n=58).

Serum sFas concentrations are expressed in pg/ml. Musculo. SLEDAI-2k stands for musculoskeletal SLEDAI-2k.

In panels A-F, horizontal bars indicate the median with [IQR].

** $p < 0.01$; * $p < 0.05$

Influence of ethnicity on sFasL in SLE

Serum sFasL was significantly higher in Asian compared to Caucasian SLE patients ($p=0.032$; **Figure 13A**). In contrast, there was no significant difference in serum sFasL according to ethnicity in HD (**Figure 13B**). No significant relationship emerged between sFasL and overall SLE disease activity according to ethnicity (**Figure 13C-F**). No significant ethnicity relationship emerged with the associations of sFasL with phenotypic SLE manifestations (data not shown).

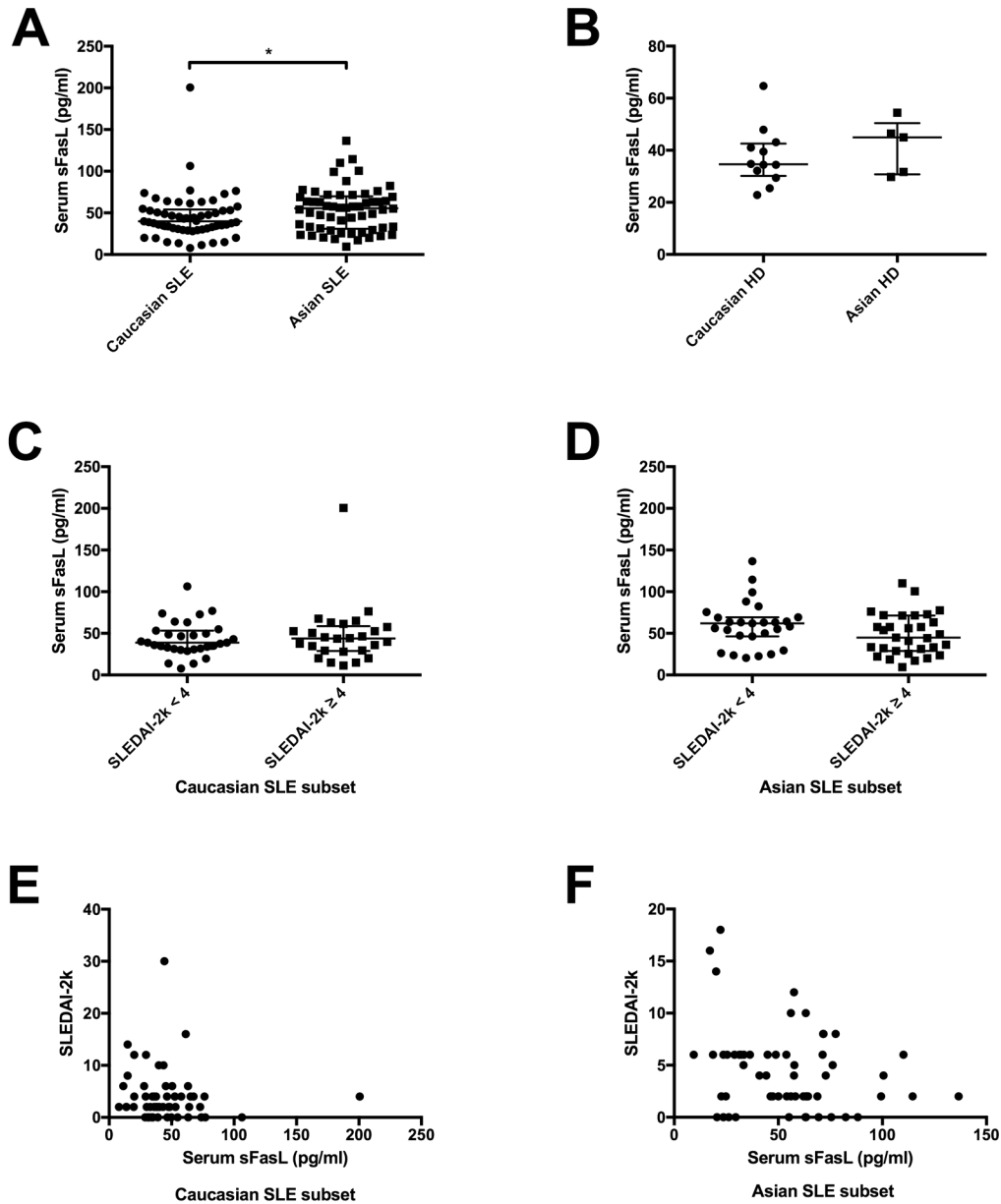


Figure 13. Influence of ethnicity on the associations between serum sFasL with SLE.

Overall SLE disease activity was assessed by the SLEDAI-2k score. (A) Serum sFasL according to ethnicity [Caucasian (n=57) vs. Asian (n=58)] in SLE. (B) Serum sFasL according to ethnicity [Caucasian (n=12) vs. Asian (n=5)] in HD, Serum sFasL concentrations according to overall SLE disease activity in (C) Caucasian (SLEDAI-2k < 4: n=31; SLEDAI-2k ≥ 4: n=26) and (D) Asian SLE subsets (SLEDAI-2k < 4: n=27; SLEDAI-2k ≥ 4: n=31). Correlation between serum sFasL concentrations with overall SLE disease activity in (E) Caucasian (n=57) and (F) Asian SLE subsets (n=58).

Serum sFasL concentrations are expressed in pg/ml.

In panels A-D, horizontal bars indicate the median with [IQR].

* $p < 0.05$

Influence of ethnicity on sFasL/sFas ratio in SLE

No significant difference was noted regarding sFasL/sFas ratio according to ethnicity in both SLE and HD (**Figure 14A-B**). In contrast to observations regarding ethnicity and sFas, the relationship between sFasL/sFas ratio and overall disease activity was not significant in Caucasians (**Figure 14C**), but was in Asians ($p=0.039$; **Figure 14D**) (**Figure 14E-F**). The potential for ethnicity to be a confounder for the association between sFasL/sFas ratio and overall SLE disease activity could not be tested in multivariable analysis, as no significant association was observed in univariable analysis using logistic regression between sFasL/sFas ratio and overall SLE disease activity. In order to correct for multiple testing, a Bonferroni correction has been applied, generating an adjusted p value of 0.025 as the threshold for statistical significance for this presented association. The relationships between sFasL/sFas ratio and overall SLE disease activity in Asians was not significant after Bonferroni correction.

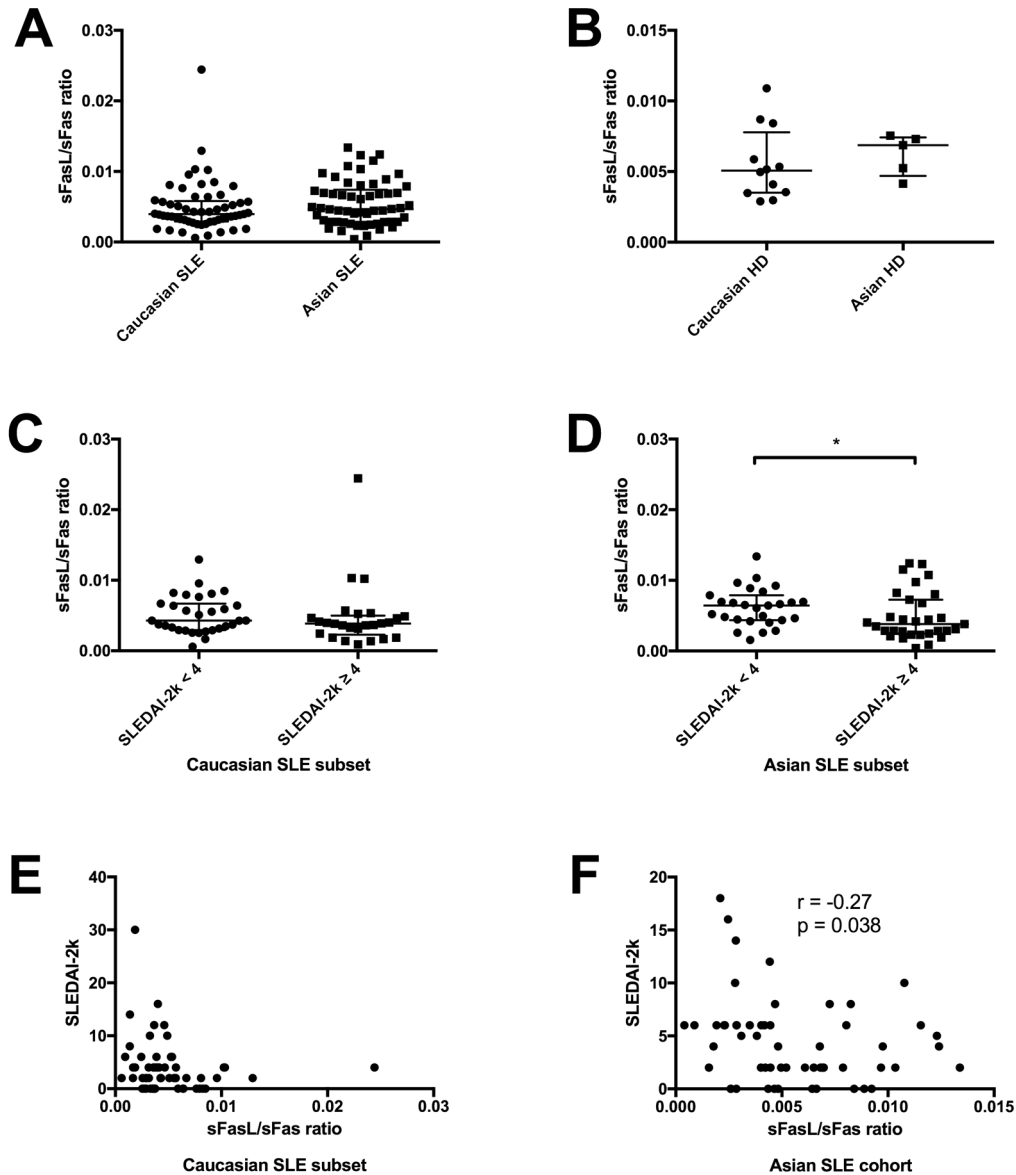


Figure 14. Influence of ethnicity on the association between sFasL/sFas ratio with SLE.

Overall SLE disease activity was assessed by the SLEDAI-2k score. (A) sFasL/sFas ratio according to ethnicity [Caucasian (n=57) vs. Asian (n=58)] in SLE. (B) sFasL/sFas ratio according to ethnicity [Caucasian (n=12) vs. Asian (n=5)] in HD, sFasL/sFas ratio according to overall SLE disease activity in (C) Caucasian (SLEDAI-2k < 4: n=31; SLEDAI-2k ≥ 4: n=26) and (D) Asian SLE subsets (SLEDAI-2k < 4: n=27; SLEDAI-2k ≥ 4: n=31). Correlation between sFasL/sFas ratio with overall SLE disease activity in (E) Caucasian (n=57) and (F) Asian SLE subsets (n=58).

Serum sFas and sFasL concentrations are expressed in pg/ml.

In panels A-D, horizontal bars indicate the median with [IQR].

* $p < 0.05$

As for serum sFas, the relationship between sFasL/sFas ratio and renal SLE disease activity appeared dependent on ethnicity. No relationship was observed in Caucasian (**Figure 15A**), but a significant relationship was present in Asians ($p=0.006$; **Figure 15B**) (**Figure 15C-D**). As reported earlier, a significant association was also found between sFasL/sFas ratio and renal disease activity in univariable analysis using logistic regression. Ethnicity was not a confounder for this association. Once again, the lower prevalence of active renal disease in Caucasian subjects means that the existence of a relationship between sFasL/sFas ratio and renal SLE in Caucasians cannot be excluded.

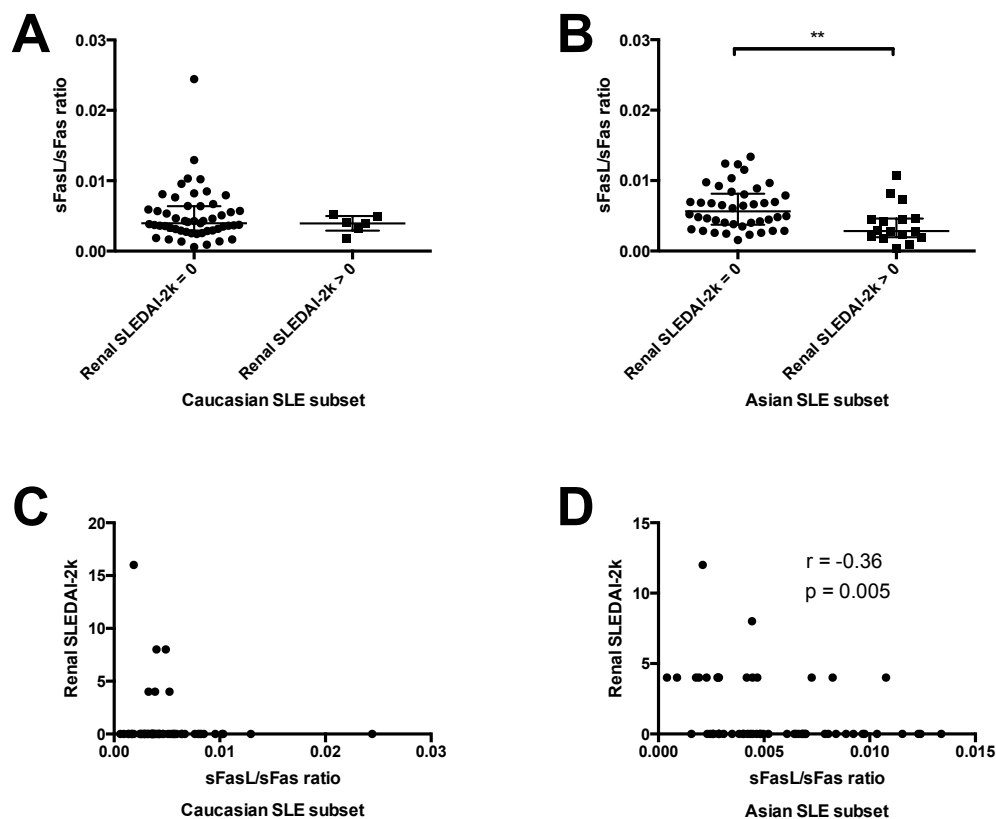


Figure 15. Influence of ethnicity on the association between sFasL/sFas ratio with organ-specific SLE.

Renal and mucocutaneous SLE disease activity was assessed by the SLEDAI-2k score. sFasL/sFas ratio according to renal SLE disease activity in (A) Caucasian (Renal SLEDAI-2k = 0: $n=51$; Renal SLEDAI-2k > 0: $n=6$) and (B) Asian subsets (Renal SLEDAI-2k = 0: $n=42$; Renal SLEDAI-2k > 0: $n=16$). Correlation between sFasL/sFas ratio and renal SLEDAI-2k in (C) Caucasian ($n=57$) and (D) Asian subsets ($n=58$).

Serum sFas and sFasL concentrations are expressed in pg/ml.

In panels A-B, horizontal bars indicate the median with [IQR].

** $p < 0.01$

Influence of ethnicity on the relationship between sFas and BAFF

Analyses of the influence of ethnicity on the relationship between serum BAFF and SLE are presented in Chapter V.

The next question was to determine whether the observed relationship between BAFF and sFas in SLE was affected by ethnicity. Serum sFas was significantly positively correlated with serum BAFF concentrations in Caucasians ($r=0.27$, $p=0.04$; **Figure 16A**), but not in Asians (**Figure 16B**). As noted earlier, a significant association was found between BAFF and sFas in univariable analysis using linear regression. Ethnicity was not a confounder. Of note, as noted earlier, after adjusting for neurological and renal domains in multivariable analysis, only a non-significant trend toward an association was observed. In the Caucasian SLE subset, there was a non-significant trend toward a correlation between serum sFasL and BAFF concentrations ($r=0.26$, $p=0.051$), which was not observed in Asians (data not shown).

Data presented in Figure 16 was reanalysed after excluding one outlier value for serum sFas in SLE. No major difference in the results or conclusions was observed after excluding this outlier, with all data analysis presented in Figure 16 remaining statistically significant at the same level (**Appendix 8**).

Collectively, the exclusion of this outlier for serum sFas concentration in one SLE patient, did not lead to major difference in data analysis and conclusions drawn regarding analysis of relationship of serum sFas and SLE presented in Figures 5, 8, 12 and 16 in this thesis.

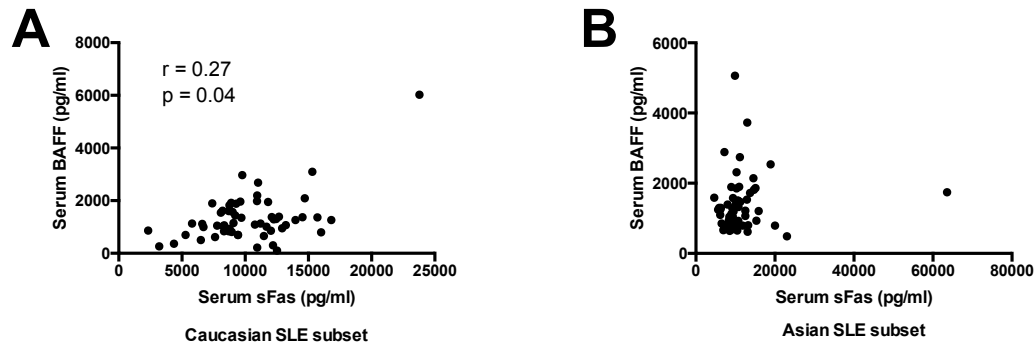


Figure 16. Influence of ethnicity on the relationship between sFas and BAFF in SLE.

Serum BAFF concentrations were defined as non-elevated (< 1438.2 pg/ml) or high (≥ 1438.2 pg/ml), as per the Methods Chapter. Correlation between serum sFas and BAFF concentrations in (A) Caucasian ($n=57$) and (B) Asian patients ($n=58$).

Serum sFas and BAFF concentrations are expressed in pg/ml.

Impact of anti-BAFF and anti-CD20 therapies on serum sFas and sFasL concentrations in SLE

No significant difference in serum sFas, sFasL, or sFasL/sFas ratio, was observed between SLE patients receiving any anti-BAFF or anti-CD20 therapy, and those not (data not shown).

4. Discussion

The present study primarily aimed to evaluate the potential of combining the analysis of serum sFas and sFasL with BAFF measurement in the stratification of SLE patients. This study also aimed to further characterize the clinical associations of sFasL/sFas ratio in SLE.

Firstly, the present study confirmed increased levels of serum sFas and sFasL in SLE compared to healthy individuals. In line with most published studies,[4, 7, 8, 17, 19, 27, 29, 30, 37, 52, 68, 72, 81, 104, 114, 118, 130, 132-134, 170-172, 174, 176, 179, 180] significantly higher concentrations of serum sFas were found in SLE patients compared to healthy subjects. With respect to serum sFasL, higher levels in SLE compared to healthy subjects were also noted, in line with most published studies.[7, 162, 168, 172, 176] In the present study, sFasL/sFas ratio was not significantly different between SLE and HD, in contrast to the sole study which investigated the use of this index in SLE.[176] Collectively, these data confirm the increased levels of both serum sFas and sFasL in SLE compared to healthy individuals.

I found serum sFas was related to overall SLE disease activity, in line with some previous published studies.[17, 68, 81, 132, 133, 168, 179] Significantly, a positive relationship was observed between serum sFas and SLE renal disease activity. These data are in line with published studies, where serum sFas was reported to be associated with proteinuria,[132] LN,[19, 164] and renal organ damage.[68, 179] Serum sFas may reflect not only its systemic production, but also local production in various organs, such as *in situ* kidney production,[68] as well as being affected by kidney function.[30] It is noteworthy that serum levels of sFas were related to renal function, irrespective of the disease responsible for kidney dysfunction.[137] Although the present study cannot demonstrate any causal link, one could speculate that, since sFas acts as a decoy for Fas-mediated apoptosis, a reduction in available mFasL through an increase in sFas would lead to less apoptosis.[29] The impact on

both infiltrated autoreactive immune cells, and on renal resident cells such as glomerular cells, may differ.[107] It is also important to note that while sFas can modulate apoptotic pathways via the Fas/FasL system, some cells undergo apoptosis via other pathways, such as TNF-related apoptosis-inducing ligand (TRAIL) via its death receptors TRAIL-R1 (DR4) and TRAIL-R2 (DR5) (reviewed in[50]). However, in the high serum sFas environment of AID, the potential presence of oligomeric sFas[42, 125, 154, 164] could lead to proapoptotic effect on mFasL-bearing immune cells and resident renal cells. The assay used in the present study did not discriminate between monomeric and oligomeric sFas forms.

A negative relationship was found between levels of serum sFas and neurological disease activity. Few published studies on serum sFas in SLE have described an association between serum sFas and CNS involvement.[132] As sFas acts as a decoy for mFasL, low sFas levels could leave more mFasL available for the Fas/FasL apoptosis pathway on autoreactive immune cells. If sFasL also plays a protective role in neurological SLE, then less sFas would also leave more sFasL available for non-apoptotic Fas/FasL effects, such as brain repair. Interestingly, *in situ* CNS production of sFas in multiple sclerosis (MS) has been suggested.[20, 32] Whether *in situ* production of sFas also occurs in neurological SLE remains unknown.

No significant relationship was observed between serum sFasL and overall SLE disease activity. However, as for sFas, a significant negative relationship was noted between serum sFasL and neurological SLE. As mentioned earlier, there are few published data on the relationship between serum sFasL and SLE disease activity, with most focussing on the relationship with overall disease activity, not phenotypic manifestations. One study showed a positive association between serum sFasL and SLE disease activity as assessed by the SLEDAI score.[168] While no causality can be determined from the present data, and extreme caution should be taken when interpreting these data in light of the small clinical

CNS SLE subset, it could, however, be speculated that in the case of neurological SLE, decreased serum sFasL may indicate Fas/FasL dysregulation in the setting of inflammation in the brain. Low levels of sFasL may leave more mFas available to interact with mFasL on autoreactive immune cells. Based on the work from Zhang *et al.* mentioned in Chapter I,[197] suggesting a potential sFasL threshold between proliferation and apoptosis, it could be speculated that a threshold in sFasL may regulate mFas function in brain repair. Again, one of the limitations of the present study is the small number of SLE patients with active neurological disease (n=4). A larger cohort focused on neurological SLE manifestations, including quantification of both serum and cerebrospinal fluid (CSF) sFas/sFasL levels, would be of interest to confirm their potential role in neurological SLE, and the usefulness of their measurement as neurological-centric SLE biomarkers and potentially therapeutic targets.

As mentioned earlier, only one previous study investigated the measurement of sFasL/sFas ratio, in a small sample of 15 SLE patients.[176] In the present study, sFasL/sFas ratio was negatively related to overall SLE disease activity, in a slightly weaker manner when compared to sFas alone. At the organ level, sFasL/sFas ratio emerged as a potential renal-centric SLE biomarker, albeit less significant than serum sFas alone. Thus, this study is the first study showing significant clinical associations of sFasL/sFas ratio in SLE, particularly with renal manifestations.

I report here a positive relationship between serum BAFF and sFas, but not sFasL, in human SLE. This relationship was also found in immunological and musculoskeletal SLE subsets, suggesting that this relationship may be more specific to these particular phenotypic manifestations of SLE. The relationship found between serum BAFF and sFas in SLE constitutes a novel finding in this thesis. As mentioned earlier, Figgett *et al.* recently showed in a SLE/SS-like mouse model, that BAFF, when overexpressed, could lead to dysregulation

of the Fas/FasL system, characterised by a defect in mFasL up-regulation, leading to impaired apoptosis of innate-like MZ B cells known to include autoreactive specificities.[49] This observed positive relationship between serum BAFF with sFas underlines the relationship of the Fas/FasL system with the BAFF/APRIL system. It could be speculated that increased sFas in a high-BAFF environment may play a role in dysregulation of the Fas/FasL system.

As outlined earlier, belimumab therapy was reported to be efficient in a subset of adult SLE patients characterised by active disease and positive autoAbs,[54, 109] underlining the presence of anti-BAFF resistant subsets. In the studies presented here, when stratifying SLE patients according to serum BAFF levels, serum sFas and sFasL/sFas ratio both appeared to be renal-centric SLE biomarkers specifically in the high BAFF subset. Strikingly, a relationship between sFasL/sFas ratio and mucocutaneous SLE emerged in patients with non-elevated BAFF, while neither serum sFas nor sFasL were individually related to this phenotypic SLE manifestation. Previous publications on the relationship between soluble form of Fas and/or FasL and mucocutaneous SLE manifestations are limited. Serum sFas levels have been reported increased in cutaneous lupus erythematosus (CLE) in some studies,[5, 55], while not in others.[179] Serum sFas levels have been also reported positively associated with oral ulcers.[164] Published data appear inconsistent with respect to elevated sFas with mucocutaneous manifestations in SLE, which could be explained by the various subtypes of LE skin disease (acute, subacute, and chronic). Associations of serum soluble components of the BAFF/APRIL in combination with components of the Fas/FasL system with clinical phenotypes in SLE constitute novel findings in this thesis.

One could speculate that non-elevated serum BAFF levels may reflect a subset with low overall disease activity level, since some studies have shown that serum BAFF might be an overall SLE disease activity biomarker (reviewed in[184]). However, it could also be

speculated that SLE patients differ according to the immunological pathway(s) involved in their disease, which in turn may lead to a particular phenotypic presentation. In the setting of non-elevated BAFF, other pathway(s) may be operative. It could then be speculated that if an SLE subset with non-elevated serum BAFF levels is characterized by non-BAFF-mediated SLE disease, then this relationship with sFasL/sFas ratio may underline a potential key role for the Fas/FasL system in this SLE subset. Thus, the combination of serum BAFF with serum sFas and/or sFasL may help to better assess SLE patients disease activity, and suggests a strategy for therapeutic intervention in patients with non-BAFF-mediated SLE, where soluble component of the Fas/FasL system may be a therapeutic target.

Using results from the work presented here, I suggest a tree algorithm whereby SLE patients could be first stratified according to serum BAFF levels, then soluble components of the Fas/FasL system could be used as biomarkers for SLE assessment and therapeutic intervention (**Figure 17**). This decision tree is proposed as a hypothetical tool to be applied in patients diagnosed with SLE. However, it is acknowledged that the findings of this thesis are insufficient to support clinical application without future validations studies.

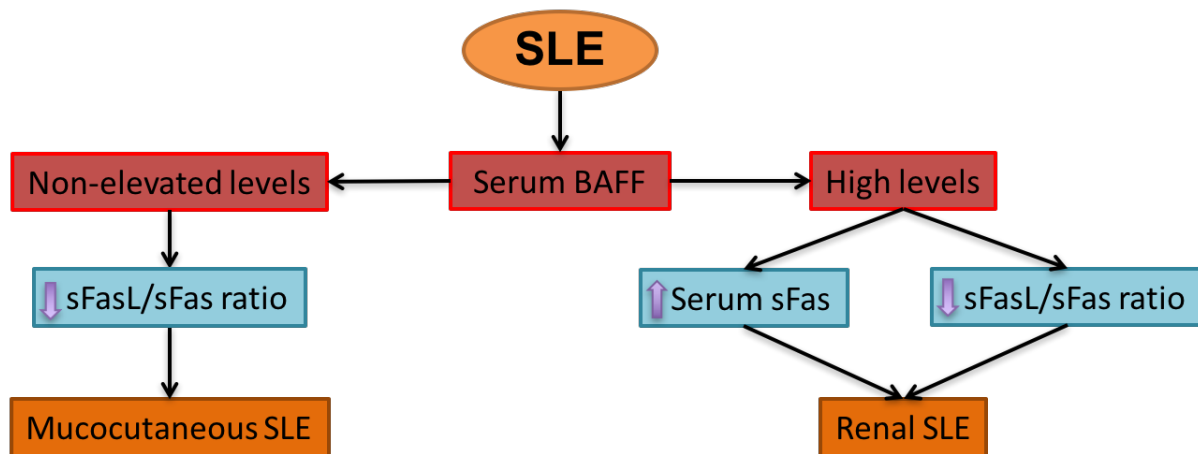


Figure 17. Decisional tree algorithm using SLE patient stratification by serum BAFF levels for emerging phenotypic SLE biomarker from the Fas/FasL system.

BAFF: B cell-activating factor from the tumour necrosis factor family; FasL: Fas ligand; sFas: soluble Fas; sFasL: soluble FasL; SLE: Systemic lupus erythematosus.

All data on influence of ethnicity on serum sFas and sFasL and their relationships with SLE disease activity are novel findings in this thesis. Ethnicity appears to influence serum levels of sFasL in SLE in the present study, underlining potential differences in Fas/FasL pathway involvement in SLE pathogenesis between ethnic groups. Further investigation is needed to determine whether the difference in serum sFasL levels observed between Asian and Caucasian SLE patients may play a role in the reported ethnic difference in SLE disease prevalence and severity (reviewed in[182, 184]). Furthermore, some relationships between soluble components of the Fas/FasL system with overall and organ disease activity were influenced by patients' ethnicity in the present study. In fact, the relationship between serum sFas and overall SLE disease activity was influenced by ethnicity, in that the relationship to disease activity was specific to Caucasian ethnicity. In contrast, the relationship between serum sFas and SLE renal disease appears influenced by Asian ethnicity. Finally, the observed relationship between serum BAFF and sFas appeared

dependent on ethnicity. Hence, stratifying SLE patients according to ethnicity may further improve the use of SLE biomarkers to direct therapy. Further larger prospective multiethnic studies would be of interest, including more ethnic groups.

Caveats to the interpretation of the present data apply. First, although this study was prospective, it was mainly monocentric. Secondly, the HD cohort was of small sample size and also monocentric. Thirdly, the number of SLE patients suffering from manifestations such as neurological, serosal or musculoskeletal disease was small. A broader range of overall SLE disease activity across the cohort would have been of interest to study the relationship between the concentrations of sFas and sFasL and SLE disease activity. However, in the study design, no selection criteria based on SLEDAI-2k score was applied to select the SLE patients enrolled in this cohort. In future studies, it will be of interest to enroll at least 30 patients in each of the following SLE subsets: inactive ($\text{SLEDAI-2k} < 4$), active ($10 > \text{SLEDAI-2k} \geq 4$), and highly active ($\text{SLEDAI-2k} \geq 10$). No sample size study has been performed to evaluate the statistical power of this study. Finally, low rates of active renal disease in Caucasian patients may have influenced the stronger relationship between sFas and sFasL/sFas ratio and renal disease in Asian patients.

In conclusion, the studies presented here show that sFas, sFasL and the ratio between them were related to SLE disease manifestations. sFas and sFasL/sFas ratio appeared to be related to specific phenotypic SLE manifestations, namely renal and mucocutaneous manifestations, respectively. Some data also suggest a potential association with neurological SLE phenotype, but this requires to be confirmed in a larger cohort. The relationship between sFasL/sFas ratio with mucocutaneous disease was only observed in patients with non-elevated serum BAFF, while the relationship between sFasL/sFas ratio and serum sFas with renal disease was observed only in SLE patients with high serum BAFF. Identifying relationships with organ disease may help understanding of the Fas/FasL system as targets for

therapeutic intervention, particularly in non-BAFF-mediated SLE where new therapies are urgently needed. An initial stratification according to serum BAFF levels may be of interest to define who could benefit from Fas/FasL targeted therapies. Further research is needed, in a larger longitudinal, multiethnic and multicentre SLE cohort, to confirm the use of these biomarkers to assess activity, and ultimately determine whether these soluble components may be valuable therapeutic targets in these SLE manifestations. This approach might also help to re-define SLE disease into biologically stratified sub-conditions, paving the way for more individualised use of specific medicine in SLE.

CHAPTER IV: Associations of sFas, sFasL and BAFF in primary Sjögren's syndrome.

1. Key findings

- 1.1. My data confirm increased levels of serum sFas in pSS compared to healthy individuals. There was no relationship between serum sFas concentrations and overall pSS disease activity, but serum sFas was negatively associated with the biological ESSDAI domain in univariable analysis.
- 1.2. My data confirm increased levels of serum sFasL in pSS compared to healthy individuals. There was no relationship between serum sFasL concentrations and overall pSS disease activity. There was a non-significant trend toward a negative association between serum sFasL with active lymphadenopathy ESSDAI domain.
- 1.3. No significant difference emerged in sFasL/sFas ratio between pSS and HD. There was no relationship between sFasL/sFas ratio and overall and organ-specific pSS disease activity.
- 1.4. There was a positive relationship between serum BAFF and sFas in pSS, particularly in patients with articular phenotype. This is of particular interest in light of the BAFF-sFas relationship observed in SLE (Chapter III), which appeared to be specific to musculoskeletal manifestations.
- 1.5. No significant relationship emerged between serum sFas concentrations and pSS disease activity when stratified according to serum BAFF levels.
- 1.6. No significant relationship emerged between serum sFasL or sFasL/sFas ratio and BAFF in pSS.

2. Introduction

The limited number of studies investigating the potential role of sFas and sFasL as biomarkers for organ-specific SLE also applies to pSS. In fact, even fewer studies have focused on this AID, with no published study investigating the relationship between sFasL or sFasL/sFas ratio with pSS disease activity.

The main aims in the studies presented in this Chapter are similar to those described in the Chapter III in relation to SLE, with the goal of discovering similarities and/or differences between the behaviour of these biomarkers in pSS.

3. Results

Patient characteristics

(Please see Chapter III regarding HD characteristics.)

The pSS cohort (N=48) was characterized by a female predominance of nearly 90%, and a median age of approximately 60 years. pSS patients were significantly older than healthy subjects in the present study ($p<0.0001$; data not shown; **Table 4**). Median disease duration was 19.4 years. Ethnicity was recorded in only one third of the cohort. Two patients with pSS (4.2%) had an associated diagnosis of MALT lymphoma of the parotid (**Table 4**). As previously mentioned in the Methods Chapter, ESSDAI data were only available in 35 pSS patients. Some clinical laboratory data were available and analysed in pSS patients with no available ESSDAI data. The median ESSDAI score was 3. Nearly 40% of pSS patients had two or more organ domains involved. More than one quarter of pSS patients had an active biological domain, whilst 29.2%, 22.9% and 18.8% suffered from articular, glandular and pulmonary manifestations of the disease, respectively. No pSS patients had active CNS organ domain, and two or fewer patients had active renal, constitutional, muscular and PNS

domains, as assessed by the ESSDAI score (**Table 5**). 83.3% (40/48) of patients were positive for anti-Ro (SSA) and/or anti-La (SSB) auto-Abs. In this pSS cohort, 33.3% (5/15), 6.3% (1/16) and 25% (4/16) of patients had increased IgG, IgA and IgM, respectively. Amongst the 28 pSS patients whom serum protein electrophoresis was performed, 7 (25%) had diffuse hypergammaglobulinaemia.

Table 4. Demographical, clinical and biological characteristics of pSS patients and HD.

Characteristics	HD (N = 17)	pSS (N = 48)	<i>p</i>
Age (years)	41 [27-45]	61.7 [55.2-67]	<0.0001
Female, n (%)	15 (88.2)	43 (89.6)	NS
<i>Ethnicity</i>	NA		
Caucasian, n (%)	NA	13 (27.1)	N/A
Asian, n (%)	NA	2 (4.2)	N/A
Hispanic, n (%)	NA	0 (0)	N/A
IA, n (%)	NA	0 (0)	N/A
Maori, n (%)	NA	0 (0)	N/A
Samoan, n (%)	NA	0 (0)	N/A
Unknown, n (%)	17 (100)	33 (73.3)	
Disease duration (years)	N/A	19.4 [14.4-6.6]	N/A
<i>Overall disease activity</i>			
ESSDAI	N/A	3 [1-5] [§]	N/A
Associated lymphoma	N/A	2 (4.2)	N/A
<i>Clinical laboratory data</i>			
Hb (g/l)	N/A	139 [128-146]	N/A
Platelets (x 10⁹/l)	N/A	233 [187.5-290]	N/A
CRP (mg/l)	N/A	2.6 [1.3-4.8]	N/A
ESR (mm/h)	N/A	24.5 [12.8-35]	N/A
C3 (g/l)	N/A	1.1 [0.92-1.4]	N/A
C4 (g/l)	N/A	0.19 [0.17-0.26]	N/A
IgG (g/l)	N/A	15.4 [11.4, 20]	N/A

Characteristics	HD (N = 17)	pSS (N = 48)	<i>p</i>
IgA (g/l)	N/A	2.94 [1.66, 4.08]	N/A
IgM (g/l)	N/A	1.17 [0.82, 2.23]	N/A
Diffuse hypergammaglobulinaemia	N/A	7 (25%)	N/A
Anti-dsDNA Ab (IU/ml)	N/A	0 [0-13.8]	N/A
Anti-dsDNA Ab +, n (%)	N/A	3 (6.3)	N/A
ANA +, n (%)	N/A	ND	N/A
Anti-Ro[†] (SSA) Ab +, n (%)	N/A	41 (85.4)	N/A
Anti-La (SSB) Ab +, n (%)	N/A	37 (77.1)	N/A
UPCR (g/mmol)	N/A	0.01	N/A
eGFR (ml/min)	N/A	84 [63.5-90]	N/A

N: number of individuals in each cohort. Data are expressed as median [IQR] or as number (percentage). Percentages (%) are calculated based on N. Mann Whitney test was used to analyse differences between two variables. Mann Whitney tests were two-tailed. Group of categorical data were analysed by Fisher's exact test. HD characteristics results are identical to those presented in Table 2 in Chapter III, and are represented here in order to contextualise the accompanying findings.

[§] Calculated in the 35 pSS patients in whom ESSDAI was assessed.

[†] Ro52 and/or Ro60 have both been assessed in pSS cohort: Anti-Ro Ab + means that at least one of these two tests was positive.

Ab: antibody; ANA: anti-nuclear antibody; C3: complement component 3; C4: complement component 4; CRP: C-reactive protein; dsDNA: double-stranded deoxyribonucleic acid; eGFR: estimated glomerular filtration rate; ESSDAI: European League Against Rheumatism Sjögren's syndrome Disease Activity Index; ESR: erythrocyte sedimentation rate; Hb: haemoglobin; HD: healthy donors; IA: Indigenous Australian; Ig: Immunoglobulin; IU, international units; NA: non available; N/A: non applicable; ND: non determined; NS: not significant; pSS: primary SS; SS: Sjögren's syndrome; SLE: systemic lupus erythematosus; SSA: Sjögren's syndrome antigen A; SSB: Sjögren's syndrome antigen B; UPCR: Urine protein/creatinine ratio.

Table 5. Activity of individual ESSDAI organ domain in pSS patients.

pSS patients with active organ domain	
<i>Organ domains</i>	(N = 35*)
Constitutional, n (%)	2 (5.7)
Lymphadenopathy, n (%)	6 (17.1)
Glandular, n (%)	11 (31.4)
Cutaneous, n (%)	4 (11.4)
Pulmonary, n (%)	9 (25.7)
Renal, n (%)	1 (2.9)
Articular, n (%)	14 (40)
Muscular, n (%)	2 (5.7)
PNS, n (%)	2 (5.7)
CNS, n (%)	0 (0)
Haematological, n (%)	7 (20)
Biological, n (%)	13 (37.1)

N: number of individuals in pSS cohort. Data are expressed as number (percentage).

**Percentages (%) are calculated based on number of individuals with available clinical (ESSDAI) data (n=35).*

CNS: central nervous system; ESSDAI: European League Against Rheumatism Sjögren's syndrome disease activity index; PNS: peripheral nervous system; pSS: primary Sjögren's syndrome.

Associations of serum sFas with pSS.

Serum sFas concentrations were detectable in all tested pSS samples, as well as in the HD samples, as previously reported in Chapter III. Serum sFas concentrations were significantly higher in pSS compared to HD ($p < 0.0001$; **Figure 18A**). There was no significant relationship between serum sFas concentrations and disease activity as measured using ESSDAI (**Figure 18B-C**). No significant association emerged between serum sFas and the presence of anti-La and/or anti-Ro (Ro52 and Ro60) (data not shown). Serum sFas concentrations were significantly lower in pSS patients with active biological domains compared to those without ($p = 0.018$; **Figure 18D**). Serum sFas was associated with active biological ESSDAI domain in univariable analysis using logistic regression (OR 0.9996; 95% CI 0.999, 0.9999; $p = 0.027$). However, after adjusting for age in multivariable analysis, there was only a non-significant trend toward an association of serum sFas and active biological ESSDAI domain (OR 0.9997; 95% CI 0.999, 1; $p = 0.051$). No other significant relationship emerged with organ domains (data not shown).

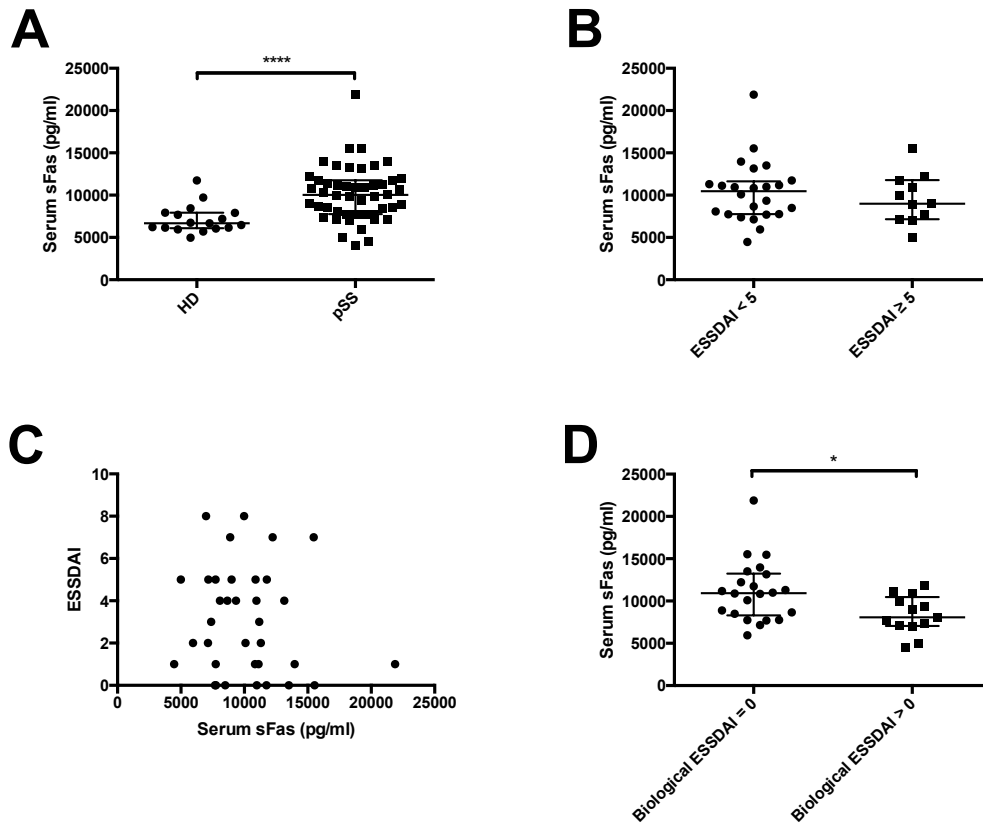


Figure 18. Associations between serum sFas with pSS disease activity.

Overall and biological pSS disease activity was assessed by the ESSDAI score. (A) Serum sFas concentrations in pSS ($n=48$) compared to HD ($n=17$). (B) Serum sFas concentrations according to overall pSS disease activity (ESSDAI < 5 : $n=24$; ESSDAI ≥ 5 : $n=11$). (C) Correlation between serum sFas concentrations and ESSDAI ($n=35$). (D) Serum sFas concentrations according to biological pSS disease activity (Biological ESSDAI = 0: $n=22$; Biological ESSDAI > 0 : $n=13$).

Serum sFas concentrations are expressed in pg/ml.

In panels A-B, and D, horizontal bars indicate the median with [IQR].

**** $p < 0.0001$; * $p < 0.05$

Associations of serum sFasL with pSS.

Serum sFasL was detectable in all tested pSS samples. Serum sFasL concentrations were significantly higher in pSS compared to HD ($p=0.007$; **Figure 19A**). There was no significant relationship between serum sFasL concentrations and ESSDAI (**Figure 19B-C**). No significant association was noted with the presence of anti-La and/or anti-Ro (Ro52 and Ro60) (data not shown). Serum sFasL concentrations were significantly lower in pSS patients

with active lymphadenopathy (ESSDAI domain) compared to those without ($p=0.0497$; **Figure 19D**). There was a non-significant trend toward an association between serum sFasL with active lymphadenopathy ESSDAI domain in univariable analysis using logistic regression (OR 0.95; 95% CI 0.9, 1; $p=0.073$). No other significant relationship with sFasL emerged with ESSDAI organ domains (data not shown).

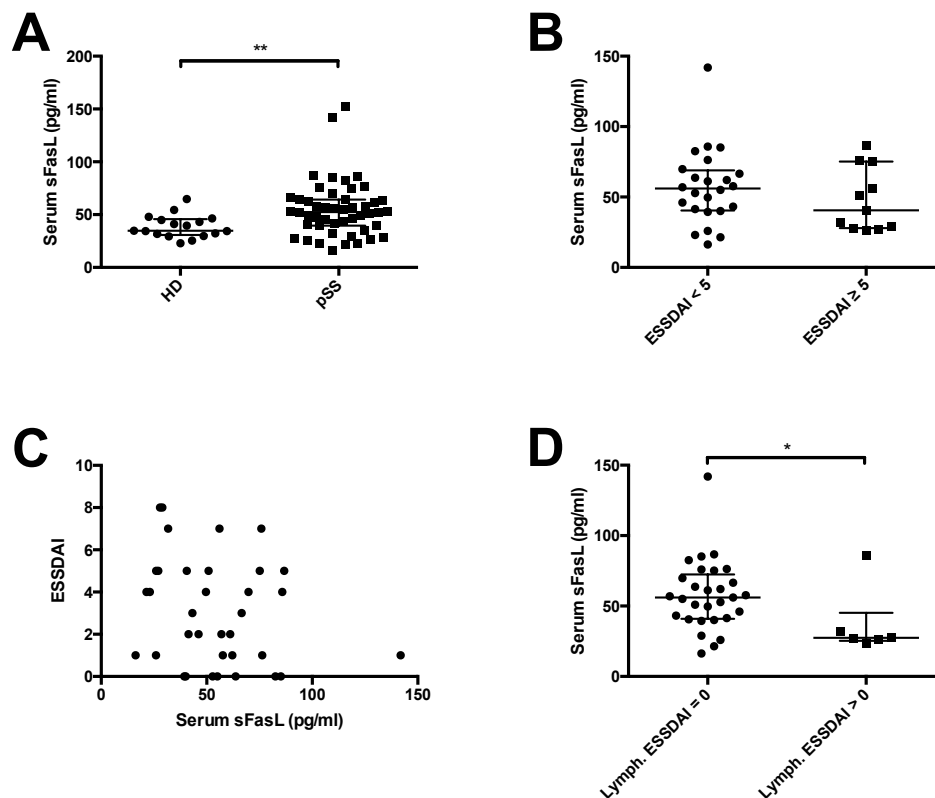


Figure 19. Associations between serum sFasL with pSS disease activity.

Overall and lymphadenopathy pSS disease activity was assessed by the ESSDAI score. (A) Serum sFasL concentrations in pSS ($n=48$) compared to HD ($n=17$). (B) Serum sFasL concentrations according to overall pSS disease activity (ESSDAI < 5 : $n=24$; ESSDAI ≥ 5 : $n=11$). (C) Correlation between serum sFasL concentrations and ESSDAI ($n=35$). (D) Serum sFasL concentrations according to pSS lymphadenopathy disease activity (Lymph. ESSDAI = 0: $n=29$; Lymph. ESSDAI > 0 : $n=6$).

Serum sFasL concentrations are expressed in pg/ml. Lymph. ESSDAI stands for lymphadenopathy ESSDAI.

In panels A-B and D, horizontal bars indicate the median with [IQR].

** $p<0.01$; * $p<0.05$

Associations of sFasL/sFas ratio with pSS.

Serum sFas and sFasL were not significantly correlated with each other in pSS (data not shown). No significant difference emerged in sFasL/sFas ratio between pSS and HD (**Figure 20A**). There was no significant relationship between sFasL/sFas ratio and ESSDAI (**Figure 20B-C**). No significant association emerged with the presence of anti-La and/or anti-Ro (Ro52 and Ro60) Abs (data not shown). sFasL/sFas ratio was significantly lower in pSS patients with active lymphadenopathy (ESSDAI domain) compared to those without ($p=0.028$; one-tailed; **Figure 20D**). However in univariable analysis, using a logistic regression model, there was no significant association between sFasL/sFas ratio with lymphadenopathy ESSDAI domain. No significant relationship with sFasL/sFas ratio emerged with other ESSDAI organ domains (data not shown).

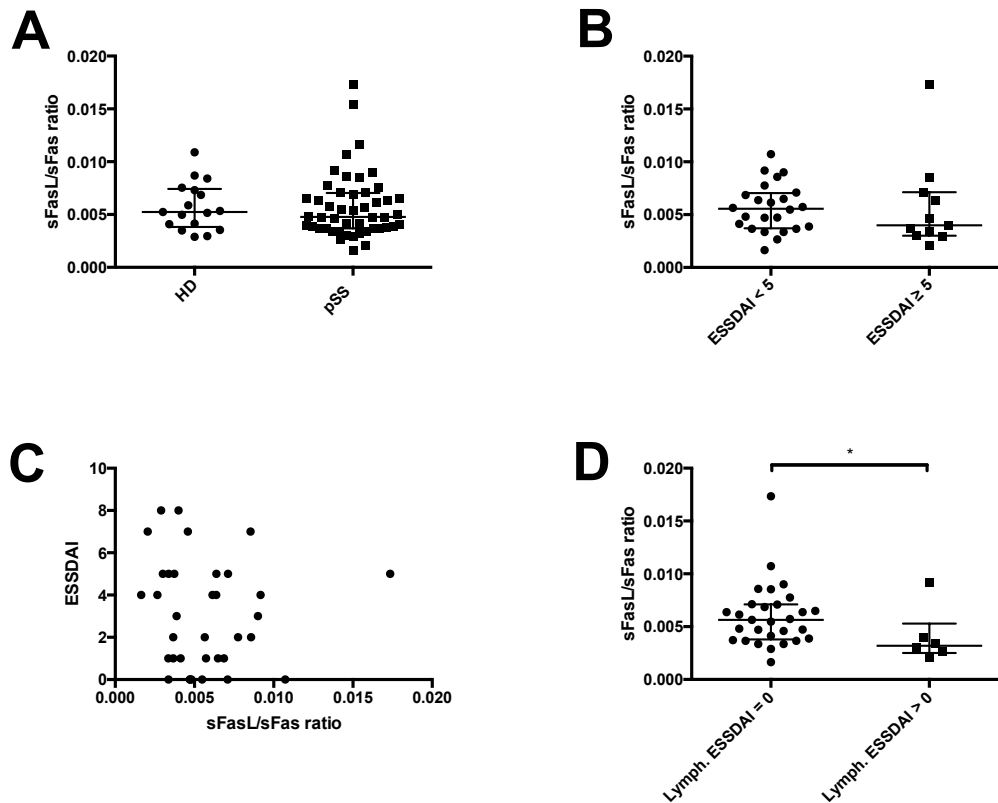


Figure 20. Associations between sFasL/sFas ratio with pSS disease activity.

Overall and lymphadenopathy pSS disease activity was assessed by ESSDAI score. (A) sFasL/sFas ratio in pSS (n=48) compared to HD (n=17). (B) sFasL/sFas ratio according to overall pSS disease activity (ESSDAI < 5: n=24; ESSDAI ≥ 5: n=11). (C) Correlation between sFasL/sFas ratio and ESSDAI in pSS (n=35). (D) sFasL/sFas ratio according to pSS lymphadenopathy disease activity (Lymph. ESSDAI = 0: n=29; Lymph. ESSDAI > 0: n=6). Serum sFas and sFasL concentrations are expressed in pg/ml. Lymph. ESSDAI stands for lymphadenopathy ESSDAI.

In panels A-B and D, horizontal bars indicate the median with [IQR].

One-tailed p value is shown in panel D.

* $p < 0.05$

Relationship between serum BAFF and sFas in pSS

Analysis of associations between serum BAFF with pSS disease are presented in Chapter VII.

Serum sFas concentrations were not significantly correlated with serum BAFF in pSS (**Figure 21A**). Nevertheless, serum sFas concentrations were significantly higher in patients with high serum BAFF levels compared to those with non-elevated BAFF levels ($p=0.011$; **Figure 21B**). When investigating this relationship in relation to clinical phenotype, this relationship was particularly specific to musculoskeletal pSS manifestations. In the subset of pSS patients with active articular disease, serum sFas concentrations were significantly positively correlated with serum BAFF concentrations ($r=0.62$, $p=0.02$; **Figure 21C**), and were correspondingly significantly higher in patients with high serum BAFF levels compared to those with non-elevated BAFF levels ($p=0.036$; **Figure 21D**). No significant correlation was noted between serum sFas and BAFF concentration in the subset of pSS patients without active articular disease ($r=0.19$, $p=0.42$) (data not shown). Serum sFas concentrations were positively correlated with serum BAFF concentrations in patients with moderate to highly active overall disease ($r=0.67$, $p=0.028$; **Figure 21E**), but not in patients with low overall activity (**Figure 21F**). Using a linear regression model, a significant association was observed between serum sFas and BAFF (coef. 0.32, 95% CI 0.01, 0.64, $p=0.043$). Of note, no confounder was found for this association.

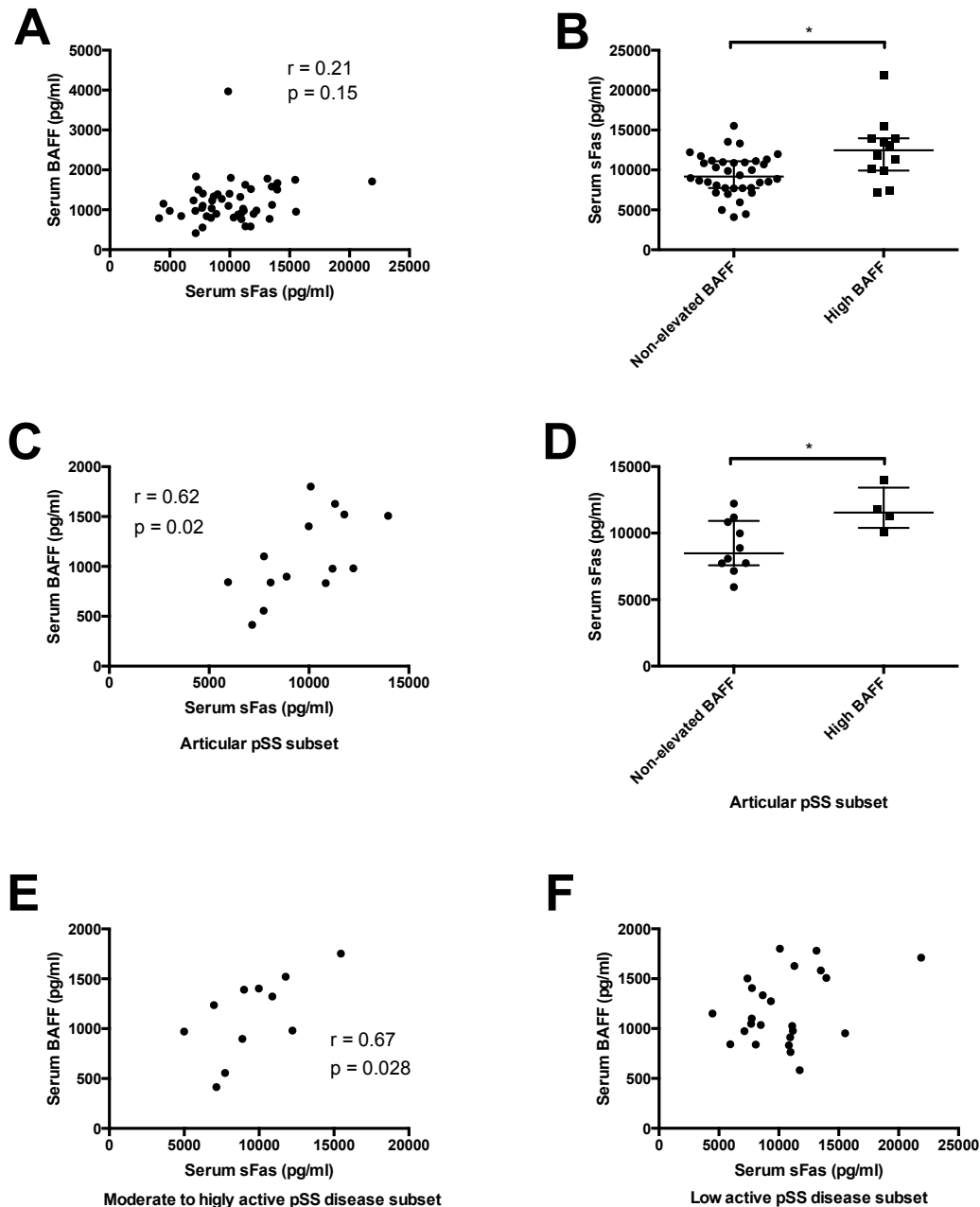


Figure 21. Relationship between serum sFas and BAFF in pSS.

Overall pSS disease activity was assessed by the ESSDAI score. Serum BAFF concentrations were defined as non-elevated (< 1438.2 pg/ml) or high (≥ 1438.2 pg/ml), as per the Methods Chapter. (A) Correlation between serum sFas and BAFF concentrations in pSS (n=48). (B) Serum sFas concentrations according to serum BAFF concentrations in pSS (Non-elevated BAFF: n=36; High BAFF: n=12). (C) Correlation between serum sFas and BAFF concentrations in the subset of pSS with active articular disease (n=14). (D) Serum sFas concentrations according to serum BAFF concentrations in the subset of pSS with active articular disease (Non-elevated BAFF: n=10; High BAFF: n=4). Correlation between serum sFas and BAFF concentrations in (E) moderate to highly active (n=11) and (F) low active pSS disease subsets (n=24).

Serum sFas and BAFF concentrations are expressed in pg/ml.

In panels B and D, horizontal bars indicate the median with [IQR].

* $p < 0.05$

Of note, no significant relationship was seen between serum sFas concentrations and ESSDAI (**Figure 22A-B**) or with any tested individual organ domain (data not shown), in either non-elevated (n=36) and high serum BAFF levels (n=12) pSS subsets.

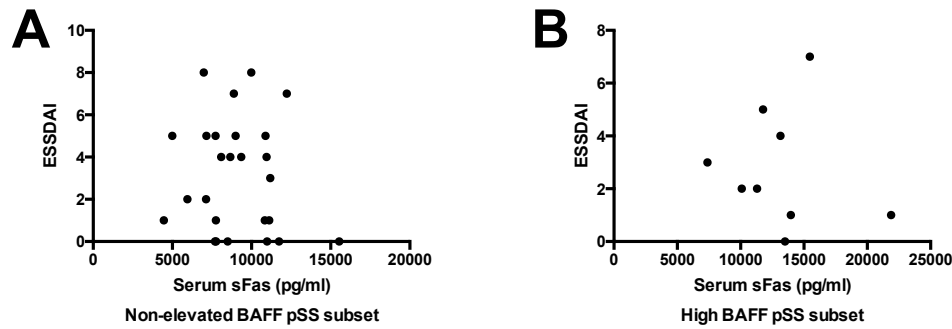


Figure 22. Relationship between serum sFas and BAFF with pSS disease activity.

Overall pSS disease activity was assessed by the ESSDAI score. Serum BAFF concentrations was defined as non-elevated (< 1438.2 pg/ml) or high (≥ 1438.2 pg/ml), as per the Methods Chapter. Correlation between serum sFas concentrations and ESSDAI in (A) non-elevated BAFF (n=26) and (B) high BAFF pSS subsets (n=9).

Serum sFas and BAFF concentrations are expressed in pg/ml.

Relationship between serum BAFF and sFasL in pSS

No significant relationship emerged between serum sFasL and BAFF in pSS (**Figure 23A-B**).

No significant relationship was noted between serum sFasL concentrations and ESSDAI (**Figure 23C-D**) or with any individual organ domain (data not shown), when patients were stratified into either non-elevated or high serum BAFF subsets.

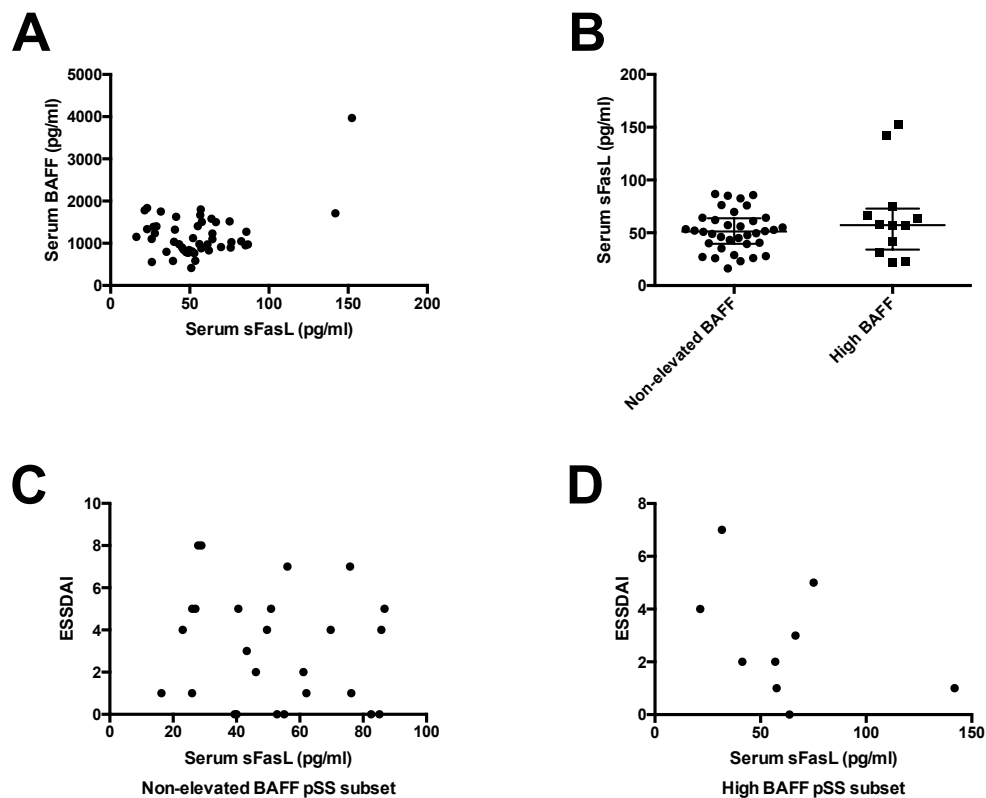


Figure 23. Relationship between serum sFasL and BAFF in pSS.

Overall pSS disease activity was assessed by the ESSDAI score. Serum BAFF concentrations was defined as non-elevated (< 1438.2 pg/ml) or high (≥ 1438.2 pg/ml), as per the Methods Chapter. (A) Correlation between serum sFasL and BAFF concentrations in pSS ($n=48$). (B) Serum sFasL concentrations according to serum BAFF concentrations in pSS (Non-elevated BAFF: $n=36$; High BAFF: $n=12$). Correlation between serum sFasL concentrations and ESSDAI in (C) non-elevated ($n=26$) and (D) high BAFF pSS subsets ($n=9$).

Serum sFasL and BAFF concentrations are expressed in pg/ml.

In panel B, horizontal bars indicate the median with [IQR].

Relationship between serum BAFF and sFasL/sFas ratio in pSS

No significant relationship emerged between sFasL/sFas ratio and BAFF in pSS (**Figure 24A-B**). No significant relationship emerged between sFasL/sFas ratio and ESSDAI (**Figure 24C-D**) or with any individual organ domain (data not shown), in either non-elevated and high serum BAFF levels pSS subsets.

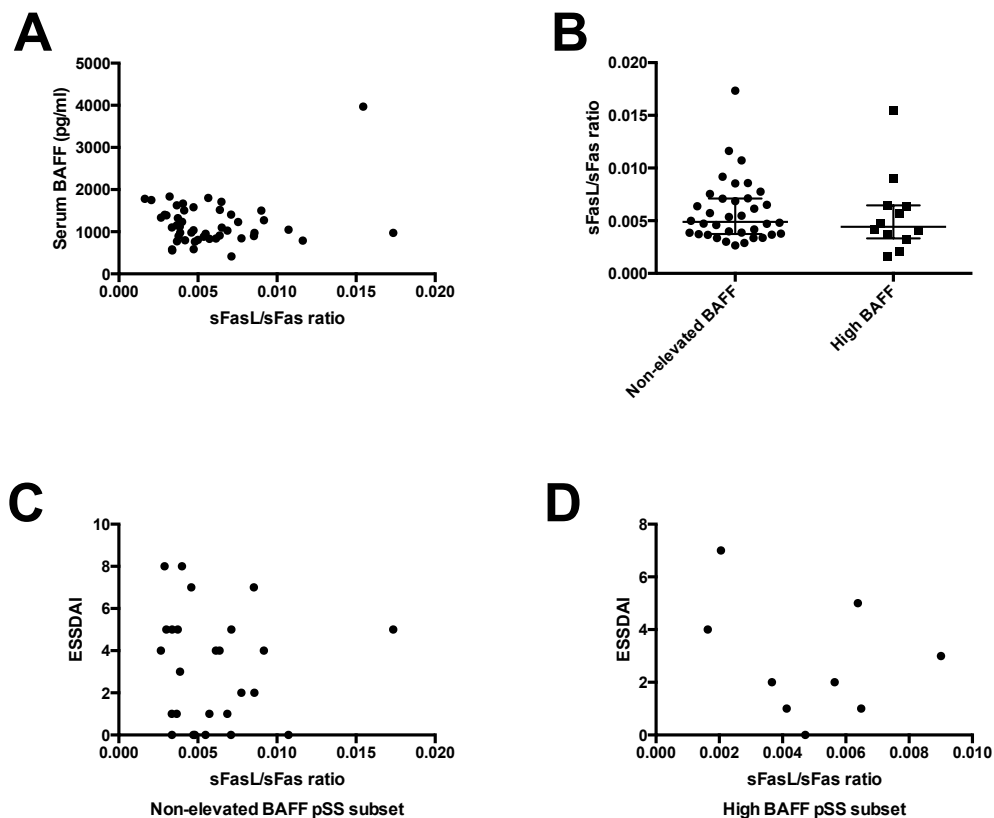


Figure 24. Relationship between sFasL/sFas ratio and BAFF in pSS.

Overall pSS disease activity was assessed by the ESSDAI score. Serum BAFF concentrations were defined as non-elevated (< 1438.2 pg/ml) or high (≥ 1438.2 pg/ml), as per the Methods Chapter. (A) Correlation between sFasL/sFas ratio and BAFF concentrations in pSS ($n=48$). (B) sFasL/sFas ratio according to serum BAFF concentrations in pSS (Non-elevated BAFF: $n=36$; High BAFF: $n=12$). Correlation between sFasL/sFas ratio and ESSDAI in (C) non-elevated BAFF ($n=26$) and (D) high BAFF pSS subsets ($n=9$).

Serum sFas, sFasL and BAFF concentrations are expressed in pg/ml.

In panel B, horizontal bars indicate the median with [IQR].

4. Discussion

This study aimed to investigate the clinical associations of serum sFas, sFasL and sFasL/sFas ratio in pSS, and to assess the utility of the combination of BAFF measurement. Another aim, in combination with the studies in Chapter III, was to characterize differences/similarities between SLE and pSS with respect to these biomarkers.

The present study is the first study of these biomarkers to use the recently validated composite pSS scoring system ESSDAI, and to also evaluate the relationship between serum soluble components from the Fas/FasL system and organ-specific pSS disease. In line with some published studies, significantly higher concentrations of both serum sFas[52, 114, 174] and sFasL[114] were found in pSS patients compared to healthy subjects. sFasL/sFas ratio was not significantly different between pSS and HD. No serum soluble component from the Fas/FasL system was related to overall pSS disease activity in the present study. To date, only one previous study investigated the relationship between pSS/SS disease activity with serum sFas, reporting no relationship.[134] No previous studies investigated sFasL/sFas ratio in SS.

A non-significant trend toward a relationship between serum sFasL and pSS lymphadenopathy emerged in univariable analysis. Although no causal link can be drawn from the present study, it could be speculated that a negative relationship between serum sFasL and lymphadenopathy in pSS may reflect a modulation of the Fas/FasL system, where decrease in serum sFasL levels might lead to increase mFas-mediated apoptosis in mFas-bearing autoreactive immune cells, particularly when they accumulate in the secondary lymphoid organs. This is of special importance in pSS, which is associated with a marked increase in risk of lymphoma.[112] This inverse relationship may, hence, reflect a protective modulation of the Fas/FasL system.

I report here for the first time a positive relationship between serum BAFF and sFas, but not sFasL, in human pSS. The relationship was dependent on the disease phenotype, and appeared specific to the articular pSS phenotype and patients with moderate to highly active overall disease. As mentioned earlier (Chapter III), a relationship between serum BAFF and sFas was also found in SLE, particularly occurring in patients with musculoskeletal SLE manifestations. These data suggest that the observed positive relationship between the BAFF/APRIL and Fas/FasL systems may be more important in musculoskeletal manifestations of both SLE and pSS conditions. Further research is needed to confirm these outcomes, in a larger cohort of pSS patients suffering from musculoskeletal manifestations.

As opposed to the SLE study presented in the Chapter III, initial patient stratification by serum BAFF levels did not reveal any specific relationship between serum soluble Fas/FasL with overall or specific organ pSS disease, although the correlation between BAFF and sFas was positive only in a moderate to high disease activity subset. These negative findings constitute novel findings in the thesis.

Caveats to the interpretation of the present data apply. Firstly, although prospective, this study was monocentric. Secondly, the pSS cohort was of modest size, and no data was available regarding treatment. A bigger sample size would have been of interest for the healthy control group. Nevertheless, although of modest size ($n < 20$), the control sample size in the present study was sufficient to demonstrate a significant difference in serum sFas and sFasL between HD and pSS. No sample size evaluation has been performed. Some clinical subsets analysed were small and therefore the presented results are preliminary. In addition, the association of BAFF with the pathogenesis of pSS has not been validated to the same degree as in SLE, where anti-BAFF therapy has proven efficacy. The pSS cohort was defined by low overall disease activity according to ESSDAI score. As was the case for the SLE cohorts used in the studies presented in the thesis, no selection criteria based on disease

activity were applied in relation to enrolment of pSS patients. In light of this limitation, future research in larger cohorts should potentially aim to enroll at least 30 pSS patients in each of the following subsets defined by disease activity state using validated ESSDAI cutoffs[141]: low (ESSDAI <5), moderate ($5 \leq \text{ESSDAI} \leq 13$) and high ($\text{ESSDAI} \geq 14$) disease activity. Finally, the use of ESSDAI domains to assign molecular causation based on serum protein measurement is, as noted, speculative.

In conclusion, I report a relationship between serum sFas and BAFF in pSS, which appears to be specific to musculoskeletal manifestations, recapitulating findings in SLE presented in Chapter III. However, in contrast to findings in SLE presented in Chapter III, no association of sFas/sFasL with overall disease activity emerged, with the exception of serum sFasL, with a non-significant trend toward a relationship with lymphadenopathy. Further research in a longitudinal pSS cohort would be of interest to evaluate the kinetics of serum sFasL levels in relation to these pSS manifestations. The potential relationship with lymphoproliferation suggests the possibility that sFasL could represent a new biomarker for monitoring pSS patients with such phenotype at risk of lymphoma, subject to confirmatory studies designed to address this.

CHAPTER V: Combining measurement of serum BAFF and IFN-gamma is associated with a renal-centric disease activity in systemic lupus erythematosus

1. Key findings

- 1.1. No significant difference emerged in serum BAFF concentrations between SLE and HD. However, a positive relationship emerged between serum BAFF and overall SLE disease activity. Serum BAFF was associated with renal SLE disease activity in univariable analysis only, and with musculoskeletal SLE disease activity.
- 1.2. No significant difference in serum BAFF levels emerged between Caucasian and Asian SLE patients. The relationship between serum BAFF and overall SLE disease activity was independent of ethnicity.
- 1.3. Serum IFN- γ concentrations were significantly higher in SLE than in HD. However, no relationship emerged between serum IFN- γ concentrations and overall SLE disease activity, or any studied organ domain disease activity.
- 1.4. Serum BAFF and IFN- γ were positively correlated in SLE, but not in HD or pSS.
- 1.5. The observed significant relationship between serum BAFF and overall SLE disease was seen regardless of the presence of detectable IFN- γ .
- 1.6. In contrast, the relationship between serum BAFF and renal SLE activity was associated with the presence of detectable serum IFN- γ . The positive relationship between serum BAFF and renal SLE was only seen in patients with detectable IFN- γ . However, in multivariable analysis, after adjusting for serosal SLEDAI-2k domain, only a non-significant trend toward an association was observed. IFN- γ was not found as a confounder for this association. A significant positive correlation emerged

between serum BAFF concentrations and proteinuria in patients with detectable IFN- γ only.

2. Introduction

As previously reviewed in Chapter I, Type II IFNs such as IFN- γ appears to play a role in SLE pathogenesis, particularly for renal disease manifestations. At the biological level, some data support an interplay between IFN- γ and the BAFF/APRIL system in autoimmune disease. In fact, BAFF is up-regulated upon stimulation with IFN- γ in human DCs, monocytes and neutrophils,[138, 184] but also in resident cells in target organs in patients with autoimmunity, such as in salivary gland epithelial cells in patients with pSS[78]. Moreover, a potential positive feedback loop between IFN- γ and BAFF production has been reported between mouse follicular helper T cells and DCs.[35] Coquery *et al.* also suggested a positive relationship between BAFF and IFN- γ in human SLE.[35] As mentioned in the Chapter I, in this PhD thesis, I will test the sub-hypothesis that clinical subsets of SLE patients can be stratified using combinations of biomarkers. I will also test the sub-hypothesis that biomarkers measured in combination may better reflect the immunological pathways involved in SLE, compared to individual markers. In light of the interplay between the BAFF/APRIL and Type II IFN systems in the immune system, and since both BAFF and IFN- γ have been previously suggested to be related to renal SLE, I sought to determine the clinical relevance of quantification of both serum BAFF and IFN- γ , as a potential composite biomarker of organ-specific disease activity, with a particular focus on renal SLE. The aim of this study was to investigate differences between Type II IFN and the BAFF/APRIL systems in relation to organ involvement, potentially informing with regard to pathway-dependent clinical phenotypic SLE manifestations. Finally, I have also tested the sub-hypothesis that ethnicity may influence these serum biomarker levels and their relationships with SLE.

3. Results

Patient characteristics

(Please see Chapter III regarding HD and SLE patient characteristics.)

As previously mentioned in the Methods Chapter, IFN- γ was not quantified in two serum samples because of sampling error or technical issue during the washing step, so the number of patients analysed was 120 for this analyte.

Associations of serum BAFF with SLE

I first aimed to evaluate the relationship between serum BAFF and overall and organ-specific SLE disease activity, and to compare to the observed serum IFN- γ outcomes. Serum BAFF data presented here are also used in Chapter VII.

BAFF was detectable in all SLE and HD samples, and was not significantly different between SLE and HD cohorts ($p=0.088$; **Figure 25A**). Serum BAFF concentrations were significantly higher in SLE patients with active (overall) disease compared to those with inactive disease ($p=0.002$; **Figure 25B**), and were also significantly positively correlated with SLEDAI-2k ($r=0.21$, $p=0.019$; **Figure 25C**). With respect to laboratory SLE markers, serum BAFF concentrations were also correlated with anti-dsDNA Abs ($r=0.21$, $p=0.022$; **Figure 25E**), and C3 levels ($r=-0.21$, $p=0.019$; **Figure 25F**), as well as with inflammation markers CRP ($r=0.25$, $p=0.007$; **Figure 25G**) and ESR ($r=0.31$, $p=0.0004$; **Figure 25H**). Thus, serum BAFF was related to overall SLE disease activity in this cohort.

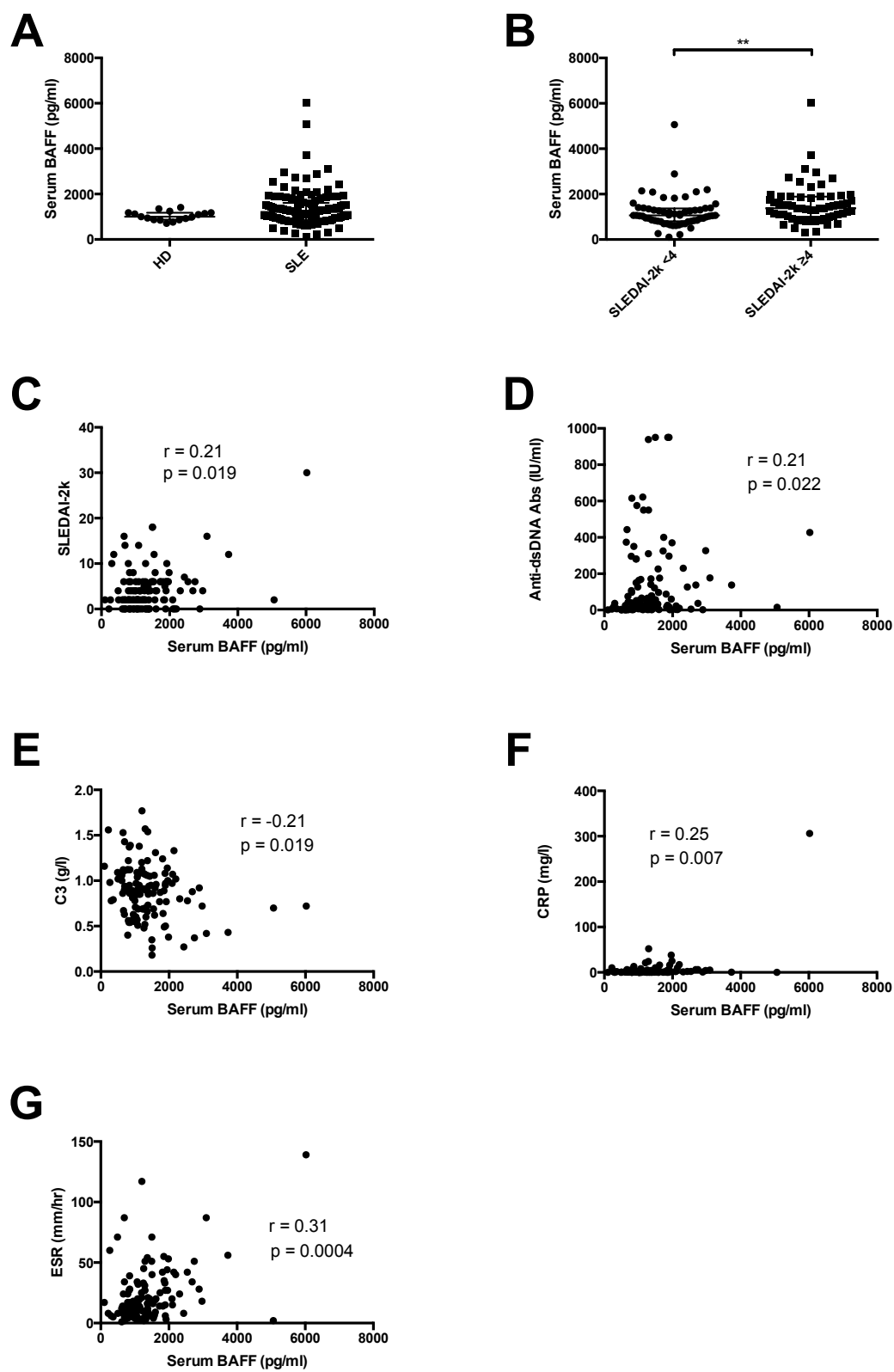


Figure 25. Associations of serum BAFF with SLE disease activity.

Overall SLE disease activity was assessed by the SLEDAI-2k score. (A) Serum BAFF concentrations in SLE (n=122) compared to HD (n=17). (B) Serum BAFF concentrations according to overall SLE disease activity (SLEDAI-2k <4: n=60; SLEDAI-2k ≥4: n=62). (C) Correlation between serum BAFF concentrations with SLEDAI-2k (n=122). Correlation between serum BAFF concentrations with (D) anti-dsDNA (n=121), (E) C3 (n=121), (F) CRP (n=120), and (G) ESR (n=122).

Serum BAFF concentrations are expressed in pg/ml. Anti-dsDNA, C3, CRP, and ESR are expressed in IU/ml, g/l, mg/l, and mm/hr, respectively.

In panels A-B, horizontal bars indicate the median with [IQR].

** $p < 0.01$

Associations of serum BAFF with organ-specific SLE

I next investigated the relationship between serum BAFF and specific organ domains of the SLEDAI-2k disease activity score.

Renal SLE

Serum BAFF concentrations were significantly higher in SLE patients with active renal disease compared to those without ($p=0.035$; one-tailed; **Figure 26A**). Serum BAFF concentrations were also weakly but significantly correlated with renal SLEDAI-2k ($r=0.18$, $p=0.042$; data not shown). Of note, serum BAFF concentrations were not correlated with SLEDAI-2k when modified by subtraction of the renal SLEDAI-2k. This suggests that the renal component of the SLEDAI-2k drove the association between serum BAFF concentrations and overall SLEDAI-2k. The association between BAFF and active renal disease was significant in univariable analysis using logistic regression (OR 1.0006, 95% CI 1.0001, 1.001; $p=0.032$). However, in multivariable analysis, after adjusting for serosal SLEDAI-2k domain, only a non-significant trend toward an association was observed (OR 1.0005, 95% CI 0.9999, 1.001; $p=0.061$). Of note, no significant relationship emerged with urine markers of disease such as UPCR.

Musculoskeletal disease

Serum BAFF concentrations were significantly higher in SLE patients with active musculoskeletal SLEDAI-2k compared to those without ($p=0.044$; **Figure 26B**). Of note, serum BAFF concentrations were still significantly correlated with SLEDAI-2k modified by subtraction of musculoskeletal domains ($r=0.19$, $p=0.033$; data not shown). This suggests that the musculoskeletal component of the SLEDAI-2k score was not the major driver of the positive correlation between serum BAFF concentrations and SLEDAI-2k. The association between BAFF and active musculoskeletal disease was significant in univariable analysis using logistic regression (OR 1.0007, 95% CI 1.0001, 1.001; $p=0.02$). No confounder was identified.”

Neurological disease

Serum BAFF concentrations were significantly lower in SLE patients with active neurological SLEDAI-2k compared to those without ($p=0.036$; one-tailed; **Figure 26C**). Only a non-significant trend toward an association was observed between BAFF and active neurological SLE in univariable analysis using logistic regression (OR 0.998, 95% CI 0.995, 1.0004; $p=0.098$). However, the subset of SLE patients with CNS disease was of small sample size ($n=4$).

No significant relationship emerged between serum BAFF concentrations with mucocutaneous, immunological, haematological, and serosal domains of SLEDAI-2k. However, numbers of patients in some of these subgroups were small.

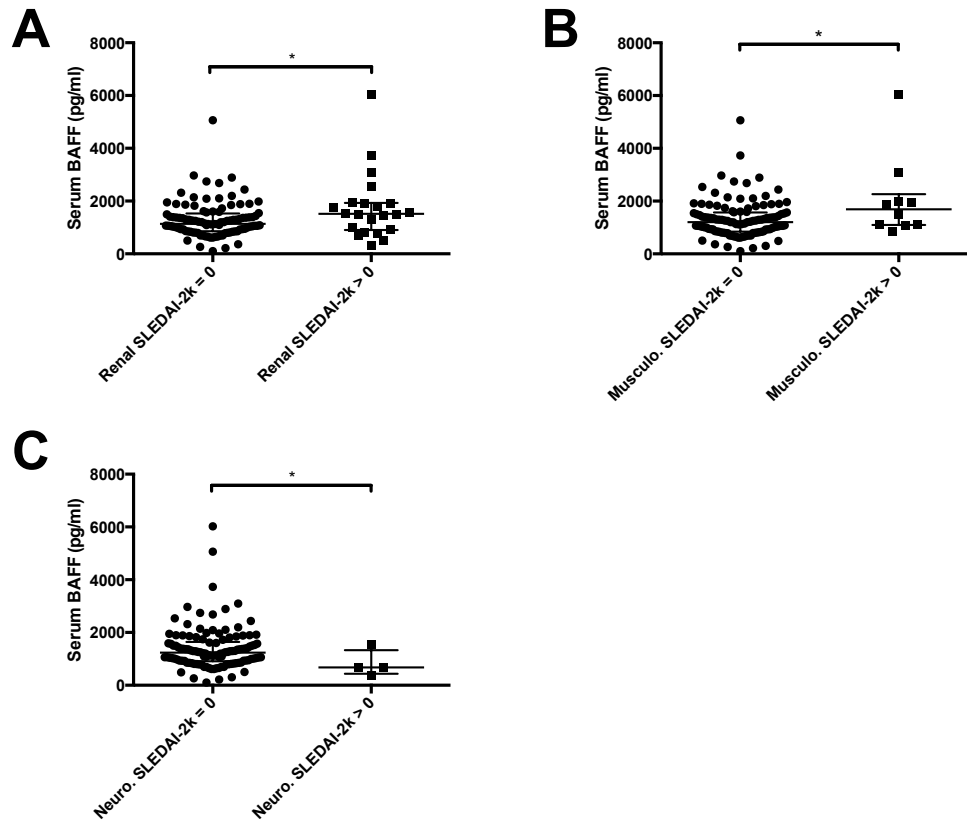


Figure 26. Associations of serum BAFF with organ-specific SLE.

Renal and musculoskeletal SLE disease activity was assessed by the SLEDAI-2k score. (A) Serum BAFF concentrations according to renal SLE disease activity (Renal SLEDAI-2k = 0: n=100; Renal SLEDAI-2k > 0: n=22). (B) Serum BAFF concentrations according to musculoskeletal SLE disease activity (Musculo. SLEDAI-2k = 0: n=112; Musculo. SLEDAI-2k > 0: n=10). (C) Serum BAFF concentrations according to neurological SLE disease activity (Neuro. SLEDAI-2k = 0: n=118; Neuro. SLEDAI-2k > 0: n=4). Musculo. and neuro. SLEDAI-2k stands for musculoskeletal and neurological SLEDAI-2k, respectively.

Serum BAFF concentrations are expressed in pg/ml.

In panels A-C, horizontal bars indicate the median with [IQR].

One-tailed p value is shown in panels A and C.

* $p < 0.05$

Serum BAFF concentrations were not significantly different between Asian and Caucasian SLE patients (**Figure 27A**). I next investigated the influence of ethnicity on the relationship between serum BAFF with overall and organ-specific SLE disease activity. The relationship between serum BAFF with overall SLE disease activity appeared independent of ethnicity, and constitutes a novel finding in this thesis. Serum BAFF concentrations were higher in Caucasian SLE patients with active disease compared to those with inactive disease ($p=0.008$; **Figure 27B**), and the same trend was seen in Asian patients ($p=0.075$; **Figure 27C**). A significant association was found between BAFF with overall SLE disease activity in univariable analysis using logistic regression (OR 1.0007, 95% CI 1.0001, 1.001; $p=0.025$). No significant confounding effect of other variables, including ethnicity, was identified.

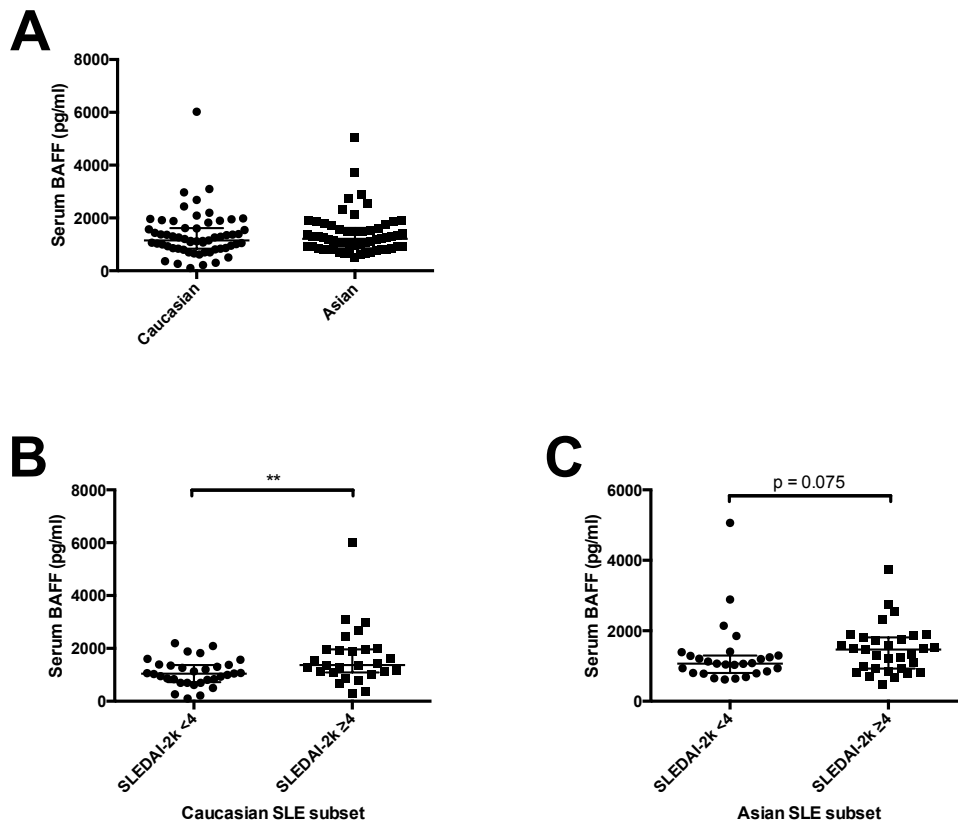


Figure 27. Influence of ethnicity on the relationship between serum BAFF and SLE disease activity.

Overall SLE disease activity was assessed by the SLEDAI-2k score. (A) Serum BAFF concentration according to ethnicity [Caucasian (n=59) vs. Asian (n=58)] in SLE. Serum BAFF concentrations according to overall SLE disease activity in (B) Caucasian (SLEDAI-2k <4: n=32; SLEDAI-2k ≥4: n=27) and (C) Asian SLE subsets (SLEDAI-2k <4: n=27; SLEDAI-2k ≥4: n=31).

Serum BAFF concentrations are expressed in pg/ml.

** p < 0.01

In panels A-C, horizontal bars indicate the median with [IQR].

Associations of serum IFN-γ with SLE

Mean serum IFN-γ concentrations were significantly higher in SLE compared to HD (p=0.032; **Figure 28A**). I next evaluated the relationship between serum IFN-γ and SLE disease activity, with a particular focus on renal disease activity. No significant relationship was observed between serum IFN-γ concentrations with overall SLE disease activity (**Figure 28B-C**). Analysis restricted only to the subset with detectable IFN-γ also did not reveal a significant association with overall disease activity (**Figure 28D-E**).

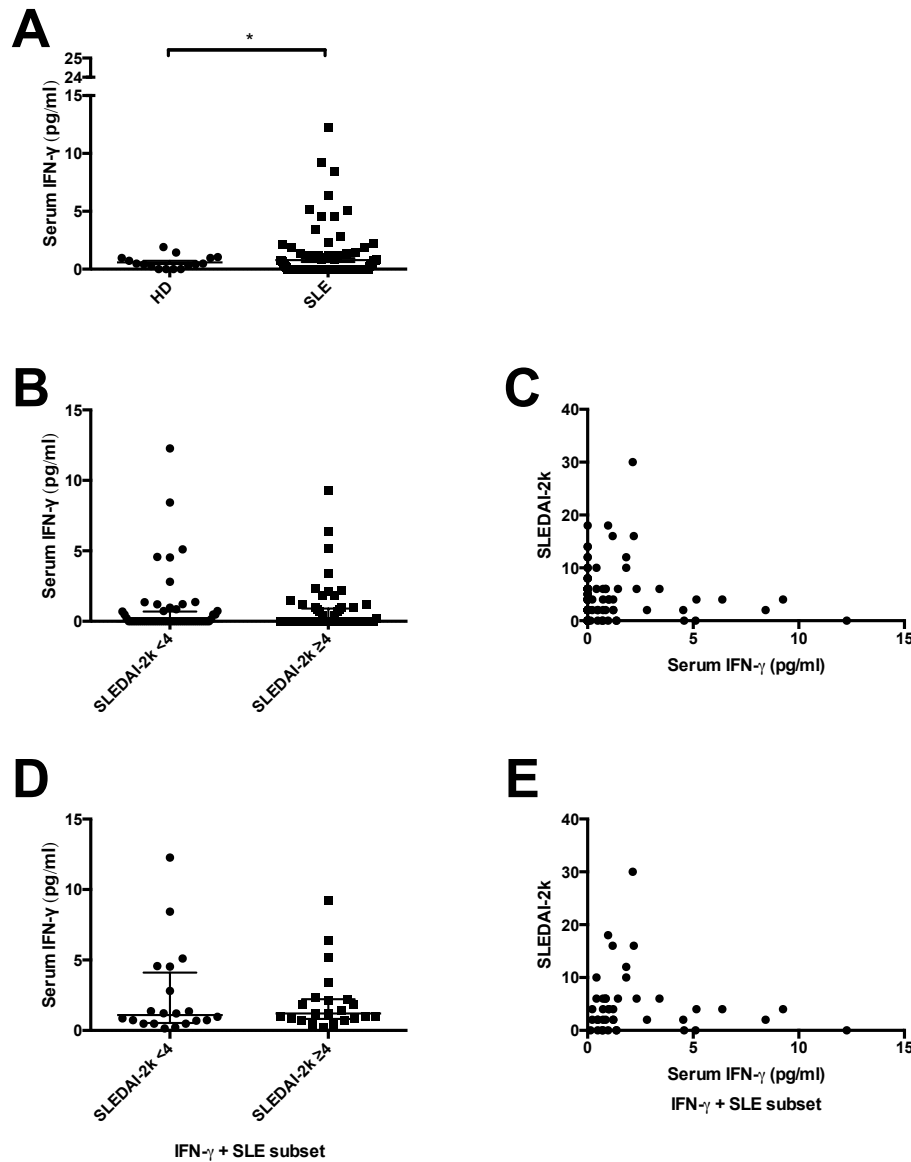


Figure 28. Associations of serum IFN- γ with SLE disease activity.

Overall SLE disease activity was assessed by the SLEDAI-2k score. (A) Serum IFN- γ concentrations in SLE ($n=120$) and HD ($n=16$). (B) Serum IFN- γ concentrations according to activity of SLE (SLEDAI-2k <4 : $n=59$; SLEDAI-2k ≥ 4 : $n=61$). (C) Correlation between serum IFN- γ concentrations and SLEDAI-2k in SLE ($n=120$). (D) Serum IFN- γ concentrations according to SLE disease activity in patients with detectable serum IFN- γ (SLEDAI-2k <4 : $n=20$; SLEDAI-2k ≥ 4 : $n=22$). (E) Correlation between serum IFN- γ concentrations and SLEDAI-2k in patients with detectable serum IFN- γ ($n=42$).

Serum IFN- γ concentrations are expressed in pg/ml.

In panel A, horizontal bars indicate the mean with SEM. In panels B and D, horizontal bars indicate the median with [IQR].

* $p < 0.05$

No significant relationship was observed between serum IFN- γ concentrations and renal SLE disease activity (**Figure 29A-B**). The same was true for relationships with other studied organ domains (data not shown). No significant relationship emerged between serum IFN- γ concentrations with renal SLE disease activity in the subset of SLE patients with detectable IFN- γ (**Figure 29C-D**).

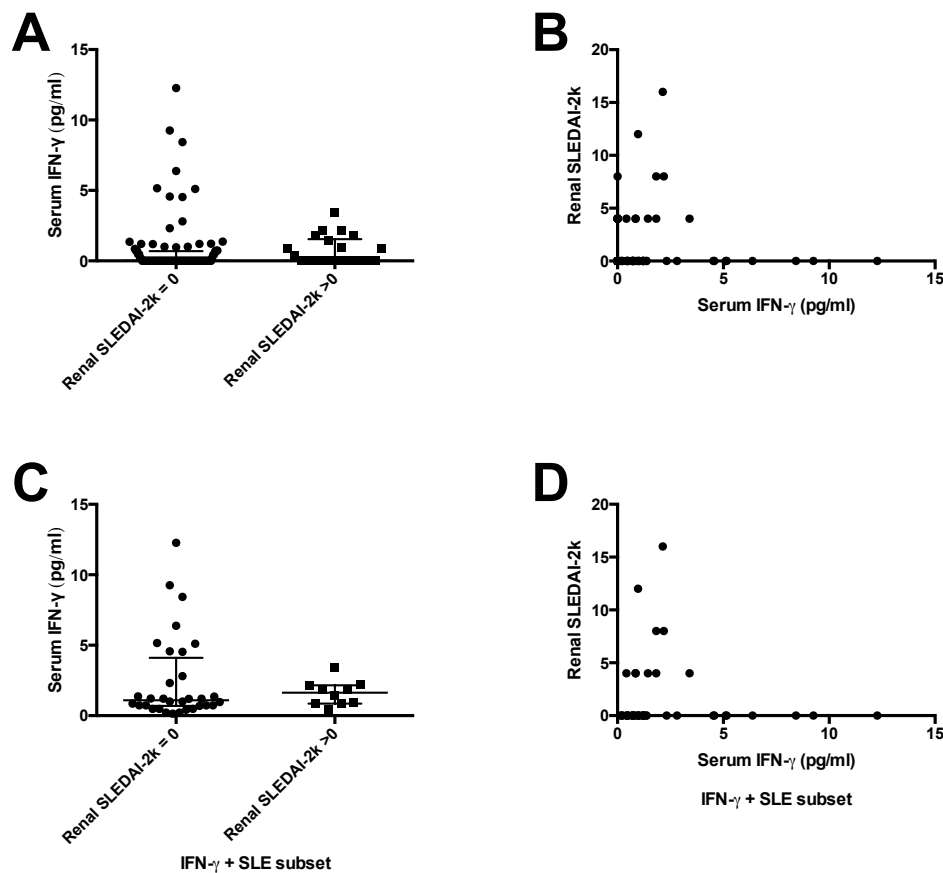


Figure 29. Associations between serum IFN- γ with renal SLE.

Renal SLE disease activity was assessed by the SLEDAI-2k score. (A) Serum IFN- γ concentrations according to renal SLE disease activity (Renal SLEDAI-2k = 0; n=98; Renal SLEDAI-2k >0; n=22). (B) Correlation between serum IFN- γ concentrations with renal SLEDAI-2k (n=120). (C) Serum IFN- γ concentrations according to renal SLE disease activity in patients with detectable serum IFN- γ (Renal SLEDAI-2k = 0; n=32; Renal SLEDAI-2k >0; n=10). (D) Correlation between serum IFN- γ concentrations with renal SLEDAI-2k in patients with detectable serum IFN- γ (n=42).

Serum IFN- γ concentrations are expressed in pg/ml.

In panels A and C, horizontal bars indicate the median with [IQR].

No significant difference in serum IFN- γ concentrations emerged between Asian and Caucasian SLE patients (**Figure 30A**). No significant relationship between serum IFN- γ with SLE disease activity emerged when SLE patients were stratified by ethnicity (**Figure 30B-E**). The same was true for association with phenotypic manifestations (data not shown).

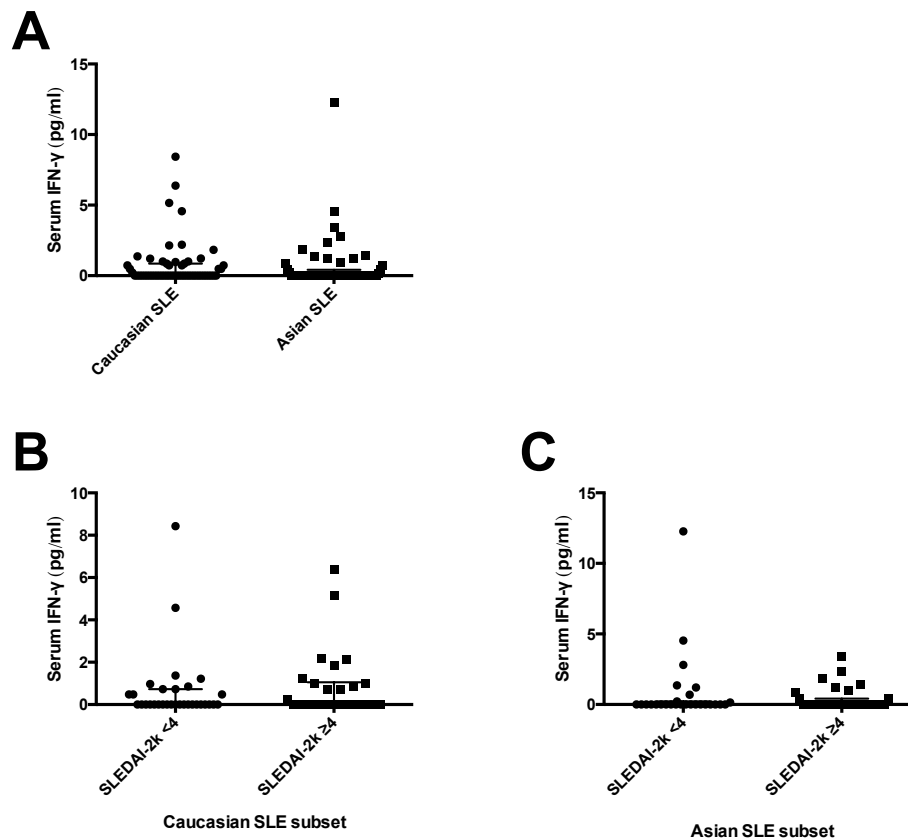


Figure 30. Influence of ethnicity on relationship between IFN- γ and SLE.

Overall SLE disease activity was assessed by the SLEDAI-2k score. (A) Serum IFN- γ concentrations according to ethnicity [Caucasian (n=57) vs. Asian (n=58)]. Serum IFN- γ concentrations according to overall SLE disease activity in (B) Caucasian (SLEDAI-2k <4: n=31; SLEDAI-2k ≥4: n=26) and (C) Asian SLE subsets (SLEDAI-2k <4: n=27; SLEDAI-2k ≥4: n=31).

Serum IFN- γ concentrations are expressed in pg/ml.

In panels A-C, horizontal bars indicate the median with [IQR].

Relationship between serum IFN- γ and BAFF in SLE

Serum BAFF and IFN- γ were significantly positively correlated in SLE ($r=0.23$, $p=0.014$; **Figure 31A**), and this association was stronger when analysis was limited to patients with detectable IFN- γ ($r=0.39$, $p=0.011$; **Figure 31B**). No significant relationship emerged between serum IFN- γ and BAFF in HD control group, whether including undetectable serum IFN- γ samples or not (data not shown).

Having shown that serum BAFF was related to both renal SLE disease activity and to serum IFN- γ , I next undertook stratification according to serum IFN- γ to assess the relationship between BAFF and renal SLE disease activity. In fact, based on the literature reviewed in Chapter I, where IFN- γ is reported to be associated with SLE, particularly with renal manifestations, even in the absence of a relationship with overall disease activity, a similar association was hypothesized in the present study. Analysing the data generated in the present study, the lack of relationship between serum IFN- γ and renal SLE disease activity was potentially explained by the modest number of patients with active renal disease in this cohort and the numbers of SLE patients with undetectable serum IFN- γ . While bearing in mind these limitations, it was further hypothesised that, since both serum IFN- γ and BAFF have been previously reported as potential renal SLE biomarkers, and since an interplay between the BAFF/APRIL and Type II IFN systems have been reported, analyzing them in combination may reveal associations with renal SLE disease activity.

The observed significant association between serum BAFF and overall SLE disease activity was seen both in patients with detectable IFN- γ (non-significant trend for association: $p=0.072$; **Figure 31C**), and in patients with undetectable IFN- γ ($p=0.017$; **Figure 31D**). As mentioned earlier, a significant association was found between BAFF and overall SLE disease activity in univariable analysis using logistic regression. No significant confounding effect of other variables, including IFN- γ , was identified. This significant relationship

between serum BAFF and overall SLE disease, observable regardless of the presence of detectable IFN- γ , is a novel finding in this thesis.

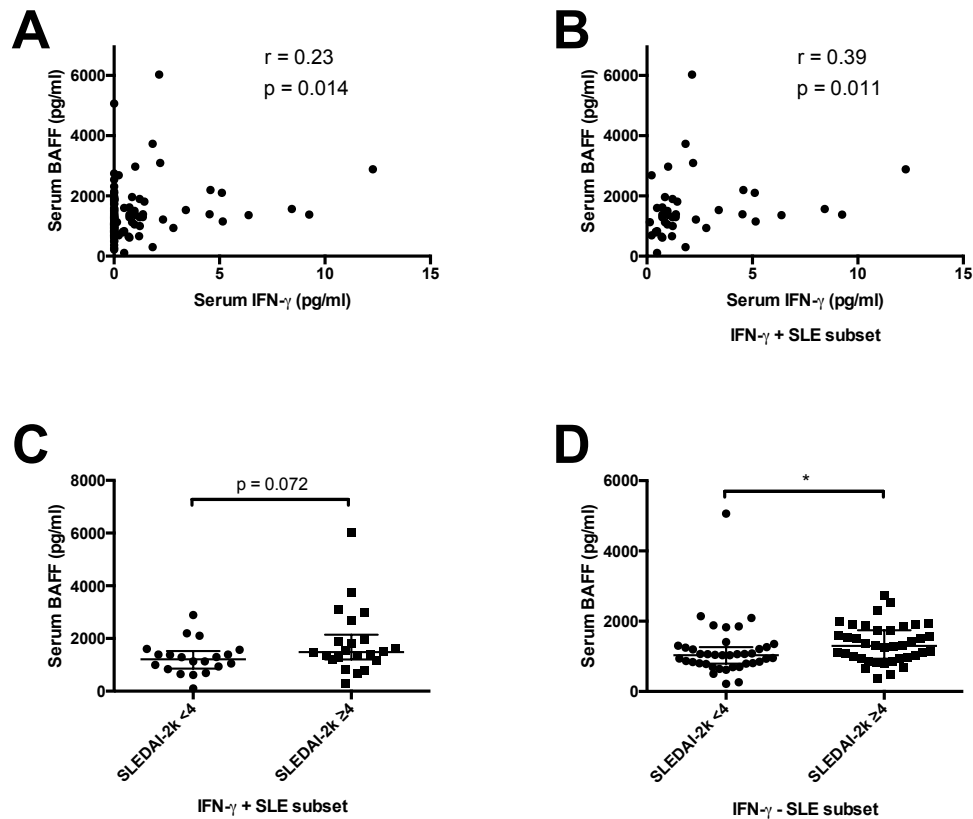


Figure 31. Relationship between serum BAFF and IFN- γ with SLE disease activity. Overall SLE disease activity was assessed by the SLEDAI-2k score. Correlation between serum IFN- γ and BAFF concentrations in the SLE cohort (A) including ($n=120$), and (B) excluding SLE patients with undetectable serum IFN- γ ($n=42$). Serum BAFF concentrations according to overall disease activity in SLE patients with (C) detectable IFN- γ (SLEDAI-2k <4 : $n=20$; SLEDAI-2k ≥ 4 : $n=22$) and (D) undetectable IFN- γ (SLEDAI-2k <4 : $n=39$; SLEDAI-2k ≥ 4 : $n=39$).

Serum BAFF concentrations are expressed in pg/ml.

In panels C-D, horizontal bars indicate the median with [IQR].

* $p < 0.05$

In contrast, the positive relationship between serum BAFF and renal SLE disease activity was seen only in SLE patients with detectable serum IFN- γ . In patients with detectable IFN- γ , serum BAFF concentrations were significantly higher in SLE patients with active renal SLEDAI-2k compared to those without ($p=0.03$; one-tailed; **Figure 32A**). In contrast, there was no significant difference in serum BAFF in relation to active renal SLE in patients with undetectable IFN- γ ($p=0.42$; **Figure 32B**). In patients with detectable IFN- γ , there was a significant correlation of serum BAFF with renal SLE disease activity ($r=0.33$, $p=0.031$; **Figure 32C**), while the scarcity of active renal disease scores did not allow correlation analysis in patients with undetectable serum IFN- γ . As mentioned earlier, the association between BAFF and active renal disease was significant in univariable analysis using logistic regression. However, in multivariable analysis, after adjusting for serosal SLEDAI-2k domain, only a non-significant trend toward an association was observed. However, IFN- γ was not found as a confounder for this association. It is noteworthy that a significant positive correlation emerged between serum BAFF concentrations with the specific renal disease activity marker UPCR in patients with detectable IFN- γ ($r=0.32$, $p=0.045$; **Figure 32D**), while this was not significant in patients with undetectable IFN- γ (**Figure 32E**). Of note, correlations of serum BAFF with both inflammation markers CRP and ESR were also stronger in patients with detectable IFN- γ (data not shown). No significant relationship between serum BAFF and other organ domains emerged after stratification with serum IFN- γ (data not shown).

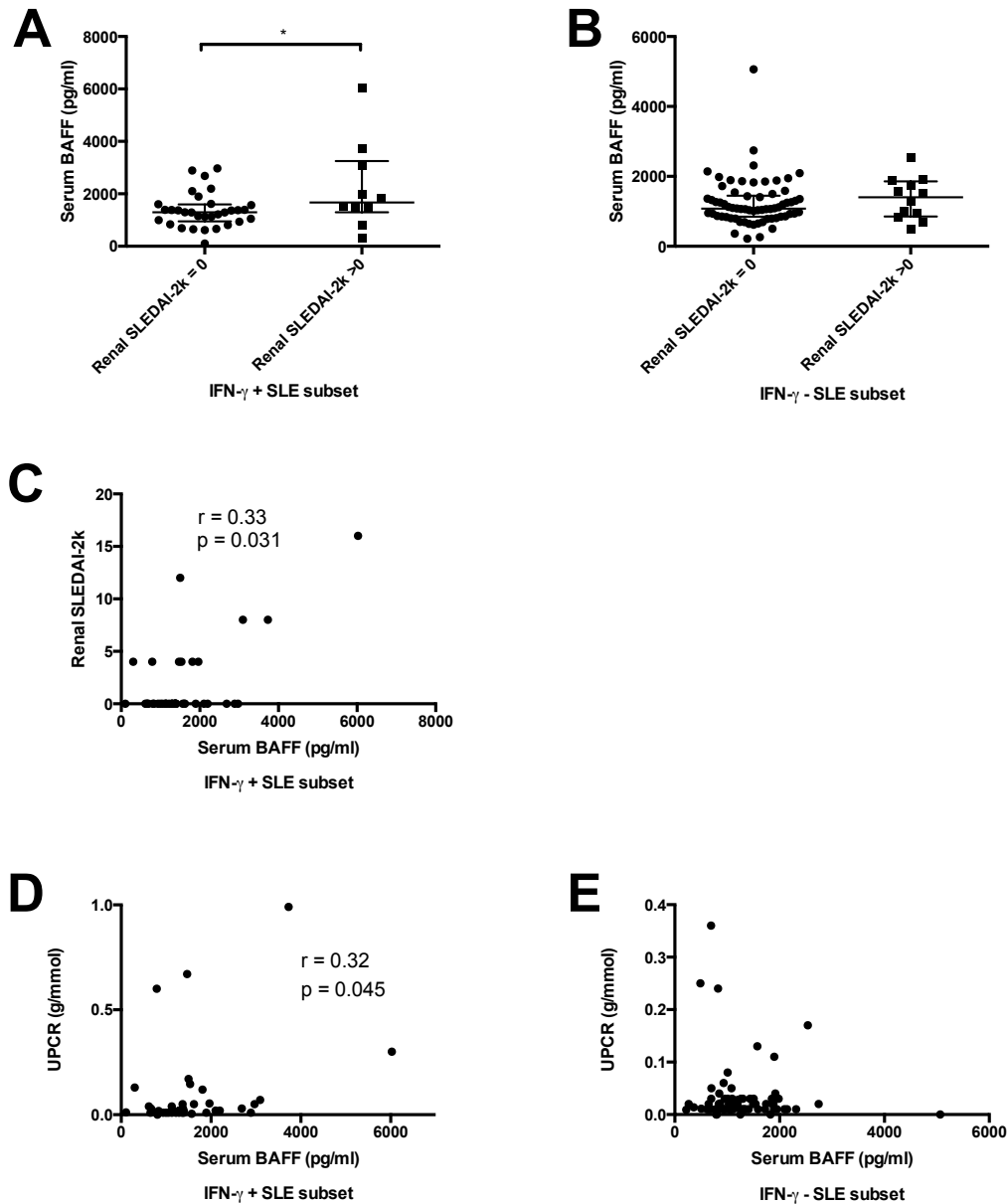


Figure 32. Relationship between serum BAFF and IFN- γ with renal SLE disease activity. Renal SLE disease activity was assessed by the SLEDAI-2k score. Serum BAFF concentrations according to renal SLE disease activity, in SLE patients with (A) detectable serum IFN- γ (Renal SLEDAI-2k = 0: $n=32$; Renal SLEDAI-2k >0: $n=10$) and (B) undetectable serum IFN- γ (Renal SLEDAI-2k = 0: $n=66$; Renal SLEDAI-2k >0: $n=12$). (C) Correlation between serum BAFF concentrations with renal SLEDAI-2k in patients with detectable serum IFN- γ ($n=42$). Correlation between serum BAFF concentrations with UPCR in patients with (D) detectable ($n=41$) and (E) undetectable serum IFN- γ ($n=74$). Serum BAFF and IFN- γ concentrations are expressed in pg/ml. UPCR is expressed in g/mmol.

In panels A-B, horizontal bars indicate the median with [IQR].

One-tailed p value is shown in panel A.

* $p < 0.05$

4. Discussion

The present study aimed to evaluate the potential of combining measurement of serum BAFF and IFN- γ as organ-specific biomarkers in SLE. In addition, I aimed to further characterize potential differences between Type II IFN and the BAFF/APRIL systems with regard to clinical associations in SLE.

In contrast with most studies (reviewed in[187]), no significant difference in serum BAFF concentrations was found between SLE and healthy subjects. However, serum BAFF was significantly related to overall SLE disease activity. In light of the unmet need for identifying therapeutic targets or biologically stratified patients for the use of anti-BAFF therapies in LN, one aim of this study was to focus on renal SLE. In line with some previous studies,[101, 127, 185] a significant association was noted between serum BAFF and renal SLE disease activity, however in univariable analysis only. In fact, these data are in line with our previous published study where we showed that serum BAFF might be a biomarker for renal and neurological disease in SLE.[185] Collectively, these data emphasize the potential role of BAFF in LN pathogenesis, and its potential as a therapeutic target. This also underlines the urgent unmet need to assess belimumab therapy in patients suffering from LN, since patients with acute LN were excluded from the two belimumab phase III clinical trials.[54, 109] Of note, three clinical trials are currently recruiting participants to investigate the use of belimumab (with or without rituximab) in lupus nephritis (ClinicalTrials.gov identifiers: NCT01639339; NCT02260934; NCT02284984), and another study of the use of blisibimod, another BAFF antagonist, in SLE with or without lupus nephritis is registered (ClinicalTrials.gov identifier: NCT02514967).

Serum BAFF was also related to musculoskeletal SLE disease in the present study, in line with one of the few published studies investigating this relationship.[101] A recent study showed no difference in plasma BAFF levels between SLE patients with arthralgia, non-

erosive and erosive arthritis, however reported a significant increase in SLE patients with erosive SLE arthritis and arthralgia compared to healthy individuals.[13] No control group of SLE patients without musculoskeletal manifestation was studied, and each SLE subset was of small size ($n \leq 20$). The authors reported a significant positive correlation with the SELENA-SLEDAI score, but did not mention any statistical analysis with the SLEDAI musculoskeletal component on its own. The authors concluded that BAFF was related more to the systemic activity of the disease rather than musculoskeletal manifestations. However, this clinical subset was of small size in the present study, hence my conclusion should be regarded as preliminary. All together, these data prompt further research in musculoskeletal SLE phenotype in a much larger cohort, to investigate the potential for BAFF as a therapeutic target for this particular phenotype.

A non-significant trend toward a negative relationship was found between levels of serum BAFF and neurological disease activity. Whilst extreme caution should be taken when interpreting these data due to the limited number of SLE patients with neurological phenotype in the present study, together with our previous published work,[185] where we showed that BAFF was increased in patients with CNS SLE, these data suggest further study of the role of BAFF in CNS SLE. In the current, but not the previous study,[185] only active CNS disease was scored. The role of BAFF in neurological SLE remains to be investigated, particularly in light of the local production of this cytokine in the brain of MS patients (reviewed in[184]). Moreover, SLE patients with severe CNS SLE were excluded from the belimumab phase III clinical trials.[54, 109] Whether this SLE phenotype could benefit from a BAFF-targeted therapy needs to be determined. However, a better understanding of the role of the BAFF/APRIL system in neurological SLE has first to be achieved, such as to understand the potential involvement of a newly described receptor for BAFF, named

reticulon-4 receptor (also known as Nogo-66 receptor or NgR) which is reported expressed in the brain (reviewed in[184]) (**Figure 1**).

Associations of serum BAFF with overall and organ-specific SLE disease activity, as well as with some laboratory markers are summarised in **Table 6**.

Table 6. Heat map of association *p* values of serum BAFF with overall and organ-specific SLE disease, and main laboratory SLE markers.

	SLE cohort (N=122)
Overall SLEDAI-2k	0.002
Renal SLEDAI-2k	<i>0.035</i>
Musculoskeletal SLEDAI-2k	0.044
Anti-dsDNA	0.022
C3	0.019
CRP	0.007
ESR	0.0004

*A 2-Colour scale has been applied, with red as the lowest *p* value, and white as *p* equal or above to 0.05. One-tailed *p* values are shown in italic. Overall and organ-specific SLE disease activity was assessed using the SLEDAI-2k score. Association *p* values of serum BAFF with overall, renal and musculoskeletal SLEDAI-2k are from Mann Whitney test. Correlation *p* values of serum BAFF with anti-dsDNA, C3, CRP and ESR are from Spearman's test.*

BAFF: B cell-activating factor from the tumour necrosis factor family; C3: complement component 3; CRP: C-reactive protein; dsDNA: double-stranded deoxyribonucleic acid; ESR: erythrocyte sedimentation rate; SLE: Systemic lupus erythematosus; SLEDAI: SLE disease activity index; SLEDAI-2K: SLEDAI 2000.

In line with most previous studies,[3, 18, 23, 31, 35, 46, 48, 61, 83, 103, 196] higher serum IFN- γ concentrations were found in SLE compared to healthy subjects. However, in contrast to most studies,[22, 23, 46, 53, 103, 196] serum IFN- γ was not observed to associate with renal SLE, or with overall SLE disease activity.

I report here for the first time in SLE a significant positive relationship between serum BAFF and IFN- γ , which was not observed in healthy subjects. As mentioned earlier, a positive relationship between these two cytokines has previously been suggested in SLE in one study, but no direct statistical test was reported.[35] This observed relationship was expected in light of the known mutual regulation of these two cytokines, occurring with up-regulation of BAFF by IFN- γ in human DCs, monocytes and neutrophils, as well as in resident salivary gland epithelial cells and FLS (reviewed in[184]),[78, 116, 138] with a potential positive feedback loop shown for both BAFF and IFN- γ between mouse T_{FH} and DCs.[35] This highlights the importance of both BAFF/APRIL and Type II IFN systems, and their interplay, in SLE, which may be crucial to take into account when developing new therapeutic interventions.

Of particular interest was the novel finding that SLE patient stratification according to serum IFN- γ detectability strengthened the relationship between serum BAFF and renal SLE, revealing a significant correlation with UPCR; no relationship between BAFF and renal disease activity was present in SLE patients with undetectable serum IFN- γ . However, in multivariable analysis, after adjusting for serosal SLEDAI-2k domain, only a non-significant trend toward an association was observed. However, IFN- γ was not a confounder for this association. Interestingly, both serum BAFF and IFN- γ have also been shown to be individually useful as predictive biomarkers for LN clinical response in some studies.[119, 196] No matching kidney biopsy data were available in the present study, and the number of SLE patients with active renal SLE disease was modest. Further research is needed, ideally

using an inception SLE cohort with biopsy-proven LN, to confirm the clinical pertinence of this novel potential renal-centric composite biomarker finding. If for example an association was found between BAFF in IFN- γ + patients and proliferative nephritis, this may help to guide or avoid the kidney biopsy for LN assessment, and potentially guide therapeutical decisions. Further studies may also help to fine-tune the use of anti-BAFF therapies, such as belimumab, as well as IFN- γ targeting therapy, such as the mAb AMG 811,[194] in SLE, and particularly in LN. For instance, a clinical trial of belimumab in SLE patients with LN stratified by detectable IFN- γ may be of particular interest. Combination therapies, eg belimumab and AMG 811, could also be tested in patients with LN, which may allow reduction of each drug dosage while potentially targeting key immunological pathways related to renal disease. Further clinical trials are needed to investigate such combination therapy strategies, particularly evaluating potential safety issues.

Caveats to the interpretation of my findings apply in this study. Firstly, for reasons outline elsewhere in this thesis, no sample size calculation has been performed to evaluate the statistical power of this study. Secondly, the healthy control group was of small size. Finally, some clinical subsets analyzed in this study were too small to draw solid conclusions; presented results are hence preliminary.

In conclusion, I have shown that immunological pathways involving both BAFF and IFN- γ appeared to be particularly related to renal SLE. Combining the use of serum BAFF and IFN- γ quantification may be a valuable strategy to potentially fine-tune the use of serum BAFF in order to assess renal SLE disease activity. Further research is needed to confirm these outcomes in a larger longitudinal SLE cohort, and to determine whether this biomarker strategy might supplant the gold standard, kidney biopsy. I also report that serum BAFF was primarily related to a particular clinical SLE phenotype characterized by renal, and potentially musculoskeletal involvement. However, the association between serum BAFF and

renal SLE was only seen in univariable analysis. Whether a biomarker strategy combining stratification by serum IFN- γ concentration can define a SLE subset with a BAFF-mediated SLE renal disease, potentially prone to respond to anti-BAFF therapies, remains to be determined. However, developing clinical trials based on biological stratification is likely to increase the power of small studies and enable rapid evaluation of such therapies. Given the availability of anti-BAFF drugs, and the paucity of proven biological therapies for SLE, such trials should be proposed.

CHAPTER VI: Soluble form of BAFF receptors in systemic lupus erythematosus

1. Key findings

- 1.1. For optimal assay performance, serum BAFF should be quantified separately from the soluble form of its receptors (sTACI and sBCMA) when using a multiplex assay. Serum BAFF detection by ELISA appeared to be affected by the presence of sBAFF-R.
- 1.2. I confirmed the presence of both sTACI and sBCMA in sera from SLE patients, in a large well-defined cohort. Serum sTACI concentrations were significantly higher in SLE patients compared to HD, and were weakly negatively correlated to overall SLE disease activity.
- 1.3. Serum sBCMA concentrations were also significantly higher in SLE compared to HD. No significant relationship emerged between serum sBCMA concentrations and overall SLE disease activity. However, serum BCMA concentrations were correlated with anti-dsDNA Abs levels, and there was a non-significant trend toward a correlation with C3 levels, and toward higher serum sBCMA concentrations in SLE patients with anti-Sm Abs compared to those without. Serum sBCMA concentrations appeared to be influenced by ethnicity, being significantly higher in Asian compared to Caucasian SLE patients. No significant relationships between serum sBCMA and overall and organ-specific SLE disease activity emerged after stratification by ethnicity.
- 1.4. I report, for the first time, the presence of sBAFF-R in sera from SLE and healthy individuals. Serum sBAFF-R concentrations were significantly lower in SLE

compared to HD. No significant relationship emerged between serum sBAFF-R concentrations and overall SLE disease activity.

- 1.5. Serum BAFF was not correlated with any of the soluble receptors in SLE. Serum sBCMA concentrations were correlated with sBAFF-R, but serum sTACI concentrations were not correlated with either sBCMA or sBAFF-R.
- 1.6. There was no significant difference in BAFF/sTACI ratio between SLE and HD. However, BAFF/sTACI ratio correlated with overall SLE disease activity, as well as anti-dsDNA Abs, C3, and ESR. BAFF/sTACI ratio was also related to musculoskeletal SLE manifestations.
- 1.7. There was no significant difference in BAFF/sBCMA ratio between SLE with HD. No significant difference emerged in BAFF/sBCMA ratio according to overall SLE disease activity. No relationship emerged between BAFF/sBCMA ratio with any organ domain of SLE disease. No significant difference emerged in BAFF/sBCMA ratio according to ethnicity in SLE.
- 1.8. BAFF/sBAFF-R ratio was significantly higher in SLE compared to HD. No significant relationship emerged between BAFF/sBAFF-R ratio and overall SLE disease activity. An association emerged between BAFF/sBAFF-R ratio with overall disease activity in Caucasian SLE patients, but not in Asian.

2. Introduction

The efficacy of the recently approved BAFF-targeting therapy, belimumab, gives weight to the fact that the cytokine BAFF plays a critical pathogenic role in SLE. Nevertheless, this therapy was reported effective only in a subset of SLE patients, suggesting a potential SLE subset characterized by BAFF-mediated disease. As previously described in Chapter I, published data regarding the potential role of serum BAFF as a SLE biomarker are inconsistent, whether at the overall or at the organ disease activity level. Amongst all potential causes for these discrepancies, the presence of soluble BAFF receptors in sera from human SLE patients needs to be considered. One group, through two recent published studies, reported the presence of sBCMA and sTACI in human SLE, however this was in a cohort of modest size, and was without evaluating clinical phenotypic associations. Another group reported the presence of serum sTACI in SLE, being particularly higher in patients with renal manifestations, in a study presented in abstract form only to date.[39] It is noteworthy that no published study has evaluated the presence of sBAFF-R in human AID. In this study, I will test the sub-hypothesis that SLE patient stratification according to clinical phenotype during analysis of associations of serum components of the BAFF/APRIL system, and particularly soluble BAFF receptors, may characterize subsets of patients with pathway-dependent SLE organ manifestations.

I first sought to evaluate whether the presence of soluble BAFF receptors may interfere with BAFF detection in patient sera. However, my main aim was to determine the association of soluble BAFF receptors with SLE phenotype, and whether they could help to fine-tune the use of serum BAFF as a SLE biomarker, by investigating their relevance as biomarkers for overall and/or organ-specific disease activity. Finally, I have also tested the sub-hypothesis that ethnicity may influence these serum soluble receptors levels and their relationships with SLE.

3. Results

Patient characteristics

(Please see Chapter III regarding SLE patient and HD characteristics.)

As previously mentioned in the Methods Chapter, sTACI and sBCMA were not quantified in two serum samples because of sampling error or technical issue during the washing step, so the number of SLE patients analysed was 120 for these analytes. sBAFF-R was not quantified in two serum samples, different from those where sTACI and sBCMA were not quantified, so the number of SLE patients analysed was 120 for this analyte. sBAFF-R was not quantified in six serum samples from HD, the number of HD analysed was 11 for this analyte.

Difference in quantifying serum BAFF: ELISA vs. multiplex assays

I first compared serum BAFF quantification using ELISA with quantification using Luminex multiplex assays including both sTACI and sBCMA. SLE (n=36) and pSS (n=36) patients, whose serum samples were measured by both ELISA and Luminex assays, were included in the analysis. Serum BAFF concentrations were significantly higher when quantified by Luminex compared to ELISA irrespective of the AID studied ($p < 0.0001$ for both SLE and pSS, **Figure 33A and B**). Nevertheless, serum BAFF concentrations measured by ELISA and Luminex were significantly positively correlated (SLE: $r = 0.72$, $p < 0.0001$; **Figure 33C**; pSS: $r = 0.8$, $p < 0.0001$; **Figure 33D**). At my request, R&D Systems the assay manufacturer performed further tests to determine the potential impact of simultaneously detecting BAFF, sTACI and sBCMA in the same multiplex assay. They reported that sBCMA and sTACI values were not affected by using a multiplex assay also quantifying BAFF. However, the detection of both sBCMA and sTACI in the standard assay cocktail led to interference with serum BAFF detection, observed as a decrease in BAFF mean fluorescence intensity values

for the standard curve, particularly impacting on higher concentration samples. The consequence of this was an increase in BAFF concentrations. While the combination of BAFF with sTACI and sBCMA in a multiplex was acceptable according to R&D Systems to generate relative values, they recommended the use of separate multiplex assays, without BAFF and sTACI and sBCMA receptors detection being combined, for optimal performance if absolute values are needed (**Appendix 1**). Thus, for optimal performance, serum BAFF should be quantified separately from the soluble form of its receptors (sTACI and sBCMA). Regarding serum BAFF concentrations quantification in this present study, and in all Chapters presented in this Thesis, only data generated by ELISA assay were used.

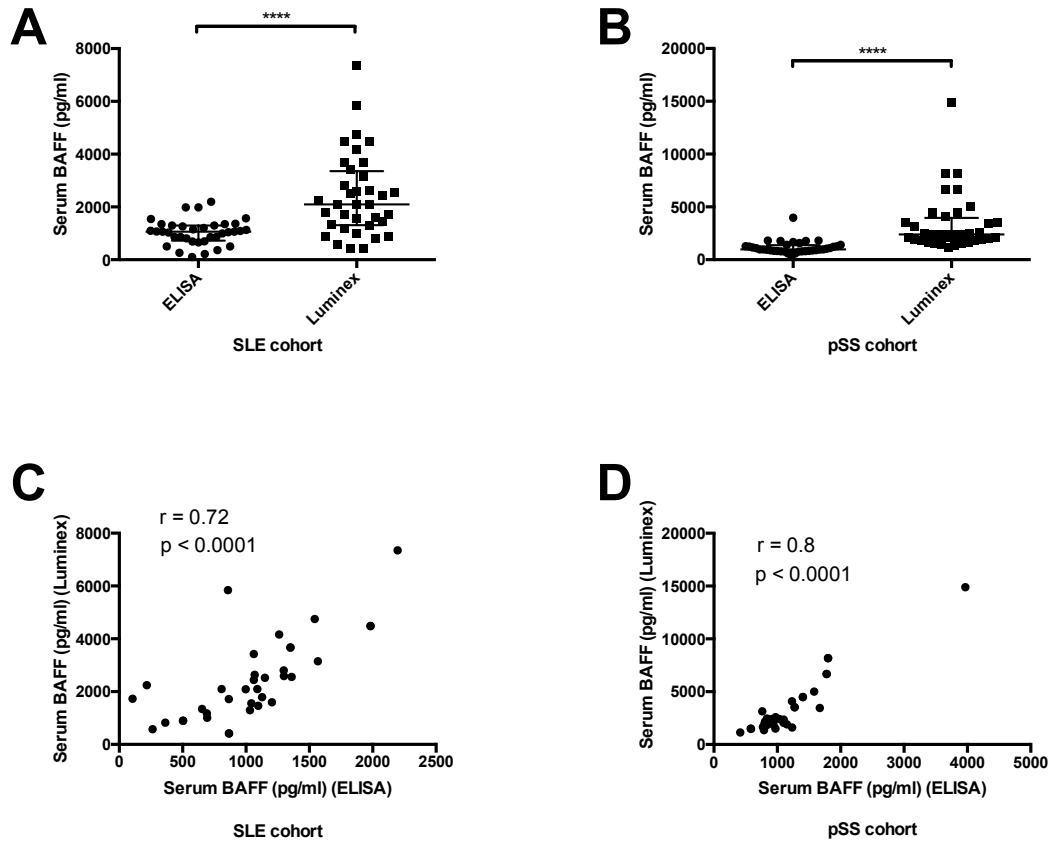


Figure 33. Quantification of serum BAFF concentrations by ELISA and Luminex assays in AID.

Both ELISA and Luminex tests have been used to quantify serum BAFF concentrations in SLE ($n=36$) and pSS ($n=36$) patients. Serum BAFF concentrations quantified by ELISA and Luminex in (A) SLE and (B) pSS cohorts. Correlation between ELISA and Luminex tests both quantifying serum BAFF concentrations in (C) SLE and (D) pSS cohorts.

Serum BAFF concentrations are expressed in pg/ml.

In panels A-B, horizontal bars indicate the median with [IQR].

**** $p < 0.0001$

Interference of sBAFF-R with serum BAFF detection by ELISA

I next investigated potential interference with serum BAFF quantification with the presence of soluble BAFF receptors in human sera. According to the BAFF ELISA kit manufacturer's booklet, rh TACI at levels >3.13 ng/ml interferes with BAFF detection. In the SLE, pSS and HD cohorts, the observed maximum concentration values were 142.4, 163.7 and 39.06 pg/ml, respectively, suggesting no interference by sTACI on serum BAFF quantification in the

present study. I therefore sought to test potential interference of sBAFF-R with BAFF detection by ELISA in human serum. Serum from one patient suffering from pSS was spiked with rh BAFF-R at different concentrations. Compared to control serum where reagent diluent was added instead of rh BAFF-R, serum BAFF concentrations were measured as lower in samples spiked with rh BAFF-R, with the reduction of detectable serum BAFF highest at a concentration $\geq 4 \mu\text{g/ml}$ rh BAFF-R (**Figure 34A-B**). Thus, serum BAFF detection by this ELISA kit also appeared to be affected by the presence of soluble BAFF-R. Based on the fact that sBCMA acts a decoy only for APRIL,[90] it can be speculated that no interference on serum BAFF quantification by the presence of endogenous sBCMA is likely, and I did not undertake specific testing of this.

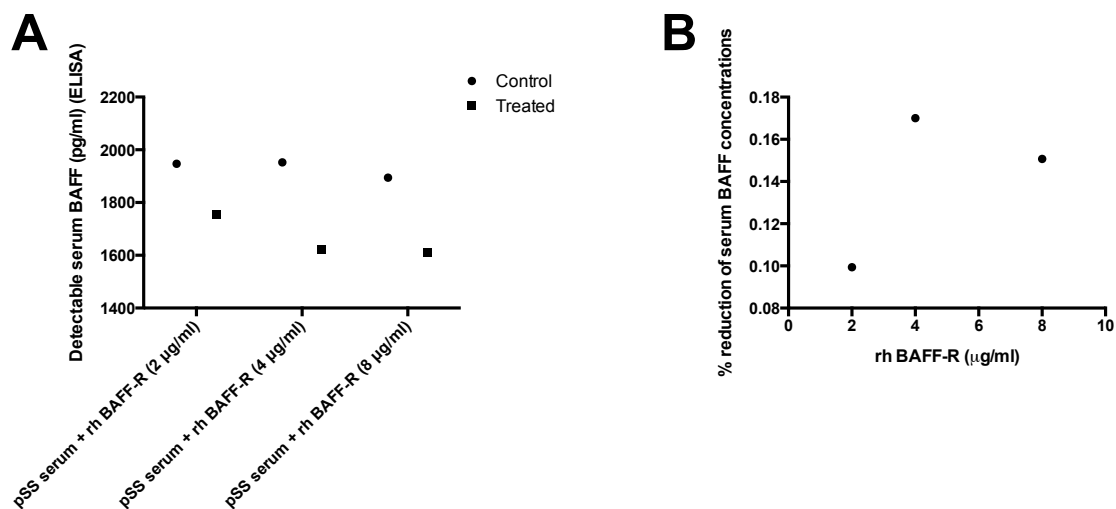


Figure 34. Interference of sBAFF-R with serum BAFF detection by ELISA.

Potential interference of the presence of sBAFF-R with BAFF detection by ELISA in human serum has been tested. (A) Serum from one patient suffering from pSS has been spiked with rh BAFF-R at different concentration (2, 4 and 8 $\mu\text{g/ml}$) and compared to control pSS serum where reagent diluent was added instead of rh BAFF-R. (B) Percentages reduction of detectable serum BAFF concentrations by ELISA according to the concentration of added rh BAFF-R have been calculated.

Serum BAFF and sBAFF-R concentrations are expressed in pg/ml and $\mu\text{g/ml}$, respectively. These data represent one experiment with serum sample from one pSS patient; all different tested rh BAFF-R concentrations and respective controls were performed in duplicates.

Effect of anti-BAFF and anti-CD20 therapies on serum BAFF and soluble BAFF receptor concentrations

I next investigated the effect of anti-BAFF therapy (tabalumab and belimumab) and of anti-CD20 therapy (rituximab) on serum BAFF and soluble BAFF receptor concentrations in SLE patient serum. As mentioned in Chapter II, four SLE patients were on clinical trials receiving tabalumab (anti-BAFF) at the time of blood and data collection. One and three SLE patients were receiving belimumab and rituximab, respectively, at the time of or within 12 months of blood sample collection. Serum BAFF concentrations were higher in SLE patients receiving anti-BAFF or rituximab compared to those without ($p=0.0003$; and $p=0.043$, respectively; **Figure 35A**). There was a non-significant trend toward a lower serum sTACI concentrations in SLE patients with anti-BAFF drugs compared to those without ($p=0.062$; **Figure 35B**), and while no significant difference emerged in serum sBCMA and sBAFF-R concentrations (**Figure 35C and D**, respectively), they were numerically lower. There was no statistically significant difference in serum sTACI, sBCMA and sBAFF-R concentrations according to the use of rituximab (**Figure 35B, C and D**, respectively), although again each was numerically lower. Based as these data showing potential effects of anti-BAFF therapies and B cell depleting agents on serum sBAFF receptors levels, patients on tabalumab, belimumab or rituximab were excluding from further analysis in this Chapter, unless otherwise stated, as described in the Methods Chapter.

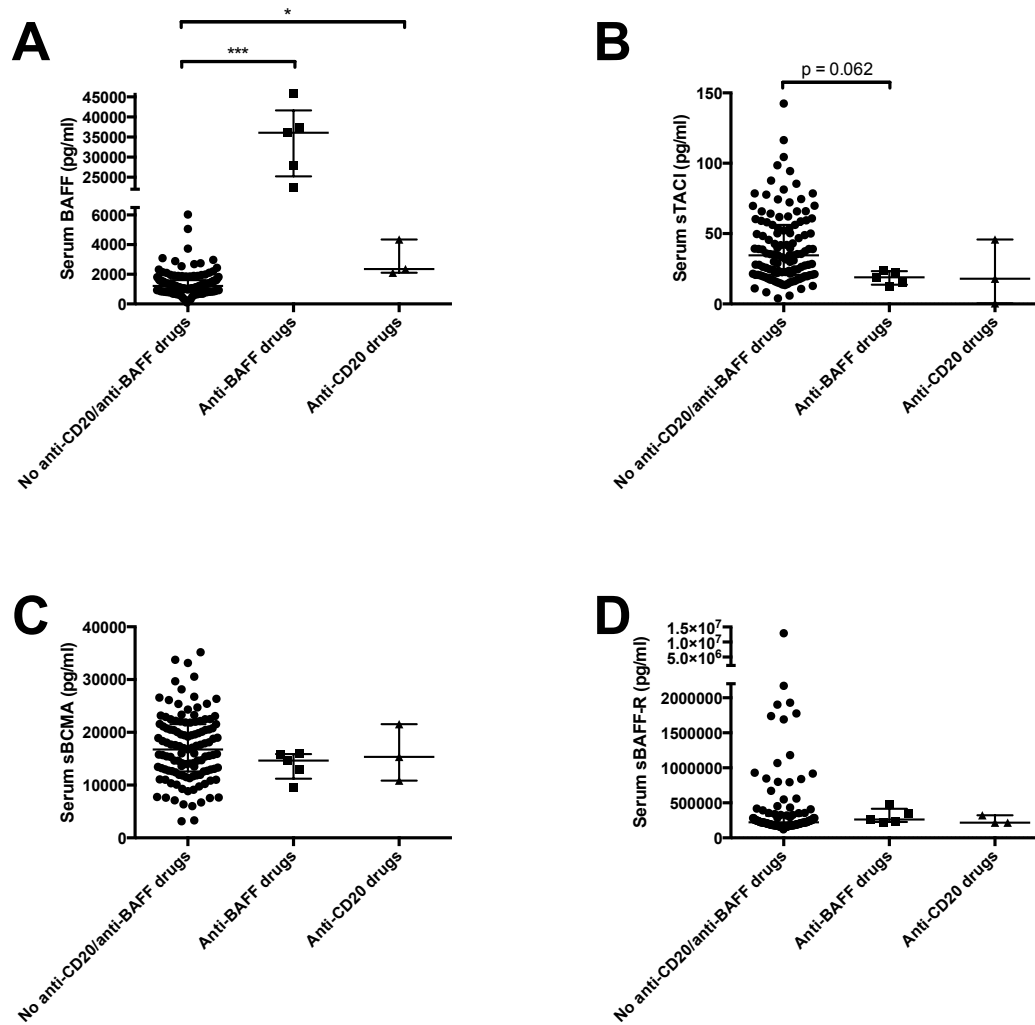


Figure 35. Effect of anti-BAFF and anti-CD20 therapies on serum BAFF and soluble BAFF receptors concentrations in SLE.

Serum (A) BAFF (No anti-CD20/anti-BAFF drugs: $n=122$; Anti-BAFF drugs: $n=5$; Anti-CD20 drugs: $n=3$), (B) sTACI (No anti-CD20/anti-BAFF drugs: $n=119$; Anti-BAFF drugs: $n=5$; Anti-CD20 drugs: $n=3$), (C) sBCMA (No anti-CD20/anti-BAFF drugs: $n=119$; Anti-BAFF drugs: $n=5$; Anti-CD20 drugs: $n=3$), and (D) sBAFF-R concentrations (No anti-CD20/anti-BAFF drugs: $n=120$; Anti-BAFF drugs: $n=5$; Anti-CD20 drugs: $n=3$), according to the use of anti-BAFF and anti-CD20 therapies in SLE patients.

Serum BAFF, sTACI, sBCMA and sBAFF-R are concentrations are expressed in pg/ml.

*** $p < 0.001$; * $p < 0.05$

In panels A-D, horizontal bars indicate the median with [IQR].

I next investigated the relationship between soluble BAFF receptors with overall and organ-specific disease activity in SLE. (*Analysis of associations of serum BAFF with SLE disease activity were presented in Chapter V.*)

Associations of serum sTACI with SLE

Serum sTACI was detectable in 99.2% (119/120) of tested SLE samples, and in all tested HD samples. Serum sTACI concentrations were significantly higher in SLE patients compared to HD ($p=0.0029$) (**Figure 36A**).

Relationship between serum sTACI and SLE disease activity

Although no significant difference was observed in mean sTACI concentration between active and inactive SLE (**Figure 36B**), serum sTACI concentrations were significantly but weakly negatively correlated with SLEDAI-2k ($r=-0.18$, $p=0.048$; **Figure 36C**). Serum sTACI concentrations were also weakly correlated with C3 ($r=0.2$, $p=0.031$; **Figure 36D**). No significant relationship was observed with tested clinical phenotype subsets (e.g. renal, musculoskeletal) when analysed based on SLEDAI-2k scores (data not shown).

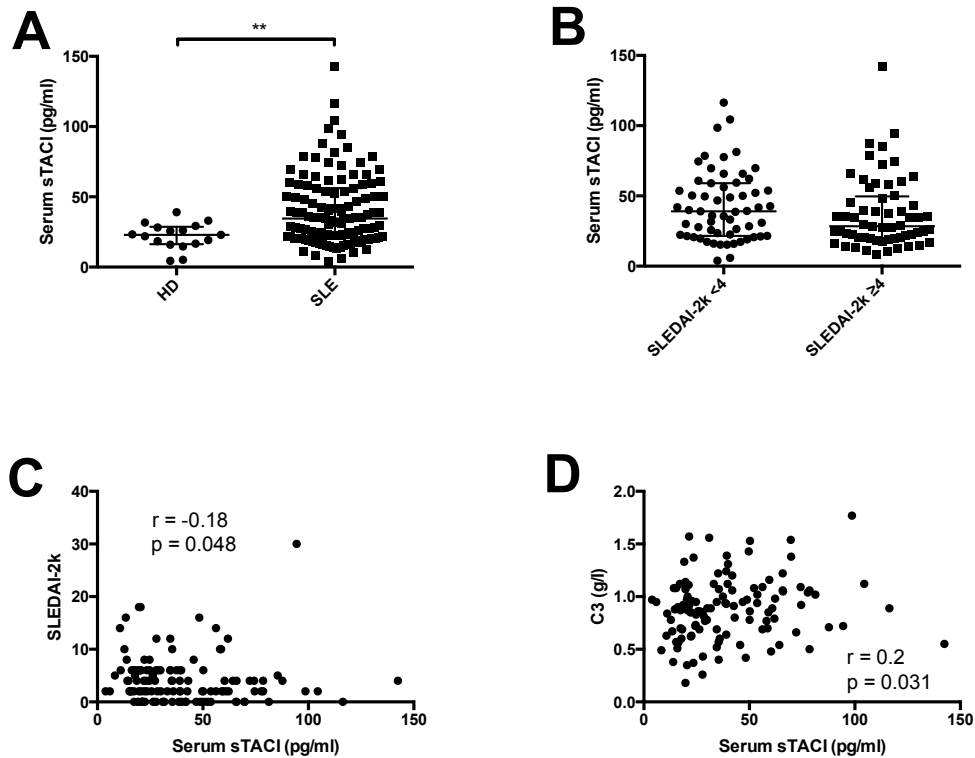


Figure 36. Associations of serum sTACI with SLE.

Overall SLE disease activity was assessed by the SLEDAI-2k score. (A) Serum sTACI concentrations in SLE (n=119) compared to HD (n=17). (B) Serum sTACI concentrations according to overall SLE disease activity (SLEDAI-2k <4: n=59; SLEDAI-2k ≥4: n=60). (C) Correlation between serum sTACI concentrations with SLEDAI-2k (n=119). (D) Correlation between serum sTACI concentrations with C3 levels (n=118).

Serum sTACI concentrations are expressed in pg/ml. C3 levels are expressed in g/l.

** $p < 0.01$

In panels A-B, horizontal bars indicate the median [IQR].

Associations of serum sBCMA with SLE

Serum sBCMA was detectable in all tested SLE and HD samples. However, as mentioned in the Methods Chapter, serum sBCMA concentrations were out of range (high) in one SLE sample, and this sample was excluded from analysis. Serum sBCMA concentrations were significantly higher in SLE compared to HD ($p=0.006$) (**Figure 37A**).

Relationship between serum sBCMA and SLE disease activity

No significant relationship emerged between serum sBCMA concentrations with overall SLE disease activity (**Figure 37B-C**). However, serum BCMA concentrations were significantly correlated with anti-dsDNA Abs levels ($r=0.24$, $p=0.01$; **Figure 37D**), and there was a non-significant trend toward a correlation with C3 levels ($r=-0.17$, $p=0.06$; **Figure 37E**), and toward higher serum sBCMA concentrations in SLE patients with anti-Sm Abs compared to those without ($p=0.064$; **Figure 37F**).

Serum sBCMA concentrations were significantly lower in patients with active neurological SLEDAI-2k compared to those without ($p=0.006$; **Figure 37G**). Serum sBCMA was also associated with neurological SLE in univariable (OR 0.9996, 95% CI 0.999, 0.9999; $p=0.012$) and multivariable analyses after adjusting for gender (OR 0.9997, 95% CI 0.999, 0.9999; $p=0.032$). However, only four patients had active neurological disease. No significant relationship was observed with other tested clinical phenotype subsets based on SLEDAI-2k scores (data not shown).

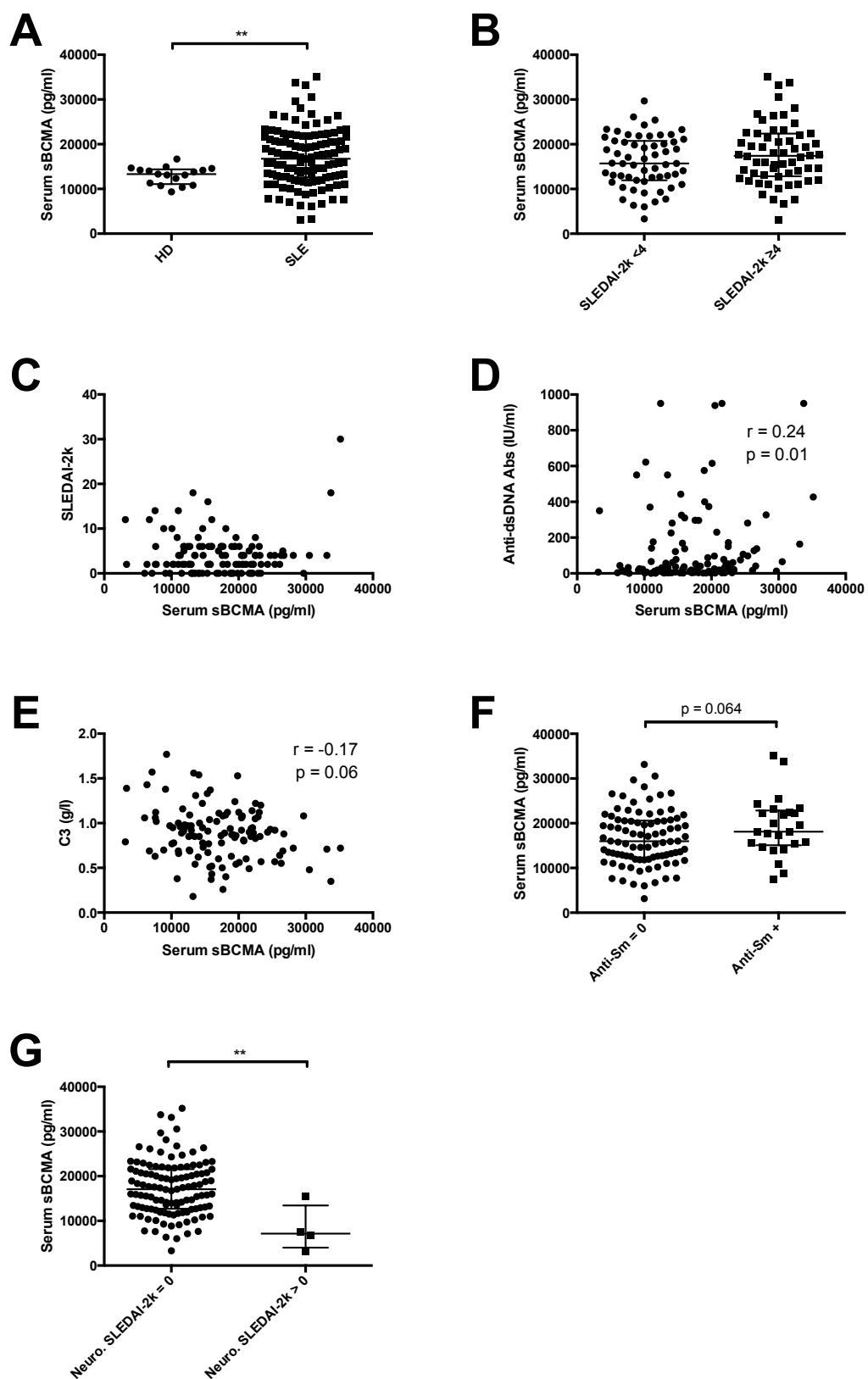


Figure 37. Associations of serum sBCMA with SLE.

Overall SLE disease activity was assessed by the SLEDAI-2k score. (A) Serum sBCMA concentrations in SLE (n=119) compared to HD (n=17). (B) Serum sBCMA concentrations according to overall SLE disease activity (SLEDAI-2k <4: n=59; SLEDAI-2k ≥4: n=60). (C) Correlation between serum sBCMA concentrations with SLEDAI-2k (n=119). Correlation between serum sBCMA concentrations with (D) anti-dsDNA (n=118) and (E) C3 levels (n=118). (F) Serum sBCMA concentrations according to the presence of anti-Sm Abs (Anti-Sm = 0: n=90; Anti-Sm +: n=25). (G) Serum sBCMA concentrations according to neurological SLE disease activity (Neuro. SLEDAI-2k = 0: n=115; Neuro. SLEDAI-2k > 0: n=4).

Serum sBCMA concentrations are expressed in pg/ml. Anti-dsDNA and C3 levels are expressed in IU/ml and g/l, respectively. Neuro. SLEDAI-2k stands for neurological SLEDAI-2k.

****** $p < 0.01$

In panels A-B, and F-G, horizontal bars indicate the median with [IQR].

Associations of serum sBAFF-R with SLE

Serum sBAFF-R concentrations were detectable in all tested SLE (n=120) and HD (n=11) samples. Serum sBAFF-R concentrations were significantly lower in SLE compared to HD ($p=0.011$; **Figure 38A**). No significant relationship emerged between serum sBAFF-R concentrations with overall SLE disease activity (**Figure 38B-C**). Serum sBAFF-R concentrations were significantly higher in SLE patients with active immunological SLEDAI-2k scores compared to those without ($p=0.039$; **Figure 38D**). However, only a non-significant trend toward an association was observed between sBAFF-R with active immunological disease in univariable analysis using logistic regression (OR 2.92, 95% CI 0.93, 9.18; $p=0.066$). No associations with other disease manifestations were detected (data not shown).

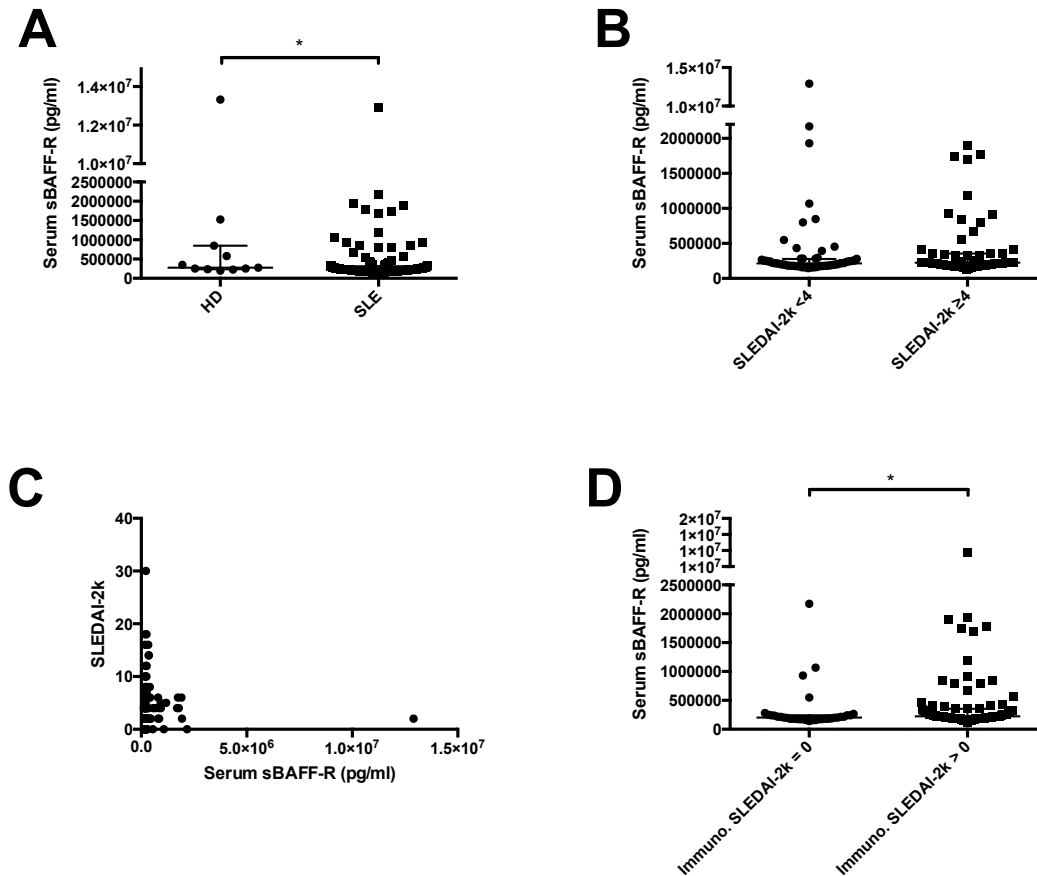


Figure 38. Associations of serum sBAFF-R with SLE.

Overall and immunological SLE disease activity was assessed by the SLEDAI-2k score. (A) Serum sBAFF-R concentrations in SLE (n=120) compared to HD (n=11). (B) Serum sBAFF-R concentrations according to overall SLE disease activity (SLEDAI-2k <4: n=60; SLEDAI-2k ≥4: n=60). (C) Correlation between serum sBAFF-R concentrations with SLEDAI-2k (n=120). (D) Serum sBAFF-R concentrations according to immunological SLE disease (Immuno. SLEDAI-2k = 0: n=33; Immuno. SLEDAI-2k > 0: n=87).

Serum sBAFF-R concentrations are expressed in pg/ml. Immuno. SLEDAI-2k stands for immunological SLEDAI-2k.

In panels A-B, and D, horizontal bars indicate the median with [IQR].

* $p < 0.05$

Relationship between serum BAFF and soluble BAFF receptors in SLE

Serum BAFF concentrations were not significantly correlated with concentrations of any soluble BAFF receptors in SLE (**Figure 39A-C**). In contrast, serum sBCMA concentrations were significantly correlated with sBAFF-R ($r=-0.29$, $p=0.001$; **Figure 39D**), while serum sTACI concentrations were not significantly correlated with either sBCMA (**Figure 39E**) or sBAFF-R (**Figure 39F**). In HD, while serum BAFF concentrations were similarly not significantly correlated with any soluble BAFF receptors (data not shown), serum sBCMA concentrations were positively correlated with both sBAFF-R and sTACI ($r=0.69$, $p=0.023$; **Figure 39G**; $r=0.67$, $p=0.004$; **Figure 39H**, respectively), while serum sTACI were not significantly correlated with sBAFF-R concentrations (**Figure 39I**). Thus, serum BAFF concentration was not significantly correlated with concentrations of any of its receptors in soluble form quantified in sera from SLE patients. The lack of correlation between serum BAFF with sTACI and sBAFF-R, and between serum sTACI with either sBCMA or sBAFF-R in SLE, are novel findings in this thesis. Also novel is the observation of a significant correlation between serum sBCMA and sBAFF-R in SLE.

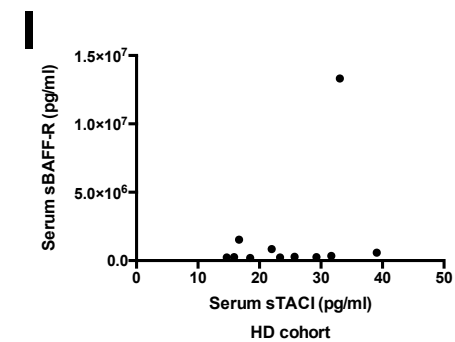
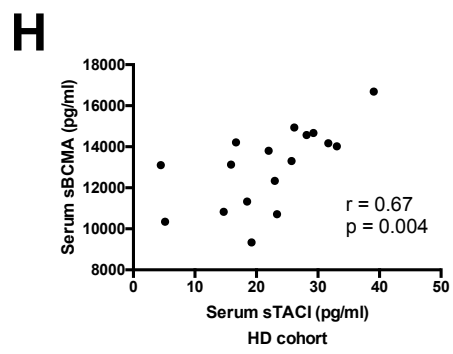
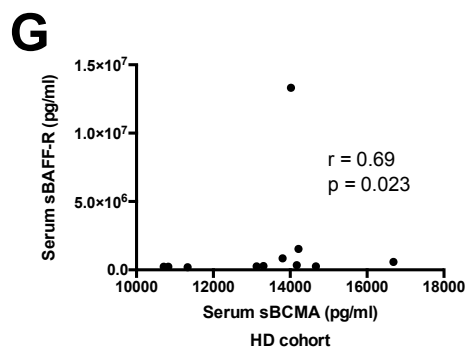
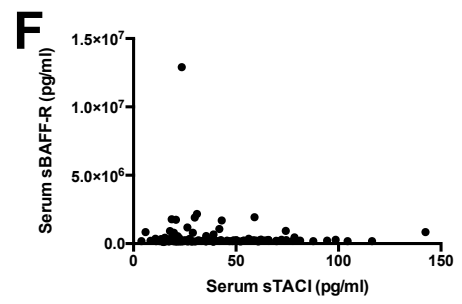
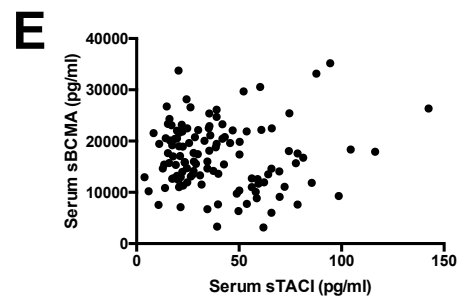
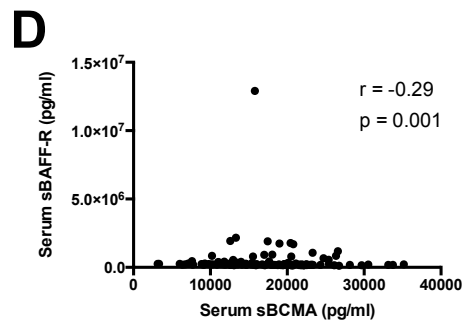
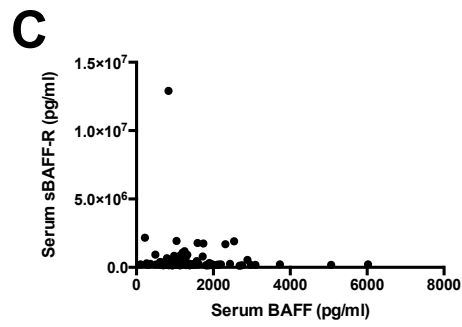
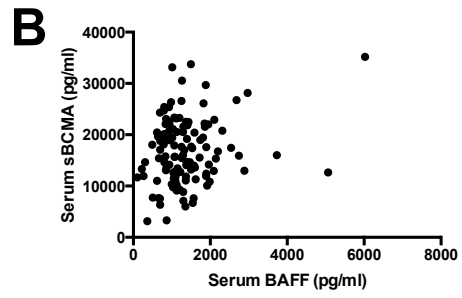
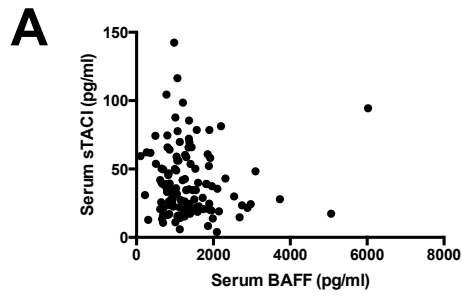


Figure 39. Relationship between serum BAFF with its soluble receptors in SLE and HD.

Correlation between serum BAFF with serum (A) sTACI (n=119), (B) sBCMA (n=119), and (C) sBAFF-R concentrations (n=120) in SLE. Correlation between serum sBCMA with (D) sBAFF-R (n=117) and (E) sTACI concentrations (n=118) in SLE. (F) Correlation between serum sTACI and sBAFF-R concentrations in SLE (n=117). Correlation between serum sBCMA with (G) sBAFF-R (n=11) and (H) sTACI concentrations (n=17) in HD. (I) Correlation between serum sTACI and sBAFF-R concentrations in HD (n=11). Serum BAFF, sTACI, sBCMA and sBAFF-R concentrations are expressed in pg/ml.

Associations of BAFF/sTACI ratio with SLE

The use of ratios between BAFF and soluble BAFF receptors might better reflect particular BAFF ligand/receptor(s) axes involved in SLE manifestations. This study is the first to investigate the clinical associations of ratios of BAFF and its soluble BAFF receptors in SLE.

Relationship between BAFF/sTACI ratio and SLE disease activity

There was a non-significant trend toward lower BAFF/sTACI ratio in SLE compared to HD (p=0.079; **Figure 40A**). BAFF/sTACI ratio was significantly higher in SLE patients with active disease compared to those without (p=0.003; **Figure 40B**), and was also significantly correlated with SLEDAI-2k (r=0.28, p=0.002; **Figure 40C**). There was also a non-significant trend toward a correlation with PGA (r=0.17, p=0.064; data not shown). BAFF/sTACI ratio was also significantly correlated with anti-dsDNA Abs levels (r=0.22, p=0.015; **Figure 40D**), C3 (r=-0.29, p=0.002; **Figure 40E**), albumin (r=-0.22, p=0.02; data not shown), and ESR (r=0.25, p=0.006; **Figure 40F**). BAFF/sTACI ratio was also significantly higher in SLE patients with anti-Ro Abs compared to those without (p=0.038; data not shown).

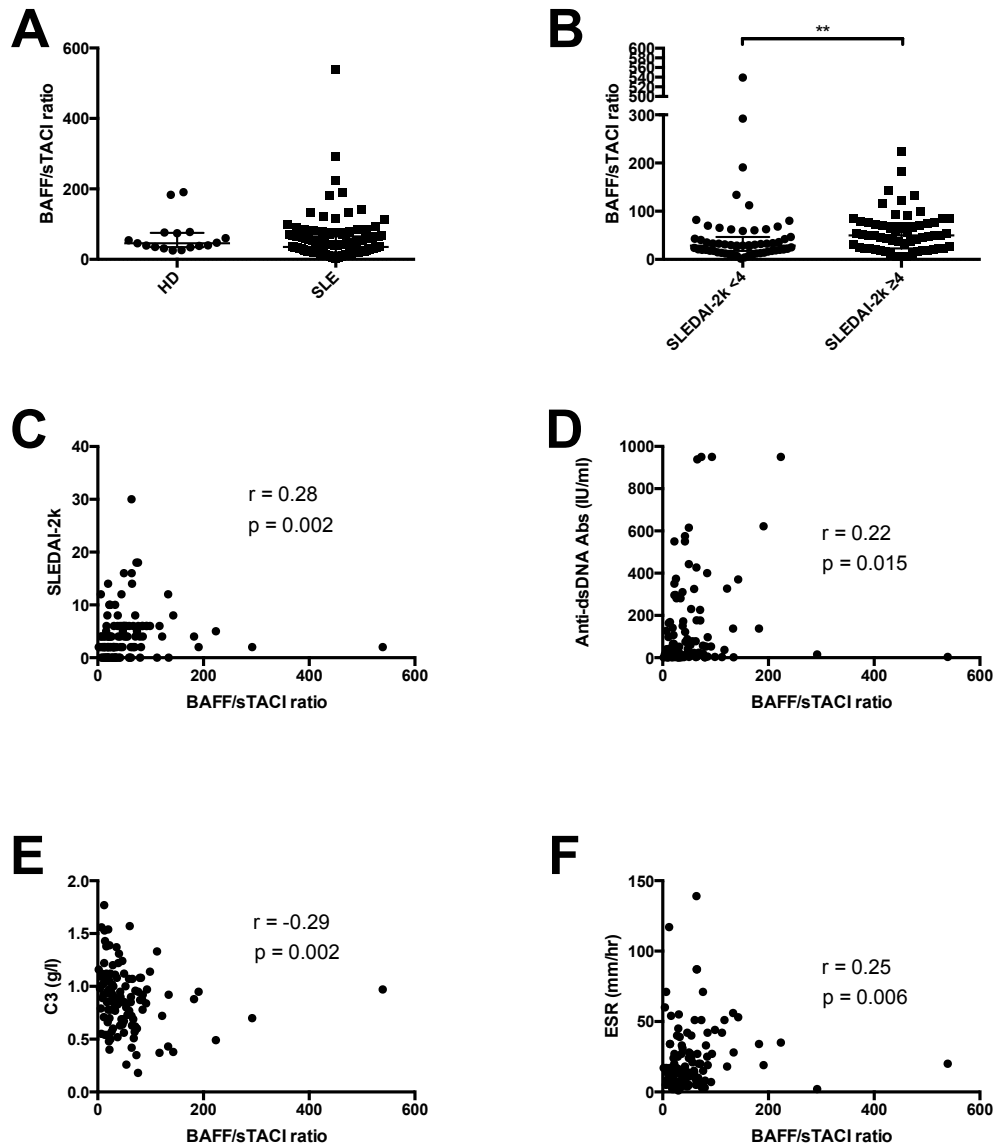


Figure 40. Associations of BAFF/sTACI ratio with SLE.

Overall SLE disease activity was assessed by the SLEDAI-2k score. (A) BAFF/sTACI ratio in SLE ($n=119$) compared to HD ($n=17$). (B) BAFF/sTACI ratio according to overall SLE disease activity (SLEDAI-2k <4: $n=59$; SLEDAI-2k ≥ 4 : $n=60$). (C) Correlation between BAFF/sTACI ratio with SLEDAI-2k ($n=119$). Correlation between BAFF/sTACI ratio with (D) anti-dsDNA Abs ($n=118$), (E) C3 ($n=118$), and (F) ESR ($n=119$) in SLE.

Serum BAFF and sTACI concentrations are expressed in pg/ml. Anti-dsDNA Abs, C3, and ESR are expressed in IU/ml, g/l, and mm/hr, respectively.

** $p < 0.01$

In panels A-B, horizontal bars indicate the median with [IQR].

Relationship between BAFF/sTACI ratio and organ-specific SLE

BAFF/sTACI ratio was significantly higher in SLE patients with active renal SLEDAI-2k compared to those without ($p=0.026$; one-tailed; **Figure 41A**). BAFF/sTACI ratio was also significantly although weakly correlated with renal SLEDAI-2k ($r=0.19$, $p=0.042$; **Figure 41B**). Of note, BAFF/sTACI ratio correlation with modified SLEDAI-2k minus renal SLEDAI-2k remained significant ($r=0.23$, $p=0.011$; data not shown). This suggests that the renal component of the SLEDAI-2k score did not entirely drive the association between BAFF/sTACI ratio and SLEDAI-2k. However, no significant association was found between BAFF/sTACI ratio and active renal disease using logistic regression.

BAFF/sTACI ratio was significantly higher in SLE patients with active musculoskeletal SLEDAI-2k compared to those without ($p=0.009$; **Figure 41C**). Of note, BAFF/sTACI ratio was still correlated with modified SLEDAI-2k minus musculoskeletal SLEDAI-2k ($r=0.22$, $p=0.015$; data not shown). This suggests that the musculoskeletal component of the SLEDAI-2k score did not entirely drive the observed positive correlations between BAFF/sTACI ratio and SLEDAI-2k. BAFF/sTACI ratio was also significantly associated with musculoskeletal SLE in univariable (OR 5.99, 95% CI 1.47, 24.39; $p=0.013$). No significant confounder was identified.

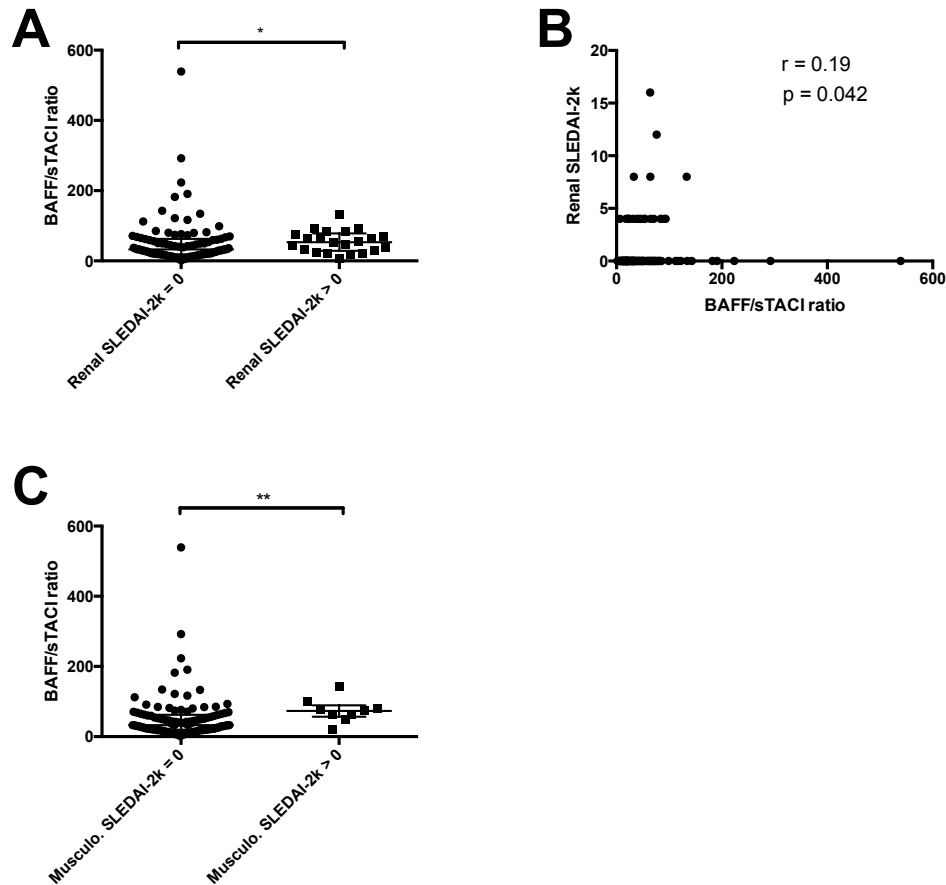


Figure 41. Associations of BAFF/sTACI ratio with organ-specific SLE.

Renal SLE disease activity was assessed by the SLEDAI-2k score. (A) BAFF/sTACI ratio according to renal SLE disease activity (Renal SLEDAI-2k = 0: n=97; Renal SLEDAI-2k > 0: n=22). (B) Correlation between BAFF/sTACI ratio with renal domain of the SLEDAI-2k score (n=119). (C) Ratio BAFF/sTACI according to musculoskeletal SLE disease activity (Musculo. SLEDAI-2k = 0: n=110; Musculo. SLEDAI-2k > 0: n=9).

Serum BAFF and sTACI concentrations are expressed in pg/ml. Musculo. SLEDAI-2k stands for musculoskeletal SLEDAI-2k.

In panel A, horizontal bars indicate the median with [IQR].

One-tailed p value is shown in panel A.

** p<0.01; * p<0.05

Associations of BAFF/sBCMA ratio with SLE

There was no significant difference in mean BAFF/sBCMA ratio between SLE and HD (**Figure 42A**). No significant difference was observed in BAFF/sBCMA ratio according to overall SLE disease activity (**Figure 42B**). However, there was a non-significant trend toward a correlation between BAFF/sBCMA ratio and SLEDAI-2k ($r=0.18$, $p=0.051$; **Figure 42C**). No significant relationship was observed with anti-dsDNA, C3 and C4 levels, but positive correlations were detected with inflammation markers CRP ($r=0.31$, $p=0.0007$; data not shown) and ESR ($r=0.21$, $p=0.025$; data not shown). No significant relationship emerged between BAFF/sBCMA ratio with any organ domain of SLE disease (data not shown).

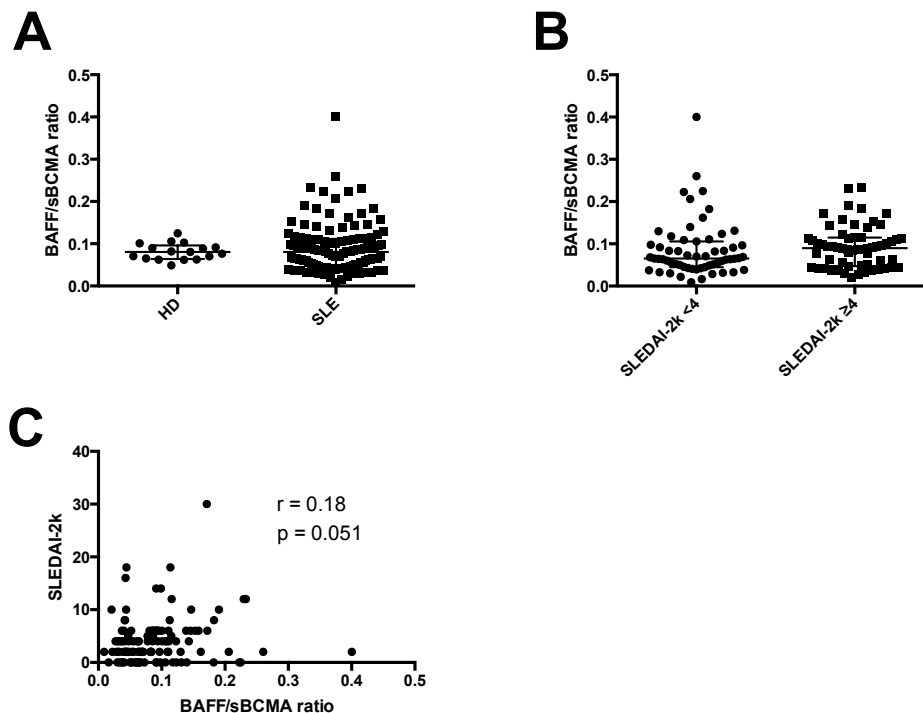


Figure 42. Associations of BAFF/sBCMA ratio with SLE disease.

Overall SLE disease activity was assessed by the SLEDAI-2k score. (A) BAFF/sBCMA ratio in SLE ($n=119$) compared to HD ($n=17$). (B) BAFF/sBCMA ratio according to overall SLE disease activity (SLEDAI-2k <4: $n=59$; SLEDAI-2k ≥ 4 : $n=60$). (C) Correlation between BAFF/sBCMA ratio with SLEDAI-2k ($n=119$).

Serum BAFF and sBCMA concentrations are expressed in pg/ml.

In panels A-B, horizontal bars indicate the median with [IQR].

Associations of BAFF/sBAFF-R ratio with SLE

BAFF/sBAFF-R ratio was significantly higher in SLE compared to HD ($p=0.036$; one-tailed; **Figure 43A**). No significant relationship emerged between BAFF/sBAFF-R ratio and overall SLE disease activity (**Figure 43B-C**). BAFF/sBAFF-R ratio was significantly higher in SLE patients with active musculoskeletal SLEDAI-2k compared to those without ($p=0.016$, **Figure 43D**). BAFF/sBAFF-R ratio was also significantly associated with musculoskeletal SLE disease activity in univariable analysis (OR 4.5, 95% CI 1.19, 17.03; $p=0.027$). No significant confounder was identified. No associations with other disease manifestations were detected (data not shown).

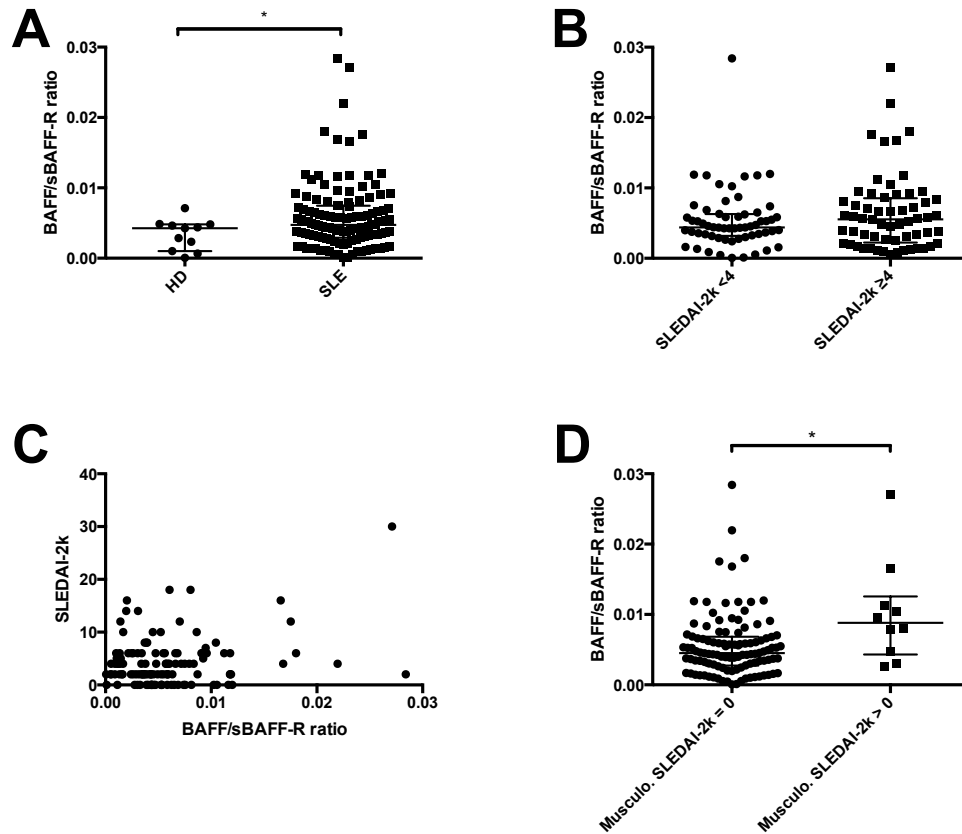


Figure 43. Associations of BAFF/sBAFF-R ratio with SLE.

Overall SLE disease activity was assessed by the SLEDAI-2k score. (A) BAFF/sBAFF-R ratio in SLE ($n=120$) compared to HD patients ($n=11$). (B) BAFF/sBAFF-R ratio according to overall SLE disease activity (SLEDAI-2k <4: $n=60$; SLEDAI-2k ≥ 4 : $n=60$). (C) Correlation between BAFF/sBAFF-R ratio with SLEDAI-2k ($n=120$). (D) BAFF/sBAFF-R ratio according to musculoskeletal SLE disease (Musculo. SLEDAI-2k = 0: $n=110$; Musculo. SLEDAI-2k > 0: $n=10$).

Serum sBAFF-R concentrations are expressed in pg/ml. Musculo. SLEDAI-2k stands for musculoskeletal SLEDAI-2k.

In panels A-B, horizontal bars indicate the median with [IQR].

One-tailed p value is shown in panel A.

* $p < 0.05$

Influence of ethnicity on serum soluble BAFF receptors in SLE

I next sought to investigate the relationship between serum sBAFF receptors and ethnicity in SLE. There was a non-significant trend toward a lower serum sTACI concentrations in Asian compared to Caucasian SLE patients ($p=0.0501$; **Figure 44A**). Of note, no significant discrepancy between the relationship of serum sTACI with overall (**Figure 44B-E**) or organ-specific (data not shown) SLE disease activity emerged when patients were stratified by ethnicity.

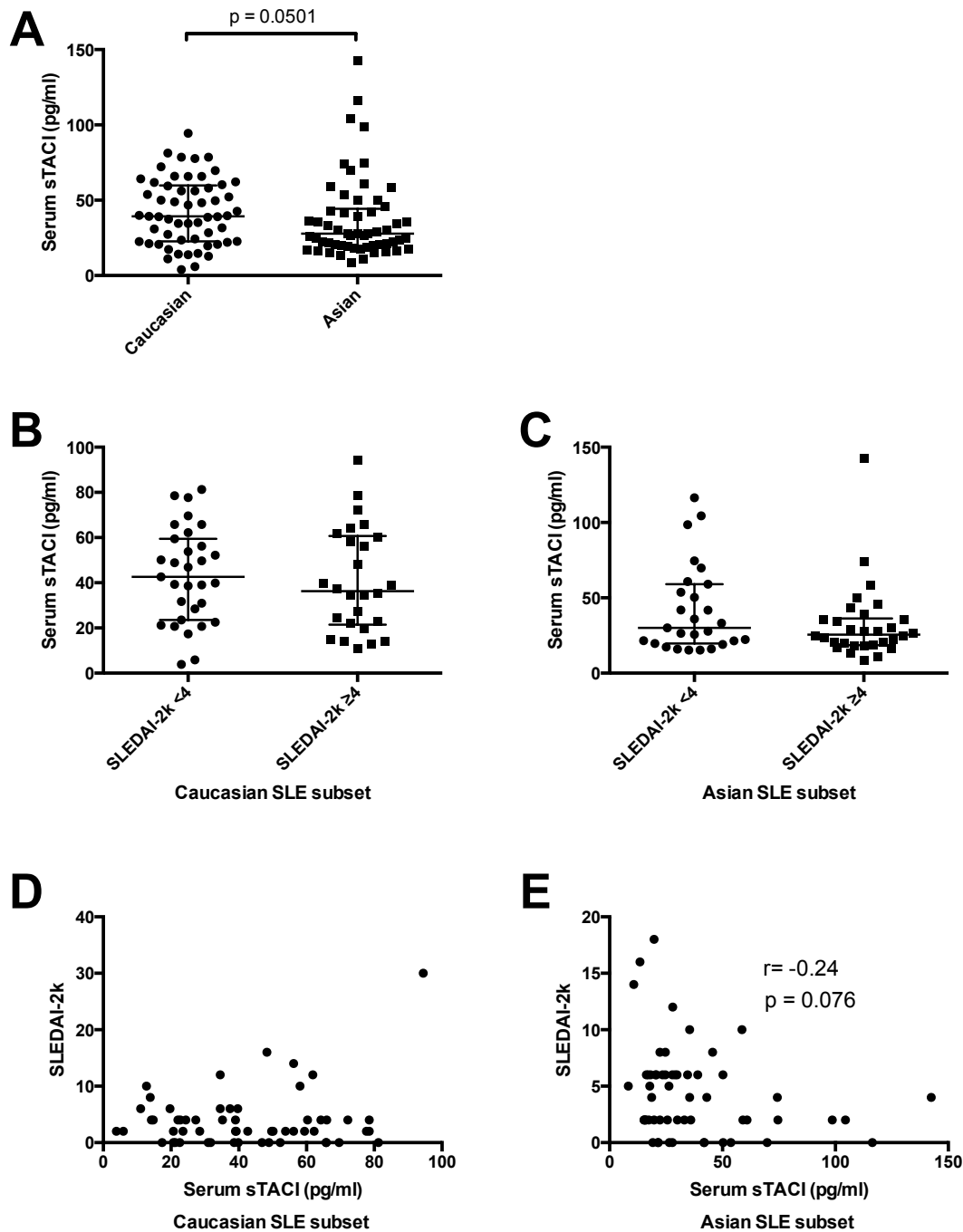


Figure 44. Influence of ethnicity on serum sTACI in SLE.

Overall SLE disease activity was assessed by the SLEDAI-2k score. (A) Serum sTACI concentrations between Caucasian (n=57) and Asian SLE patients (n=57). Serum sTACI concentrations according to overall SLE disease activity in (B) Caucasian (SLEDAI-2k <4: n=31; SLEDAI-2k ≥4: n=26) and (C) Asian SLE subsets SLEDAI-2k <4: n=27; SLEDAI-2k ≥4: n=30). Correlation between serum sTACI concentrations with SLEDAI-2k in (D) Caucasian (n=57) and (E) Asian SLE patients (n=57).

Serum sTACI concentrations are expressed in pg/ml.

In panels A-C, horizontal bars indicate the median with [IQR].

Serum sBCMA was significantly higher in Asian compared to Caucasian SLE patients ($p=0.043$; **Figure 45A**). However, no significant relationships between serum sBCMA with overall (**Figure 45B-E**) and organ-specific (data not shown) SLE disease activity emerged after ethnicity stratification.

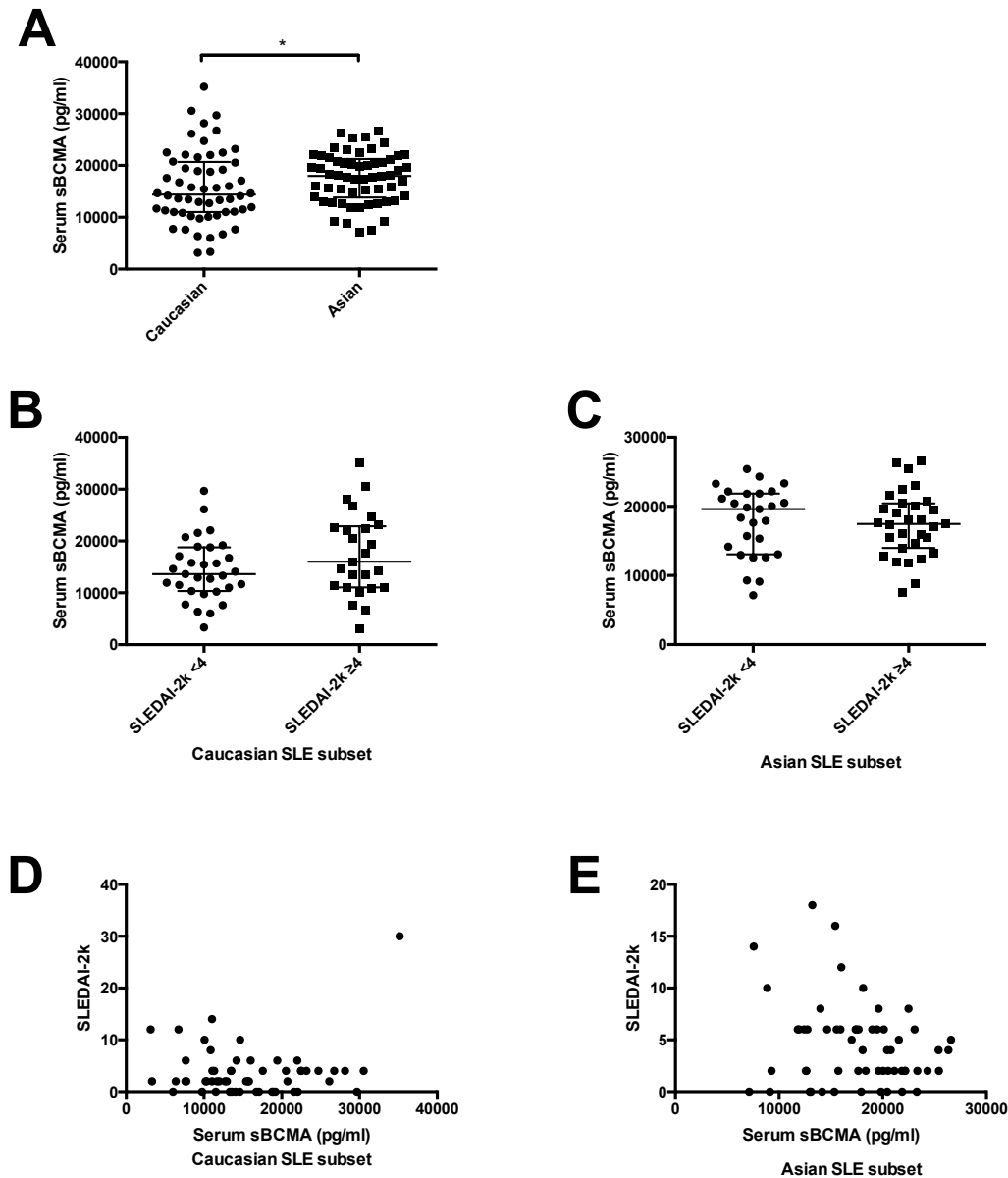


Figure 45. Influence of ethnicity on serum sBCMA in SLE.

Overall SLE disease activity was assessed by the SLEDAI-2k score. (A) Serum sBCMA concentrations between Caucasian (n=56) and Asian SLE patients (n=58). Serum sBCMA concentrations according to overall SLE disease activity in (B) Caucasian (SLEDAI-2k <4: n=31; SLEDAI-2k ≥4: n=25) and (C) Asian SLE subsets (SLEDAI-2k <4: n=27; SLEDAI-2k ≥4: n=31). Correlation between serum sBCMA concentrations with SLEDAI-2k in (D) Caucasian (n=56) and (E) Asian SLE subsets (n=58).

Serum sBCMA concentrations are expressed in pg/ml.

In panels A-C, horizontal bars indicate the median with [IQR].

* p < 0.05

There was a non-significant trend toward higher serum sBAFF-R concentrations in Asian compared to Caucasian SLE patients ($p=0.051$; **Figure 46A**). While not seen in Caucasians (**Figure 46B**), a significant association between serum sBAFF-R concentrations with SLEDAI-2k emerged in the Asian subset, wherein concentrations were higher in Asian SLE patients with active overall disease compared to those without ($p=0.025$; **Figure 46C**). Nevertheless, no significant correlation between serum sBAFF-R concentrations and overall SLE disease activity emerged after ethnicity stratification (**Figure 46D-E**). Only a non-significant trend toward an association between serum sBAFF-R and overall SLE disease activity was found in univariable analysis (OR 2.32, 95% CI 0.97, 5.53; $p=0.058$). After Bonferroni correction, this association was not significant. No significant relationship emerged after ethnicity stratification with SLE phenotype (data not shown).

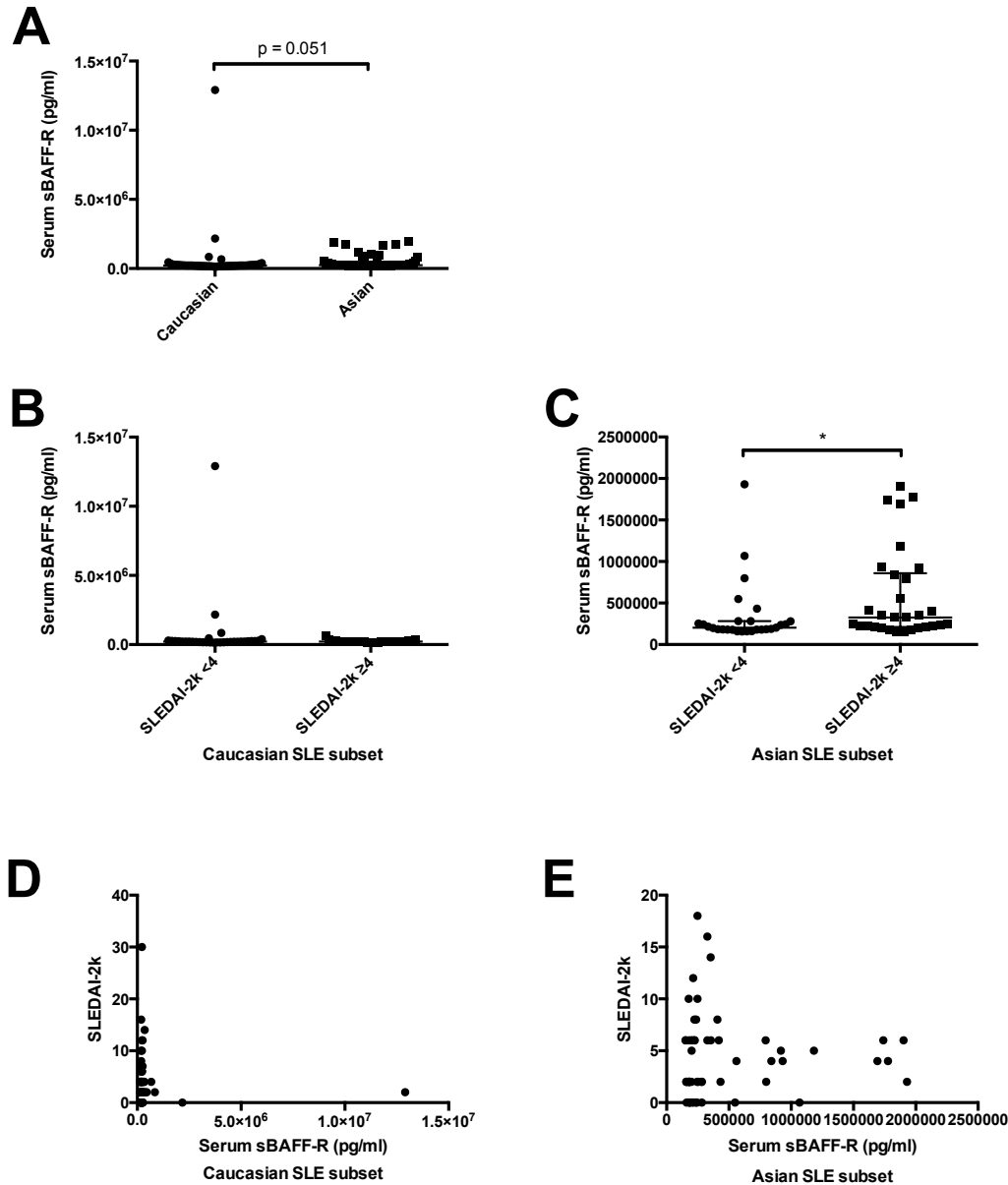


Figure 46. Influence of ethnicity on serum sBAFF-R in SLE.

Overall SLE disease activity was assessed by the SLEDAI-2k score. (A) Serum sBAFF-R concentrations between Caucasian (n=59) and Asian SLE patients (n=57). Serum sBAFF-R concentrations according to overall SLE disease activity in (B) Caucasian (SLEDAI-2k <4: n=32; SLEDAI-2k ≥ 4 : n=27) and (C) Asian SLE subsets (SLEDAI-2k <4: n=27; SLEDAI-2k ≥ 4 : n=30). Correlation between serum sBAFF-R concentrations with SLEDAI-2k in (D) Caucasian (n=59) and (E) Asian SLE subsets (n=57).

Serum sBAFF-R concentrations are expressed in pg/ml

In panels A-C, horizontal bars indicate the median with [IQR].

* $p < 0.05$

There was a non-significant trend toward a higher BAFF/sTACI ratio in Asian compared to Caucasian SLE patients ($p=0.052$; **Figure 47A**). The relationship between BAFF/sTACI ratio with overall SLE disease activity appeared independent of ethnicity. BAFF/sTACI ratio was higher in SLE patients with active overall disease in both Caucasian ($p=0.03$; **Figure 47B**) and Asian SLE subsets ($p=0.044$; **Figure 47C**). There was also a non-significant trend toward a correlation between BAFF/sTACI ratio with SLEDAI-2k in Caucasian SLE patients ($r=0.24$, $p=0.074$; **Figure 47D**), and BAFF/sTACI ratio was significantly correlated with SLEDAI-2k in Asian SLE patients ($r=0.28$, $p=0.038$; **Figure 47E**). BAFF/sTACI ratio was also associated with overall SLE disease activity in univariable analysis using logistic regression (OR 2.94, 95% CI 1.12, 7.74; $p=0.029$). No significant confounder was identified. No significant relationship emerged by ethnicity stratification with SLE phenotype (data not shown).

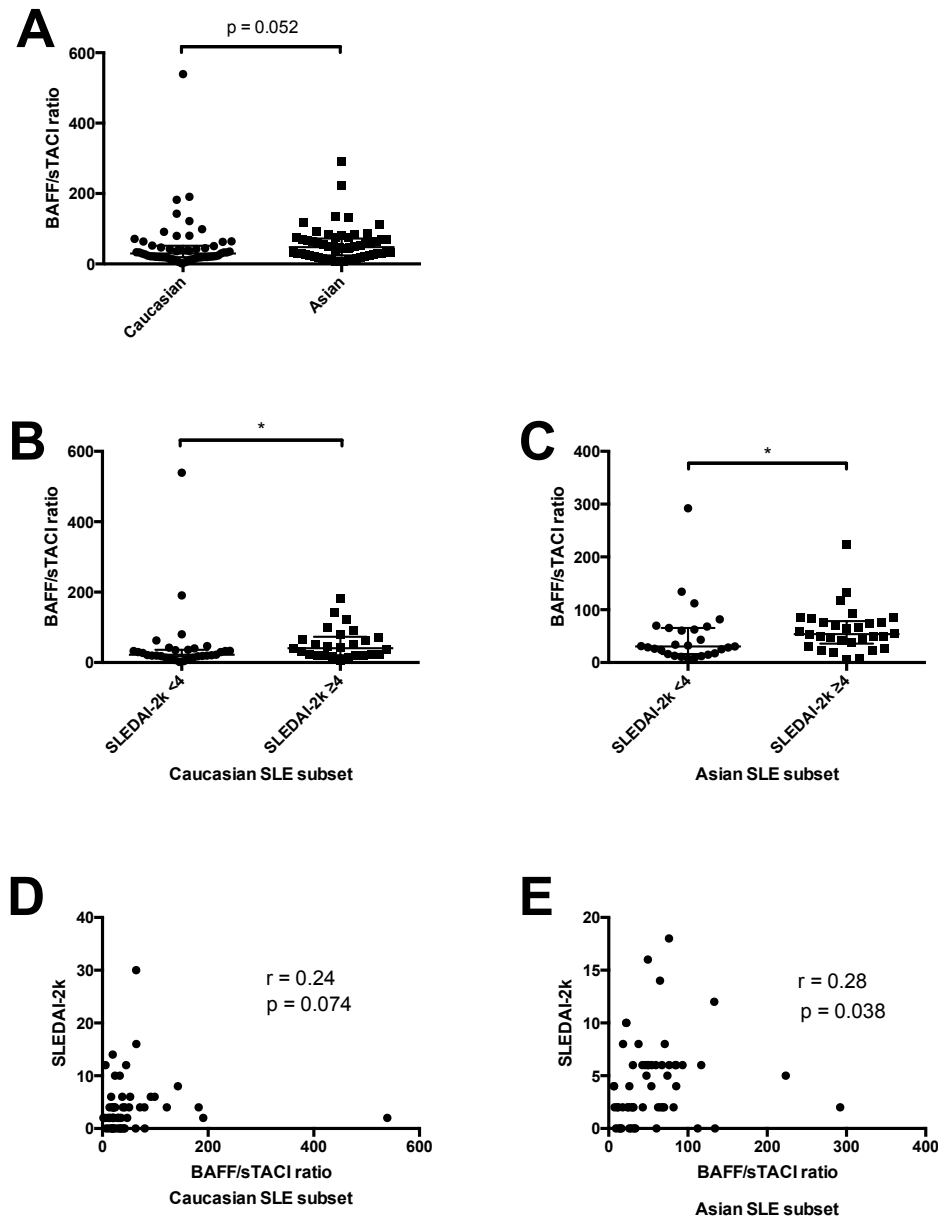


Figure 47. Influence of ethnicity on BAFF/sTACI ratio in SLE.

Overall SLE disease activity was assessed by the SLEDAI-2k score. (A) BAFF/sTACI ratio between Caucasian (n=57) and Asian SLE patients (n=57). BAFF/sTACI ratio according to overall SLE disease activity in (B) Caucasian (SLEDAI-2k <4: n=31; SLEDAI-2k ≥4: n=26) and (C) Asian SLE subsets (SLEDAI-2k <4: n=27; SLEDAI-2k ≥4: n=30). Correlation between BAFF/sTACI ratio with SLEDAI-2k in (D) Caucasian (n=57) and (E) Asian SLE subsets (n=57).

Serum BAFF and sTACI concentrations are expressed in pg/ml.

In panels A-C, horizontal bars indicate the median with [IQR].

* $p < 0.05$

No significant difference emerged in BAFF/sBCMA ratio according to ethnicity (**Figure 48A**), and no significant relationships between BAFF/sBCMA ratio and overall (**Figure 48B-E**) or organ-specific (data not shown) SLE disease activity emerged when patients were stratified by ethnicity.

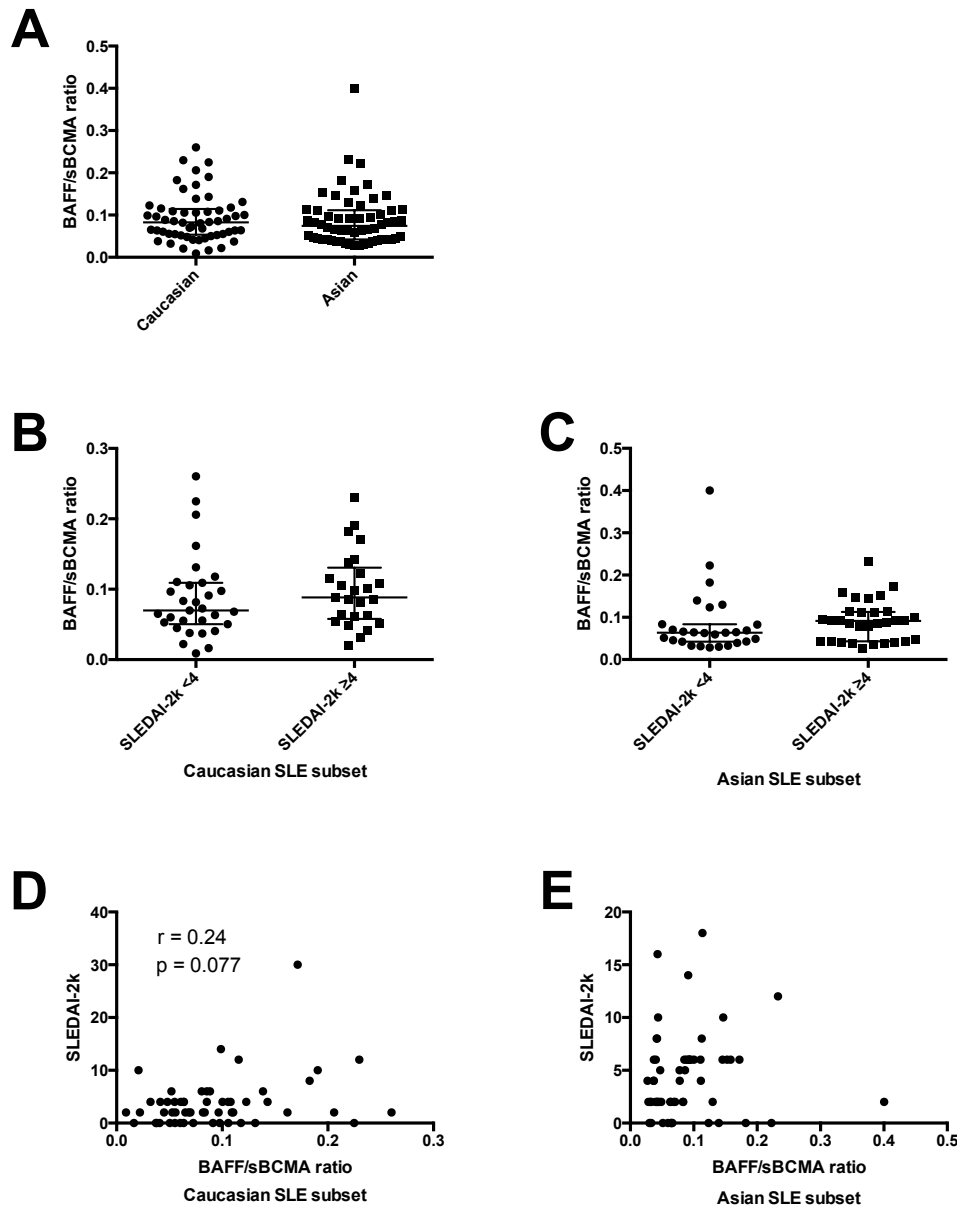


Figure 48. Influence of ethnicity on BAFF/sBCMA ratio in SLE.

Overall SLE disease activity was assessed by the SLEDAI-2k score. (A) BAFF/sBCMA ratio between Caucasian (n=56) and Asian SLE patients (n=58). BAFF/sBCMA ratio according to overall SLE disease activity in (B) Caucasian (SLEDAI-2k <4: n=31; SLEDAI-2k ≥4: n=25) and (C) Asian SLE subsets (SLEDAI-2k <4: n=27; SLEDAI-2k ≥4: n=31). Correlation between BAFF/sBCMA ratio with SLEDAI-2k in (D) Caucasian (n=56) and (E) Asian SLE subsets (n=58).

Serum sBCMA concentrations are expressed in pg/ml.

In panels A-C, horizontal bars indicate the median with [IQR].

There was a non-significant trend toward a lower BAFF/sBAFF-R ratio in Asian compared to Caucasian SLE patients ($p=0.06$; **Figure 49A**). A new association emerged, when patients were stratified by ethnicity, where BAFF/sBAFF-R ratio was significantly higher in Caucasian SLE patients with active overall disease compared to those without ($p=0.02$; **Figure 49B**), while this was not seen in Asian SLE subset (**Figure 49C**). Nevertheless, no significant correlation between BAFF/sBAFF-R ratio with overall SLE disease activity emerged after ethnicity stratification (**Figure 49D-E**). No association was found between BAFF/BAFF-R ratio and overall SLE disease activity in the whole cohort using logistic regression models, in line with the data presented above. Hence, ethnicity could not be tested as a potential confounder. However, after Bonferroni correction, the above presented association between BAFF/BAFF-R ratio and overall SLE disease activity in Caucasians remained significant.

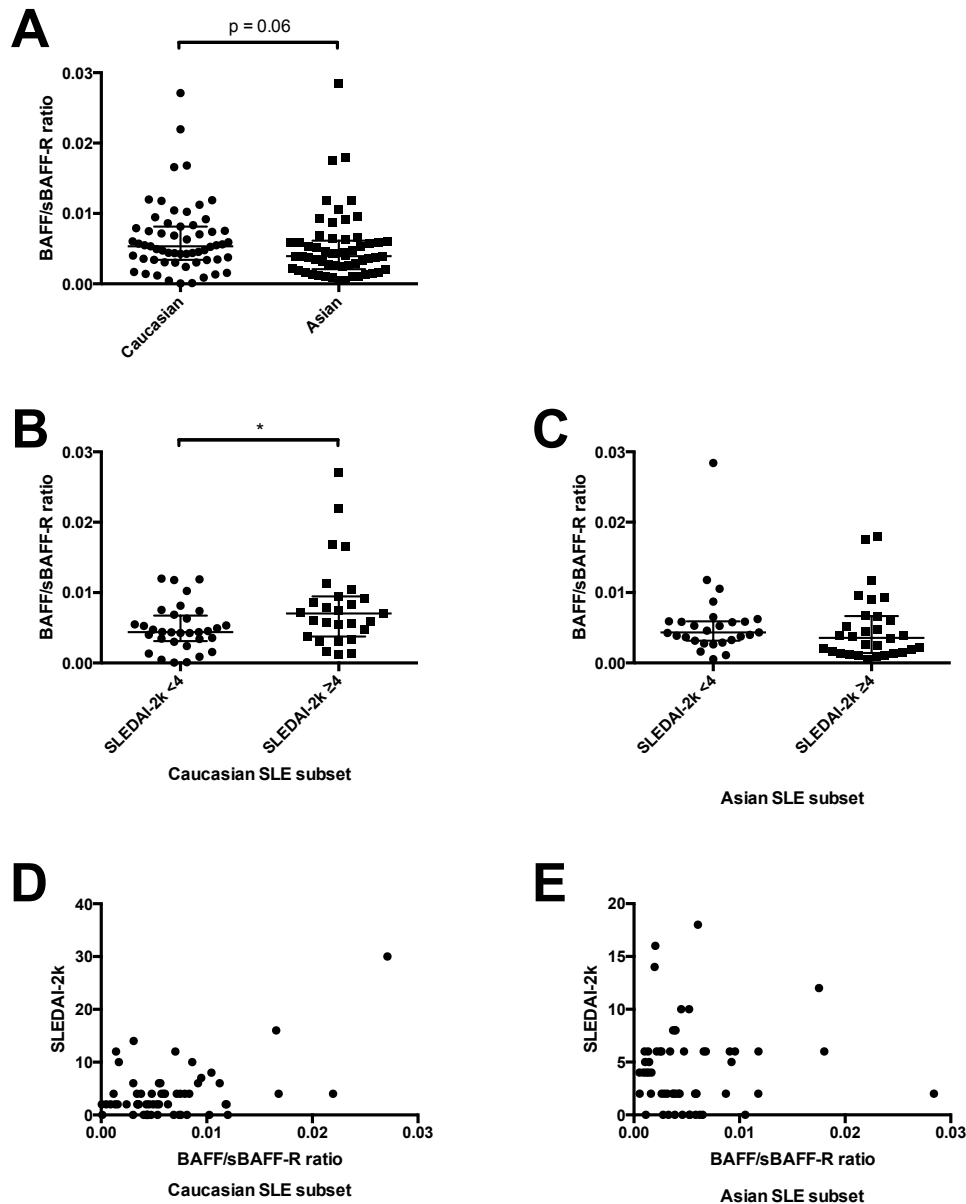


Figure 49. Influence of ethnicity on BAFF/sBAFF-R ratio in SLE.

Overall SLE disease activity was assessed by the SLEDAI-2k score. (A) BAFF/sBAFF-R ratio between Caucasian ($n=59$) and Asian SLE patients ($n=57$). BAFF/sBAFF-R ratio according to overall SLE disease activity in (B) Caucasian (SLEDAI-2k <4: $n=32$; SLEDAI-2k ≥ 4 : $n=27$) and (C) Asian SLE subsets (SLEDAI-2k <4: $n=27$; SLEDAI-2k ≥ 4 : $n=30$). Correlation between BAFF/sBAFF-R ratio with SLEDAI-2k in (D) Caucasian ($n=59$) and (E) Asian SLE subsets ($n=57$).

Serum BAFF and sBAFF-R concentrations are expressed in pg/ml.

In panels A-C, horizontal bars indicate the median with [IQR].

* $p < 0.05$

4. Discussion

This study aimed to evaluate the presence of all soluble BAFF receptors in a large well-defined SLE cohort, and to characterize their clinical associations with SLE phenotype. The present study also investigated potential interference of the presence of soluble BAFF receptors when detecting BAFF in patient sera.

The present study is supported by data I received from R & D Systems reporting interference of both sTACI and sBCMA with serum BAFF detection when combined into the same multiplex assay. It could be extrapolated that the use of a multiplex assay to measure BAFF and sBAFF-R in the same sample will face technical issues. I also found interference by the presence of sBAFF-R with serum BAFF quantification, being a novel finding in the thesis. Based on the reported sBCMA decoy activity being restricted to APRIL,[90] it can be speculated that no impact on serum BAFF detection would be observed. To summarize, interference between soluble form of BAFF receptors with serum BAFF detection were observed either indirectly through impact on the generation of standard curves when using a multiplex assay, or directly when detecting BAFF by ELISA with the concomitant presence of soluble form of BAFF receptors in the serum. Therefore, here and throughout this thesis, ELISA assay was used to quantify serum BAFF concentrations separately.

The main limitation of the use of Luminex technology in the present study, which also constitutes a novel finding in this thesis, is that it cannot be used to quantify both ligand and soluble receptors from the BAFF system within the same assay. Rather, it is necessary for BAFF ligand and its cognate soluble receptors to be quantified in separate assays using the Luminex platform. Another option, as in the present study, is that receptors can be assayed together, while BAFF can be measured by another technology, such as ELISA. This avoids the main limitation of protein multiplexing using Luminex technology, namely interference between analytes. Another limitation of the Luminex assay, that also applies to other

multiplex technologies such as Quantibody, is the potential differences in dilution required for each analyte. Luminex assays are also limited by bead region interference, where two analytes of interest may have the same bead region assigned. In this case, discussion with the manufacturer is needed to try to assign a different bead region to each analyte. Otherwise, as for dilution factor difference, analytes cannot be assayed together, and multiple Luminex plates have to be run using a lower plexing approach. The other limitation discussed in this study is the presence of soluble BAFF receptors in human sera, which may hide the epitope recognized by the capture/detection antibodies used in Luminex and ELISA assays for BAFF quantification. However, this is not inherent to the Luminex assay, as it can apply to any assay used to detect BAFF in human sera.

Whether the detected levels of BAFF and soluble BAFF receptors in this study include complexed forms with their respective cognate soluble receptors/ligands is not known. I received additional information from the manufacturer that Abs used in the Luminex assay for sTACI and sBCMA, as well as the detection Ab for BAFF ELISA were all neutralizing Abs. I do not, however, have information about epitope mapping for capture and detection Abs used in these commercial assays, hence, it remains unknown if these are blocking Abs, thus interfering with ligand/receptor binding. The presence of BAFF/receptor complexes, for instance BAFF/sTACI complexes, could be determined by performing co-immunoprecipitation (co-IP) on serum samples by using anti-BAFF antibodies, then performing Western blot to detect sTACI. Another way would be to perform a sandwich ELISA with anti-BAFF capture antibody and anti-TACI detection antibody, and vice versa. It would be of interest to know whether detected levels of complexed and/or free soluble BAFF receptors or serum BAFF are of particular relevance in human AID. The fact that some assays may have used blocking Abs for BAFF detection and/or capture, while other not, may explained, at least in part, some observed discrepancies between many published studies

quantifying human serum BAFF. Hence, when assessing serum BAFF levels, it appears important to take into account the presence of endogenous soluble form of BAFF receptors that may interfere with serum BAFF detection. Future research investigating the role of soluble receptors and/or ligands of the BAFF/APRIL system in autoimmunity should identify and distinguish complexed and free forms of these soluble receptors and ligands.

Here, I describe the presence of soluble forms of the three BAFF receptors in SLE. I report, for the first time, the presence of sBAFF-R in sera from SLE and healthy individuals. The present study also confirmed the presence of both sTACI and sBCMA in sera from SLE patients, in a larger prospective and better defined cohort than any reported before.[39, 70, 90] Both serum sTACI and sBCMA concentrations were significantly higher, while serum sBAFF-R concentrations were significantly lower, in SLE compared to HD. Since no significant difference in serum BAFF concentrations was observed between SLE and HD (see Chapter V), this suggests that soluble BAFF receptors may be more specific than BAFF for SLE. Higher serum sTACI and sBCMA concentrations were previously reported in SLE patients, when compared to healthy individuals.[39, 70, 90] Of note, concentrations of serum sTACI in Hoffmann *et al.* study ranged from about 0 to about 9,500 pg/ml, while in the present study serum sTACI ranged from 3.9 to 142.4 pg/ml. There was also an approximately 3-fold difference in serum sBCMA concentrations between Laurent *et al.* and the present studies. Concentration differences may be explained by the difference between ELISA and Luminex assays, with potential assay sensitivity variations, and/or the potential detection of the complex of BAFF-sTACI.

As reported in Chapter V, serum BAFF concentrations were positively related to overall SLE disease activity, in line with some published studies (reviewed in[184]). The observed significant negative relationship between serum sTACI and overall SLE disease activity in the present study is consistent with the decoy function of sTACI for both BAFF

and APRIL reported by Hoffmann *et al.*.[70] The presence of sTACI may lead to a reduction in free BAFF, hence less functional (pathogenic) signalling. Hoffmann *et al.* suggested the usefulness of sTACI as a biomarker in both human MS and SLE. However, they reported a positive correlation between serum sTACI and SLEDAI in untreated patients, while a negative correlation was observed in the present study. Considering the decoy function of sTACI for BAFF, a negative correlation was, in fact, expected. This study is the first to report a correlation between serum sTACI with C3 component in SLE. Of particular interest, a relationship between BAFF/sTACI ratio with overall SLE disease activity emerged, characterized by a stronger correlation than that of either serum BAFF and sTACI alone. Further research is needed to confirm whether a biomarker strategy integrating both BAFF and sTACI might help physician to stratify SLE patients to guide therapeutic intervention, particularly for belimumab (anti-BAFF) therapy.

Serum sTACI has previously been reported higher in SLE patients with renal manifestations, in a study presented in abstract form only in 2001,[39] however this was not confirmed in the present study, where no association was found with any other clinical phenotypes, constituting novel negative findings in this thesis. In the present study, significant positive relationships emerged between serum BAFF/sTACI ratio with renal SLE disease using non-parametric test. However, this association was not confirmed using logistic regression model.

The present study also suggests a potential association between BAFF/sTACI ratio and musculoskeletal SLE activity. As reported in Chapter V, BAFF was also potentially associated with this phenotype. It is worth to note that BAFF/sTACI ratio had a stronger association with this SLE phenotype compared to BAFF alone. As underlined in Chapter V, due to the limited number of patients with an active musculoskeletal disease in the present study, these data need to be confirmed in a larger cohort, while also prompting to investigate

the combination of BAFF and sTACI as potential biomarkers to guide anti-BAFF treatment for this phenotype.

sBCMA has recently been reported acting as a decoy only for APRIL.[90] In a pooled cohort of SLE patients, sBCMA was positively correlated with SLEDAI.[90] Conversely, the present data on a much larger cohort did not show any significant correlation with SLEDAI-2k. Consistent with the decoy function of sBCMA being restricted to APRIL, no significant relationship emerged with overall or organ-specific SLE disease when using the BAFF/sBCMA ratio. Amongst the novel findings in this study, it is of note that a negative relationship was found between levels of serum sBCMA and neurological disease activity, while not with other clinical phenotypes. Notwithstanding that this subset of SLE patients with active neurological disease is too small to draw any solid conclusions from, it could be speculated that the decoy role of sBCMA for APRIL may be particularly important in neurological SLE, to optimally regulate systemic and/or *in situ* free-form of soluble APRIL. Both sTACI and sBCMA have been detected in high levels in CSF of MS patients, but not in their blood, suggesting an *in situ* CNS production and/or a CNS influx from systemic production.[70, 90] Both BAFF and APRIL have been reported detectable and in higher levels in CSF of SLE patients compared to healthy controls. Interestingly, CSF APRIL, but not BAFF, was particularly higher in SLE patients with neuropsychiatric SLE disease compared to those without.[56] These authors found no correlation between serum and CSF APRIL, and suggested an *in situ* production of CSF-detected APRIL. Moreover, both BAFF and APRIL have been reported being produced in CNS by astrocytes, in MS.[87, 165] Interestingly, BCMA was also reported increased in CNS of MS patients compared to normal brain.[87] All together, this emphasizes the potential important role for the APRIL-BCMA axis in CNS SLE disease pathogenesis, and these data prompt further investigation on sBCMA in this SLE phenotype. Determining the role of soluble BAFF receptors in

neurological SLE and MS conditions might improve our knowledge on the mechanism of action of atacicept, a fusion protein consisting of the extra-cellular domain of TACI fused to a human IgG Fc domain, which can bind BAFF, APRIL and BAFF-APRIL heterotrimers (reviewed in[184]).

As mentioned above, this work constitutes the first study to show the presence of sBAFF-R in sera from SLE and healthy individuals, but also to investigate its clinical associations in SLE. There was a non-significant trend toward an association between serum sBAFF-R and immunological domains of the SLEDAI-2k score, although not to the overall disease activity. Some data also suggest a relationship between BAFF/sBAFF-R ratio and musculoskeletal disease activity, albeit being weaker than the association of BAFF/sTACI ratio with this phenotype. As mentioned above, this phenotypic subset was of small size in the present study, making hard to draw any solid conclusion, and my findings will need confirmation in larger cohort. Whether BAFF, sTACI and/or sBAFF-R play a role in this disease phenotype and could be targeted therapeutically remains to be determined.

SLE is a multifactorial disease, where genetic factors are acknowledged to play an important role in disease pathogenesis (reviewed in[190]). Consistent with this, SLE is reported of a higher prevalence and more severe in different ethnicities, including Asian and Indigenous Australian (IA), when compared to Caucasians, even when studied at the same centre (reviewed in[182, 184]). Serum BAFF levels have been reported potentially different between African-American and White American SLE patients, with potential differences with regard to its relationship with disease activity only observed in White Americans (reviewed in[184]). Here, Asian SLE patients displayed significantly higher serum sBCMA, and a trend toward both lower sTACI and higher BAFF-R levels, compared to Caucasians. As reported in Chapter V, no difference in serum BAFF was found between Asian and Caucasian, in line with our previous published work.[185] It is hence unlikely that observed

differences in BAFF receptor concentrations were the consequences of variations in serum BAFF concentrations between ethnic groups. These novel findings suggest that ethnicity might influence serum sBAFF receptor levels in SLE patients.

Amongst the novel findings shown in this thesis, a significant relationship was seen between BAFF/sBAFF-R ratio and overall SLE disease activity in Caucasians, while this relationship was not seen in the un-stratified cohort. This emphasises that the lack of relationship between biomarker candidates and overall disease activity does not preclude the possibility of relationship in patient subsets stratified by ethnicity or other profiles. The same is true when comparing clinically un-stratified SLE patients versus SLE patient subsets stratified by clinical phenotype. These observations support the need for highly nuanced analysis of cytokine biomarker associations in SLE, and point to the heterogeneity of the disease biology across ethnic and clinical subsets.

Data from the present study are also supported by some clinical trials where anti-BAFF therapies (belimumab and tabalumab) showed efficacy, in SLE cohorts characterised by a high predominance of musculoskeletal and mucocutaneous manifestations.[54, 77, 102, 109] Interestingly, in a *post-hoc* analysis of the two BLISS-52 and BLISS-76 phase III clinical trials of belimumab in SLE,[54, 109] Manzi *et al.* reported that SLE patients in these trials were predominantly suffering from mucocutaneous and musculoskeletal manifestations along with immunological domains.[98] Improvement was observed in response to belimumab therapy compared to placebo for those three particular organ domains. Of note, patients with acute LN and severe CNS disease were excluded from the two trials.[54, 109] However, *post-hoc* analysis suggested that belimumab therapy might benefit SLE patients with kidney disease (reviewed in[184]). This suggests that the immunological pathway involving the BAFF/APRIL system in SLE may be associated with clinical phenotypes involving mucocutaneous, musculoskeletal, renal and immunological domains. The present

data revealed relationships between soluble components of the BAFF/APRIL system particularly with renal, and musculoskeletal and neurological disease activity. Collectively, this gives more weight to the hypothesis that the BAFF/APRIL system could be primarily involved in such phenotypic SLE manifestations. Using these BAFF/APRIL system biomarkers to stratify SLE patients may help to guide anti-BAFF therapeutic strategy. This also underlines the need to assess therapies targeting the BAFF/APRIL system in SLE patients suffering from renal and/or neurological SLE in clinical trials.

Serum BAFF concentrations have been reported to be affected by the use of anti-CD20 (rituximab) and anti-BAFF (belimumab and tabalumab) therapies.[77, 102, 124, 178] The increased levels of serum BAFF in patients receiving anti-BAFF therapies could be considered counterintuitive. It is, however, in line with previous published studies,[77, 102] where an increase in serum BAFF levels was observed in SLE patients receiving tabalumab, and explained by the fact that the assay used can detect the complex of BAFF and tabalumab. Regarding belimumab, in the reported clinical trials,[54, 109, 191] serum BAFF was only quantified prior to belimumab dosing, hence data on belimumab effects on serum BAFF in SLE are lacking. Studies in pSS, however, did not show any sustained increase in serum BAFF after belimumab therapy, but rather a transient decrease at week 4 post therapy, restored to baseline levels by week 24.[124] Differences between serum BAFF levels in pSS patients receiving belimumab therapy in this latter study and in SLE patients in the present study may be explained by differences in the assay used, as well as the delay between anti-BAFF therapy administration and serum BAFF measurement. Whether the use of these drugs also leads to variation in serum sBAFF receptors concentrations was previously unknown. In the present study, non-significant differences were found in serum sBAFF receptors concentrations according to the use of these drugs. Serum BAFF has been reported not to be a predictive biomarker for belimumab therapy effectiveness,[149] but was reported to be a

predictive biomarker for atacicept therapy.[64] Based on the present data, and while taking into account the small size of the anti-BAFF- and anti-CD20-treated SLE cohorts, as well as the absence of pre- and post-treatment samples, it could be speculated that serum sBAFF receptors may be interesting candidates as pharmacodynamic biomarker(s) for anti-BAFF therapies and B-cell depleting agents. Hoffmann *et al.* also suggested the potential usefulness of sTACI as a biomarker for optimization of B-cell targeting drugs.[70] However, surface expression of these receptors has to also be considered. TACI is mostly expressed on CD27⁺ memory B cells, BCMA is predominantly expressed on plasma cells and plasmablasts, while BAFF-R is expressed early in B cell development, from immature B cell stage up to plasma cells (reviewed in[50, 183]). Since belimumab affects activated and naïve B cells, as well as short-lived plasma cells, but not CD27⁺ memory B cells,[54] sTACI may be of interest as a target for patients receiving belimumab. Further research in larger prospective and longitudinal cohorts is needed to investigate the potential usefulness of serum sBAFF receptors as biomarkers for biological drugs in SLE.

This study has some limitations. As mentioned in the Chapter III, recruitment was mainly monocentric for SLE patients, the HD cohort was of small size, and numbers of patients with specific active SLE organ manifestations were also small. However, the data draw on one of the best characterised cohorts of SLE patients worldwide. In light of the small sample size of the SLE patients treated with biologics in this study, caution should be taken when interpreting these data, awaiting confirmation in independent studies in larger cohorts. Finally, no sample size study has been performed to evaluate the statistical power of this study.

In conclusion, technical pitfalls were discovered in this work, where I show the potential for assay interference in multianalyte systems, resulting in the requirement to measure BAFF separately, and importantly the potential confounding effect of anti-B cell and

anti-BAFF therapies on these measures. I report the clinical associations of the three soluble BAFF receptors in SLE in a single study for the first time. All three BAFF receptors appeared more specific to SLE compared to serum BAFF. A significant negative relationship was observed between serum sTACI and overall disease activity in SLE, consistent with the decoy activity of sTACI previously demonstrated by Hoffmann *et al.*[70] Accordingly, a significant relationship was observed between BAFF/sTACI ratio and overall SLE disease activity, which appears stronger when compared to BAFF or sTACI alone, and was independent of ethnicity. Finally, some data also suggest a potential association between both BAFF/sTACI and BAFF/sBAFF-R ratios with musculoskeletal SLE, and between sBCMA and neurological SLE phenotype, but these observations will need to be confirmed in a larger cohort. These data provide more insights into BAFF/APRIL system-mediated SLE phenotypes. These findings may help to define new therapeutic approaches within the BAFF/APRIL system, which may fine-tune the use of anti-BAFF therapies in SLE, for example by stratifying using BAFF/sTACI ratio. Whether BAFF/sTACI ratio could help guide therapeutic intervention approaches should be studied in prospective trial.

CHAPTER VII: Soluble TACI as a biomarker in primary Sjögren's syndrome

1. Key findings

- 1.1. I report the presence of both sTACI and sBCMA in sera from primary Sjögren's syndrome (pSS) patients. Serum sTACI concentrations were significantly higher in pSS patients compared to healthy donors (HD). Of note, serum sTACI concentrations were higher in pSS patients with anti-Ro60 Abs compared to those without. Serum sTACI concentrations were negatively associated with overall pSS disease activity, consistent with the decoy activity of sTACI. No significant relationship emerged between serum sTACI with any of the organ domain of the ESSDAI score.
- 1.2. No significant difference emerged in serum sBCMA concentrations between pSS with HD cohorts. No significant relationship emerged between serum sBCMA and overall pSS disease activity, or sBCMA and any of the organ domains of the ESSDAI score.
- 1.3. Serum BAFF concentrations were not significantly different between pSS and HD cohorts. No significant relationship emerged between serum BAFF with overall pSS disease, or with any of the organ domain of the ESSDAI score.
- 1.4. No significant relationship was observed between BAFF and either sTACI or sBCMA concentrations, or between sTACI and sBCMA.
- 1.5. BAFF/sTACI ratio was significantly lower in pSS patients compared to HD. There was a non-significant trend toward a relationship between BAFF/sTACI ratio and overall pSS disease activity, and with the presence of anti-Ro60 and anti-La Abs.

1.6. No significant difference emerged in BAFF/sBCMA ratio between pSS and HD. No significant relationship emerged between BAFF/sBCMA ratio and overall pSS disease activity, or BAFF/sBCMA ratio and any of the organ domains of the ESSDAI score.

2. Introduction

The main aims in this Chapter are similar to the ones explored in Chapter VI in SLE, to identify clinical associations of cytokines and receptors of the BAFF/APRIL system in pSS. By studying pSS in parallel, another aim is to test the sub-hypothesis that these potential pathway-dependent clinical phenotypes and the composite biomarker strategies may operate differently in pSS to SLE. So far, no published study has investigated soluble BAFF receptors in pSS.

3. Results

Patient characteristics

(Please see Chapter III regarding HD characteristics, and Chapter IV regarding pSS patient characteristics.)

As previously mentioned in the Methods Chapter, ESSDAI data were only available in 35 pSS patients. Some pSS patients in whom ESSDAI score was not assessed, had clinical laboratory data available, which were used for analysis in these studies.

I first sought to investigate relationships between serum sTACI and sBCMA concentrations, and overall disease activity in pSS. Serum sBAFF-R concentrations were not quantified in pSS.

Associations of serum sTACI with pSS

Serum sTACI was detectable in all pSS samples, as well as in HD. Serum sTACI concentrations were significantly higher in pSS patients compared to HD ($p < 0.0001$; **Figure 50A**). There was no significant difference in sTACI according to gender (data not shown). Serum sTACI concentrations were significantly lower in pSS patients with moderate to high

disease activity compared to those with low disease activity ($p=0.036$; **Figure 50B**). Serum sTACI concentrations were significantly negatively correlated with C4 ($r=-0.69$, $p=0.004$; **Figure 50C**). Serum sTACI concentrations were higher in pSS patients with anti-Ro60 Abs, compared to those without ($p=0.0499$; **Figure 50D**). No significant relationship emerged between serum sTACI and any of the organ domains of the ESSDAI score (data not shown).

Serum sTACI concentrations were numerically although not significantly lower in pSS patients with associated lymphoma (median [IQR] sTACI: 31.9 [17.8-46.1] pg/ml) vs. those without (median [IQR] sTACI: 55.3 [26.2-72.6] pg/ml).

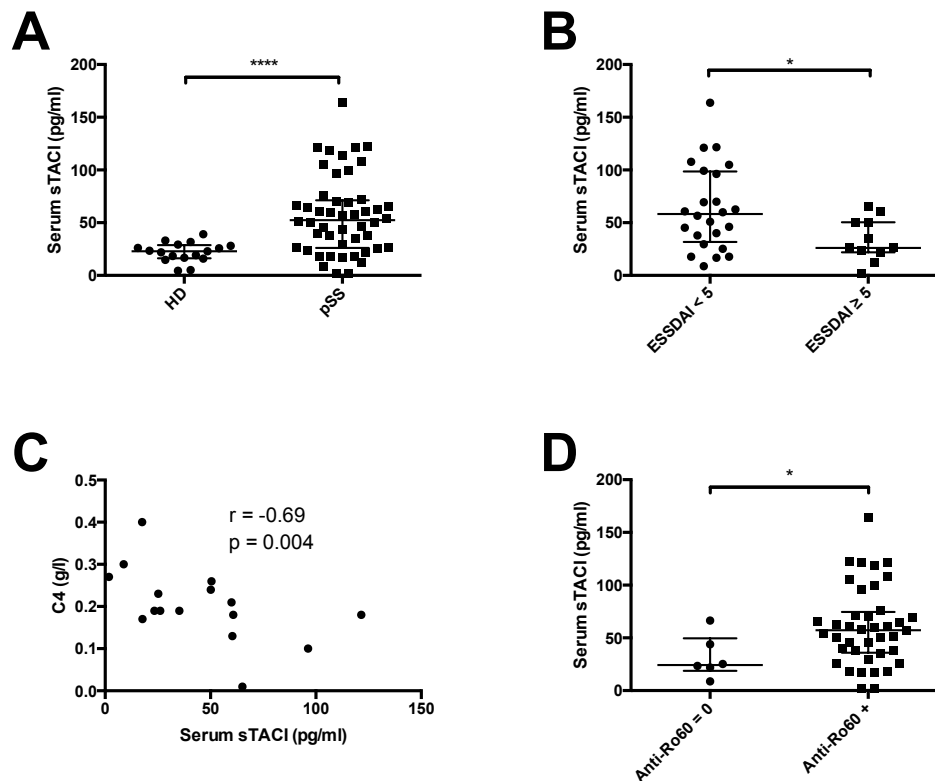


Figure 50. Associations of serum sTACI with pSS disease activity.

Overall pSS disease activity was assessed by the ESSDAI score. (A) Serum sTACI concentrations in pSS ($n=48$) compared to HD ($n=17$). (B) Serum sTACI concentrations according to overall pSS disease activity (ESSDAI < 5 : $n=24$; ESSDAI ≥ 5 : $n=11$). (C) Correlation between serum sTACI concentrations with C4 levels in pSS ($n=16$). (D) Serum sTACI concentrations according to the presence of anti-Ro60 Abs (Anti-Ro60 = 0: $n=6$; Anti-Ro60 +: $n=40$).

Serum sTACI concentrations are expressed in pg/ml. C4 levels are expressed in g/l.

In panels A-B, and D, horizontal bars indicate the median with [IQR].

**** $p < 0.0001$; * $p < 0.05$

Associations of serum sBCMA with pSS

Serum sBCMA was detectable in all pSS samples, as well as in HD (see Chapter VI). No significant difference emerged in serum sBCMA concentrations between pSS and HD (**Figure 51A**). No significant relationship was observed between serum sBCMA and overall pSS disease activity (**Figure 51B-C**). No significant relationship emerged between serum sBCMA concentrations and any of the studied organ domains of the ESSDAI score (data not shown).

Serum sBCMA concentrations were similar between pSS patients with and without associated lymphoma (median [IQR] sBCMA: 11062 [6936-15188] vs. 12180 [8907-16358], respectively).

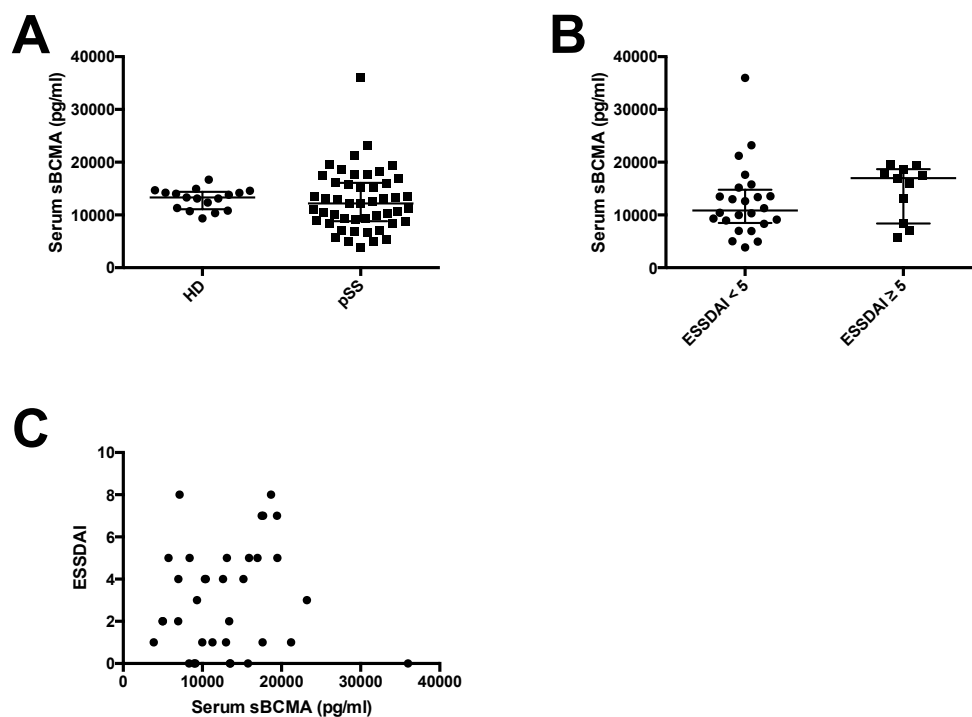


Figure 51. Associations of serum sBCMA with pSS disease activity.

Overall pSS disease activity was assessed by the ESSDAI score. (A) Serum sBCMA concentrations in pSS (n=48) compared to HD (n=17). (B) Serum sBCMA concentrations according to overall pSS disease activity (ESSDAI < 5: n=24; ESSDAI ≥ 5: n=11). (C) Correlation between serum sBCMA concentrations with ESSDAI (n=35).

Serum sBCMA concentrations are expressed in pg/ml.

In panels A-B, horizontal bars indicate the median with [IQR].

Associations of serum BAFF with pSS

Serum BAFF concentrations were detectable in all pSS samples, as well as in HD samples, and were not significantly different between pSS and HD (**Figure 52A**). No significant difference emerged in serum BAFF concentrations according to gender in pSS (data not shown). No significant relationship emerged between serum BAFF with overall pSS disease activity (**Figure 52B-C**), or with any of the organ domains of the ESSDAI score (data not shown). Of note, however, serum BAFF concentrations were significantly correlated with CRP ($r=0.45$, $p=0.047$; **Figure 52D**).

Serum BAFF concentrations were numerically similar between pSS patients with and without associated lymphoma (median [IQR] BAFF: 1154 [974.2-1334] vs. 1073 [841.4-1503] pg/ml, respectively).

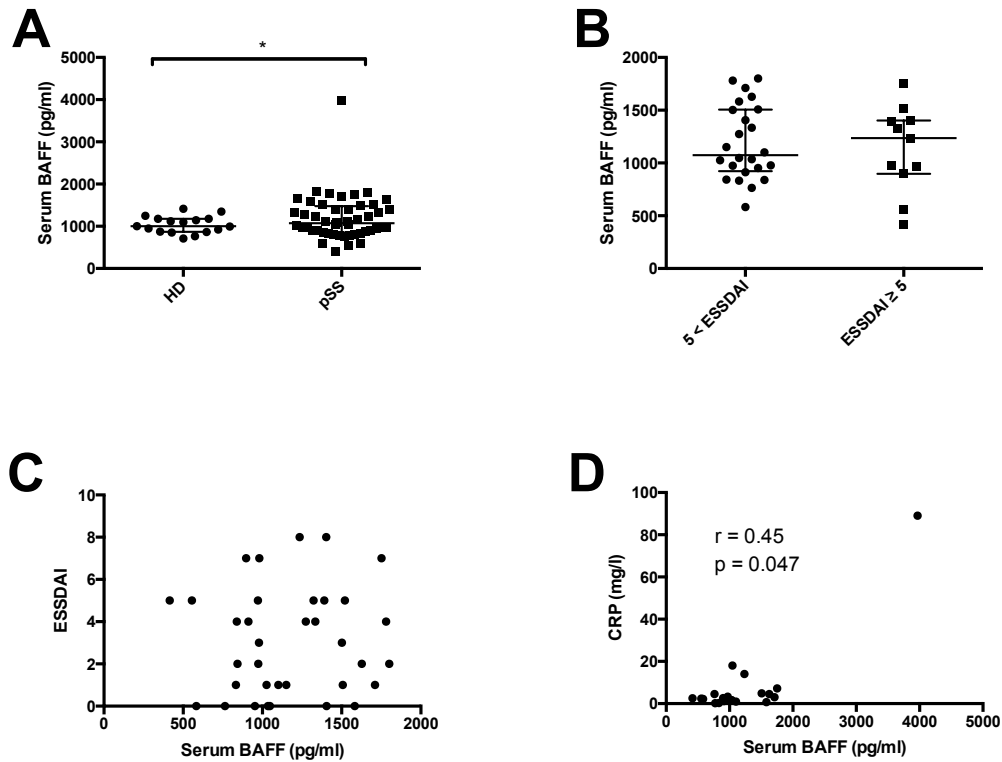


Figure 52. Associations of serum BAFF with pSS disease activity.

Overall pSS disease activity was assessed by the ESSDAI score. (A) Serum BAFF in pSS patients ($n=48$) compared to HD ($n=17$). (B) Serum BAFF concentrations according to overall pSS disease activity ($5 < \text{ESSDAI}$: $n=24$; $\text{ESSDAI} \geq 5$: $n=11$). (C) Correlation between serum BAFF concentrations and ESSDAI ($n=35$). (D) Correlation between serum BAFF concentrations with CRP levels in pSS ($n=20$).

Serum BAFF concentrations are expressed in pg/ml. CRP levels are expressed in mg/l.

In panels A-B, horizontal bars indicate the median with [IQR].

* $p < 0.05$

Relationship between serum BAFF and soluble BAFF receptors in pSS

No significant relationship was observed between serum concentrations of BAFF and either sTACI or sBCMA, or between sTACI and sBCMA (**Figure 53A-C**). In HD, serum BAFF concentrations were not correlated with sBCMA and sTACI, while unlike in pSS, serum sBCMA concentrations were significantly positively correlated with sTACI in HD, as already reported in Chapter VII.

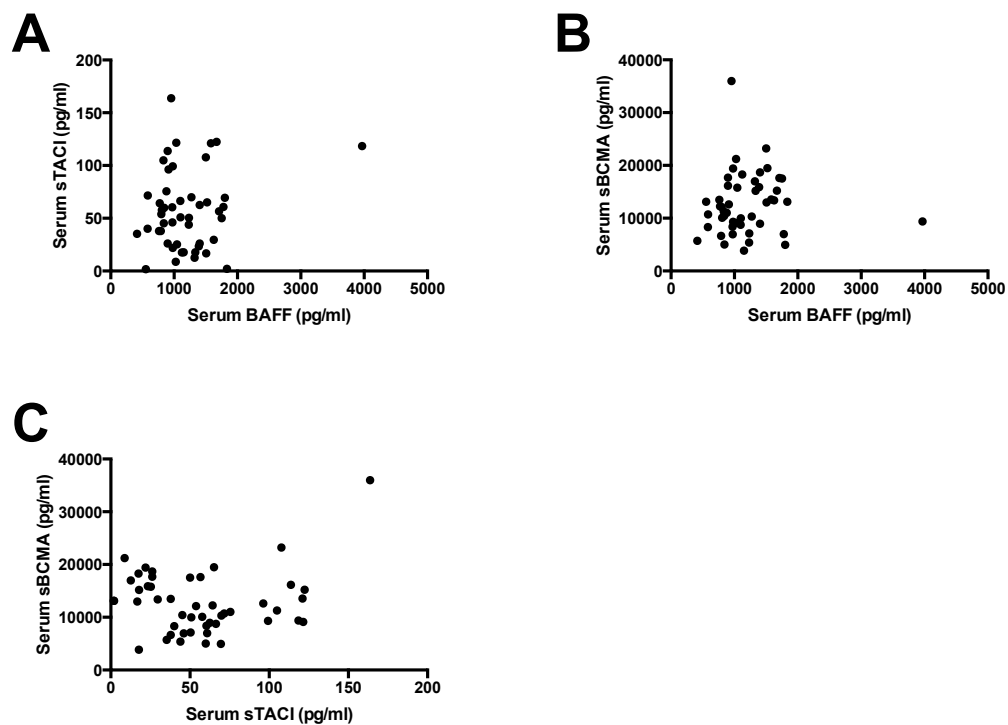


Figure 53. Relationship between serum BAFF and its soluble receptors in pSS.
Correlation between serum BAFF and serum (A) sTACI ($n=48$) and (B) sBCMA concentrations ($n=48$) in pSS. (C) Correlation between serum sTACI and serum sBCMA concentrations in pSS ($n=48$).
Serum BAFF, sTACI, and sBCMA concentrations are expressed in pg/ml.

Associations of BAFF/sTACI ratio and pSS

BAFF/sTACI ratio was significantly lower in pSS patients compared to HD ($p=0.0005$; **Figure 54A**). There was a non-significant trend toward a higher BAFF/sTACI ratio in pSS patients with moderate to high disease activity compared to those with low disease activity ($p=0.061$; **Figure 54B**), and toward a correlation between BAFF/sTACI ratio and ESSDAI ($r=0.31$, $p=0.072$; **Figure 54C**). BAFF/sTACI ratio was correlated with C4 ($r=0.58$, $p=0.021$; **Figure 54D**) and CRP ($r=0.56$, $p=0.01$; **Figure 54E**). There was a non-significant trend toward lower BAFF/sTACI ratio in pSS patients with anti-Ro60 Abs compared to those without ($p=0.051$; **Figure 54F**), and in pSS patients with anti-La compared to those without ($p=0.058$; **Figure 54G**).

BAFF/sTACI ratio was significantly higher in pSS patients with active lymphadenopathy compared to those without ($p=0.034$; one-tailed; **Figure 54H**). However, no significant association was observed in univariable analysis using logistic regression. No significant relationship emerged between BAFF/sTACI ratio and any other organ domains of the ESSDAI score (data not shown).

Of note, BAFF/sTACI ratio was numerically although not significantly higher in pSS patients with associated lymphoma (median [IQR] BAFF/sTACI ratio: 48.1 [21.1-75]) vs. those without (median [IQR] BAFF/sTACI ratio: 21.3 [13.5-42.3]).

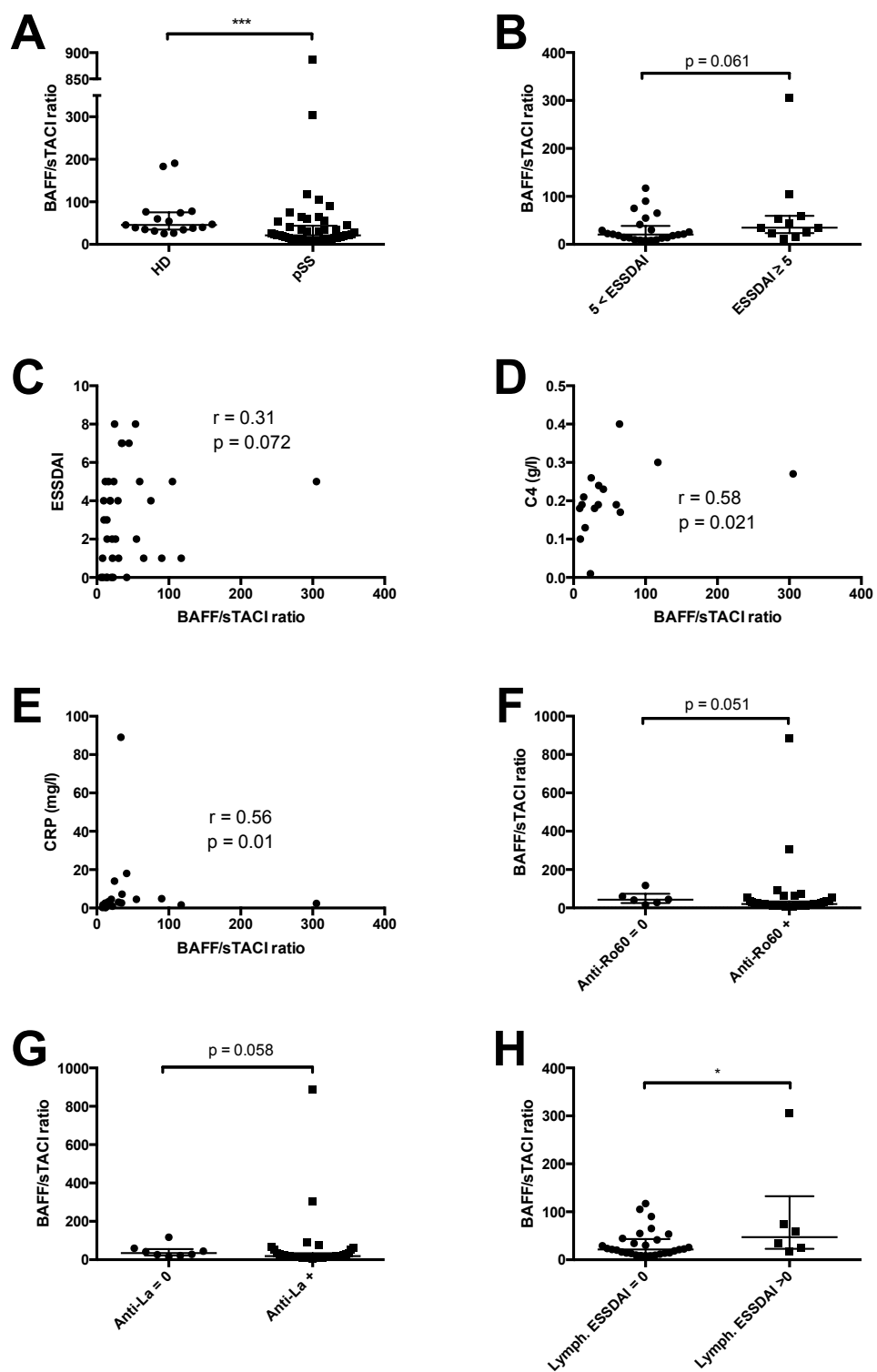


Figure 54. Associations of BAFF/sTACI ratio with pSS disease activity.

Overall and lymphadenopathy pSS disease activity was assessed by the ESSDAI score. (A) BAFF/sTACI ratio in pSS (n=48) compared to HD (n=17). (B) BAFF/sTACI ratio according to overall pSS disease activity ($5 < \text{ESSDAI}$: n=24; $\text{ESSDAI} \geq 5$: n=11). (C) Correlation between BAFF/sTACI ratio with ESSDAI (n=35). (D) Correlation between BAFF/sTACI ratio with C4 levels in pSS (n=16). (E) Correlation between BAFF/sTACI ratio with CRP levels in pSS (n=20). BAFF/sTACI ratio according to the presence of (F) anti-Ro60 (Anti-Ro60 = 0: n=6; Anti-Ro60 +: n=40) and (G) anti-La Abs (Anti-La = 0: n=8; Anti-La +: n=37) in pSS. (H) BAFF/sTACI ratio according to the presence of lymphadenopathy in pSS (Lymph. ESSDAI = 0: n=29; Lymph. ESSDAI >0: n=6).

Serum BAFF and sTACI concentrations are expressed in pg/ml. C4 and CRP levels are expressed in g/l and mg/l, respectively.

In panels A-B, and F-H, horizontal bars indicate the median with [IQR].

One-tailed p value is shown in panel H.

*** $p < 0.001$; * $p < 0.05$

Associations of BAFF/sBCMA ratio with pSS

No significant difference was observed in BAFF/sBCMA ratio between pSS and HD (**Figure 55A**). No significant relationship was seen between BAFF/sBCMA ratio and overall pSS disease activity (**Figure 55B-C**). No significant relationship was noted between BAFF/sBCMA ratio with any of the studied organ domains of the ESSDAI score (data not shown).

BAFF/sBCMA ratio was similar between pSS patients with and without associated lymphoma (median [IQR]: 0.11 [0.09-0.14] vs. 0.08 [0.07-0.12], respectively).

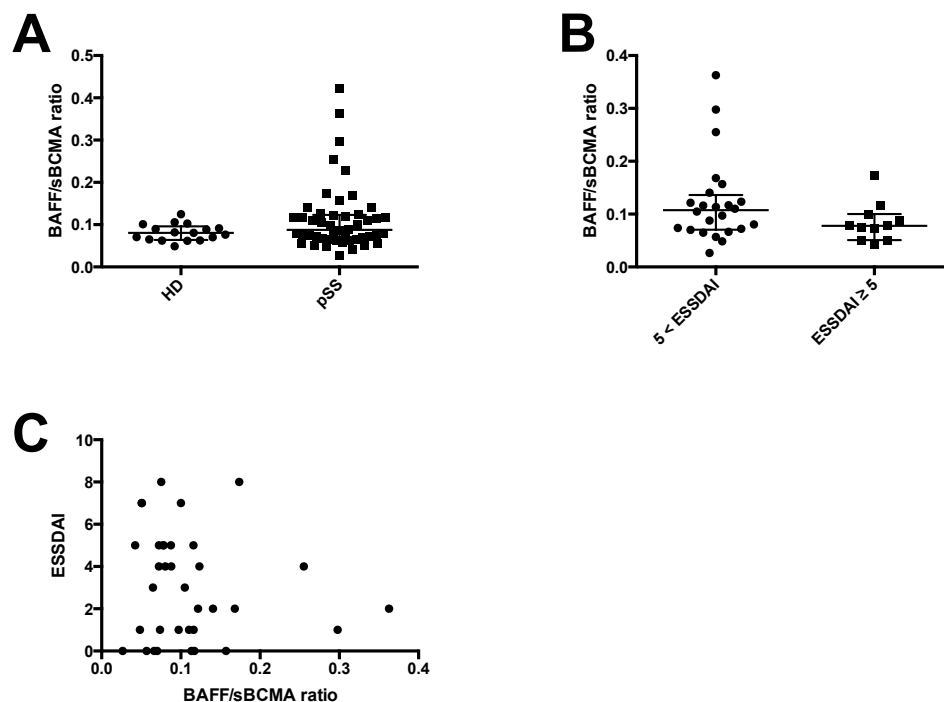


Figure 55. Associations of BAFF/sBCMA ratio with pSS disease activity.

Overall pSS disease activity was assessed by the ESSDAI score. (A) BAFF/sBCMA ratio in pSS (n=48) compared to HD (n=17). (B) BAFF/sBCMA ratio according to overall pSS disease activity ($5 < \text{ESSDAI}$: n=24; $\text{ESSDAI} \geq 5$: n=11). (C) Correlation between BAFF/sBCMA ratio with ESSDAI (n=35).

Serum BAFF and sBCMA concentrations are expressed in pg/ml.

In panels A-B, horizontal bars indicate the median with [IQR].

4. Discussion

This study aimed to characterize the presence and clinical association of soluble BAFF receptors sTACI and sBCMA in pSS, and to investigate if their quantification could be combined with the use of serum BAFF as a biomarker in this AID. Ultimately, the aim was to characterise potential similarities and/or differences between the behaviour of these biomarkers in pSS compared to SLE.

As mentioned in Chapter I, BAFF is acknowledged to play a crucial role in pSS pathogenesis (reviewed in[111]). BAFF-Tg mice develop a SLE/SS-like syndrome,[96] and human suffering from pSS have been reported to have elevated serum levels of BAFF which correlate with markers of disease activity (reviewed in[187]).[65, 99, 126] Encouraging results from a phase II clinical trial of belimumab in pSS[40] further underline the key role of the BAFF/APRIL system in this AID. In contrast to some published studies (reviewed in[184]), no difference in serum BAFF concentrations emerged between pSS patients and HD in the present study. Serum BAFF levels have been reported to significantly positively correlate with ESSDAI score, and with some ESSDAI individual organ domains, namely constitutional, glandular, lymphadenopathy and biological domains.[126] Discrepancies with the present study may be explained by a major difference in the study populations. In fact, as opposed to the present study where only 4.2% (2/48) of pSS patients had an associated lymphoma, 18.4% (14/76) of pSS patients enrolled in Quartuccio *et al.* study had overt lymphoma and 36.8% (28/76) harboured prelymphomatous manifestations; hence, less than half of the whole pSS cohort in that study had no such associated disorder.[126] Such differences, particularly considering that variations in serum BAFF concentrations have been reported related to the presence of lymphoma associated with pSS (reviewed in[112]), may have influenced these outcomes for serum BAFF as potential biomarkers for overall and organ-specific disease activity in the Quartuccio *et al.* study.[126] We consider that our

cohort more accurately reflects populations with pSS overall, where a lymphoma incidence of 4.2% is reported.[112]

As reported in Chapter VI, and in line with published data,[70] I found that serum sTACI was significantly higher in SLE compared to HD. The present study is the first to investigate the presence of sTACI in pSS. I found that serum sTACI was significantly higher in pSS compared to healthy controls. Also in line with outcomes reported in Chapter VI, wherein sTACI was significantly negatively associated with SLE disease activity, I found that serum sTACI was significantly negatively associated with overall pSS disease activity. As for SLE, this negative relationship in pSS is consistent with the decoy activity of sTACI for BAFF and APRIL reported by Hoffmann *et al.*[70] In this construct, in the presence of sTACI in patient's sera, levels of free BAFF would be decreased, leading to less pathogenic signalling. This clinical association of sTACI but not BAFF with pSS disease activity is in contrast to outcomes reported in Chapters V and VI, where both serum BAFF and sTACI were associated with SLE disease activity. This is of particular relevance with regards to clinical trials of anti-BAFF therapies in pSS, and raises the question of refining therapeutic targeting within the BAFF/APRIL system in pSS.

The occurrence of associated lymphoma in pSS is of major clinical relevance in daily clinical practice. Interestingly, 0.03 to 2.9 % of aged BAFF-Tg mice (heterozygous and homozygous, respectively) developed associated lymphoma, suggesting that the BAFF/APRIL system plays a role in lymphomagenesis in these SLE/SS-prone mice.[14] Higher serum BAFF concentrations have been reported in pSS patients having a history of, or an associated current, lymphoma compared to those without lymphoma (reviewed in[112]). So far, no published study reported serum BAFF as a predictive biomarker of lymphomagenesis in human pSS, but the multicentre 5-year prospective French ASSESS cohort might have generated enough data so far, to investigate this question.[65]

Interestingly, Quartuccio *et al.*,[126] when reporting similar outcomes found by Gottenberg *et al.*,[65] also showed that pSS patients with prelymphomatous manifestations also harboured increased, although not significantly so, serum BAFF levels compared to patients without lymphoma.

In the present study, two pSS patients were previously diagnosed with an associated MALT lymphoma of the parotid. Numerical differences were found in serum sTACI, being almost half as much lower in pSS with associated MALT lymphoma, compared to pSS patients without, in contrast with both serum BAFF and sBCMA concentrations, which appeared not to be affected. Median [IQR] ESSDAI in pSS patients with and without associated lymphoma was similar (3 [2-4] and 3 [1-5], respectively). Thus, this observed numerical decrease in serum sTACI in pSS patients with associated lymphoma might not be related to overall pSS disease activity, suggesting a potential relationship with lymphomagenesis. Although extreme caution must be taken considering the very small size of the pSS subset with associated lymphoma, these preliminary data prompt further investigation of the potential use of serum sTACI as disease activity and predictive biomarker for lymphomagenesis in pSS, as well as to unveil potential mechanism(s) through which sTACI may be involved in lymphomagenesis in pSS pathogenesis.

I reported in Chapter VI higher levels of serum sBCMA in SLE compared to healthy subjects, in line with the previous published study of Laurent *et al.*,[90] In contrast to my findings in SLE, I observed no significant difference in serum sBCMA levels in pSS compared to HD. Similarly, no significant relationship was found between sBCMA and disease activity in pSS, a finding similar to my findings in SLE where no relationship to SLEDAI-2k was seen. This was in contrast to Laurent *et al.*, wherein a relationship to SLEDAI was reported.[90] Laurent *et al.* also reported the decoy function of sBCMA only for APRIL.[90] The absence of clinical association of sBCMA in pSS in the present study

has to be considered bearing in mind the phenotype of pSS patients enrolled in this study, particularly the absence of CNS phenotype. While one could speculate that sBCMA has no clinical association in pSS, suggesting no interest as a potential biomarker or therapeutic target, it could also be speculated that the APRIL-BCMA axis may be important particularly in CNS pSS pathogenesis and that the cohort studied here precluded investigation of this possibility. Further research is needed in a larger pSS cohort to investigate sBCMA in pSS, particularly in phenotypes that are underrepresented in the present study.

There was a non-significant trend toward a relationship between BAFF/sTACI ratio and overall pSS disease activity. The lack of statistical significance may be explained by the relatively modest size of the pSS cohort studied. It is noteworthy that a significant relationship emerged between BAFF/sTACI ratio and activity of the lymphadenopathy organ domain, when analysed using non-parametric tests. However, this was not confirmed in univariable analysis using logistic regression. In the BELISS phase II study, De Vita *et al.* showed promising results with the use of belimumab in pSS, with disease activity improvement at 52 weeks, particularly in glandular, lymphadenopathy and articular organ-related domains of the ESSDAI score.[40] Within a small subset of 15 pSS patients enrolled in this BELISS study, authors reported that baseline serum BAFF levels quantified before belimumab induction was not predictive of response to belimumab.[142] The reported efficacy of belimumab in pSS patients with glandular, lymphadenopathy and articular manifestations underline the potential link between the BAFF/APRIL pathway and these phenotypic manifestations of the disease. Further research is needed to determine if these phenotypic manifestations in pSS are primarily BAFF-mediated, and whether BAFF/sTACI ratio could be used as a biomarker to guide therapeutic intervention in pSS patients with such phenotypes.

This study has some limitations. Firstly, although prospective, the pSS cohort was monocentric and of modest size. Secondly, the HD cohort was of small sample size and also monocentric. Thirdly, some phenotypes were underrepresented in the pSS cohort. Some clinical subsets analysed in this study were too small to draw solid conclusions; the presented results are hence preliminary. As mentioned in Chapter IV, the association of BAFF with the pathogenesis of pSS has not been validated to the same degree as in SLE, where anti-BAFF therapy has proven efficacy. The pSS cohort was defined by low overall disease activity. Finally, the use of ESSDAI domains to assign causation based on serum protein measurement is, as previously noted, speculative.

In conclusion, I report here for the first time the presence of both sTACI and sBCMA in sera from pSS patients. Serum sTACI was negatively related to overall pSS disease activity, consistent with the decoy activity of sTACI. Further research is needed to determine whether serum sTACI, in combination with BAFF, may help to stratify pSS patients for therapeutic intervention. These data may help to optimise future clinical trial design or patient selection for studies of anti-BAFF therapies and therapies targeting other components of the BAFF/APRIL system in pSS.

CHAPTER VIII: Urinary BAFF and APRIL in lupus nephritis

1. Key findings

- 1.1. BAFF was detectable in less than 10% of SLE patient urine samples. However, uBAFF was significantly higher in SLE compared to both HD and IgA nephropathy cohorts. uBAFF was significantly higher in Asian compared to Caucasian SLE patients. No significant relationship was seen between serum BAFF concentrations and uBAFF.
- 1.2. uBAFF was not detected in pSS patients, while serum BAFF was detectable.
- 1.3. A significant positive relationship emerged between uBAFF and overall SLE disease activity. SLE patients experiencing a flare of disease had higher uBAFF compared to those without. uBAFF was significantly higher in SLE patients with anti-Sm Abs compared to those without. A significant positive relationship emerged between uBAFF and renal SLEDAI-2k, but not proteinuria. uBAFF was not related to histological class of LN or interstitial inflammation. However, uBAFF was significantly higher in LN patients with moderate interstitial fibrosis compared to those with very mild or mild interstitial fibrosis.
- 1.4. In a study of disease activity over a period subsequent to uBAFF measurement, overall SLEDAI-2k at 18 months was significantly higher, and both C3 and C4 levels at 18 months were significantly lower, in SLE patients with detectable uBAFF at baseline compared to those with no uBAFF detectable. Time adjusted mean SLEDAI-2k integrating all visits over one year following baseline assessment was significantly higher in SLE patients with detectable baseline uBAFF compared to those without. Baseline uBAFF was significantly higher in SLE patients with active renal disease at 18 months.

- 1.5. APRIL was detectable in nearly a third of SLE patient urine samples. No significant difference in uAPRIL was observed between SLE and HD. Although no significant relationship was seen between serum APRIL concentrations and uAPRIL, a non-significant trend toward a correlation was seen in SLE patients with detectable uAPRIL.
- 1.6. uAPRIL was significantly positively related to overall SLE disease activity. There was also a significant positive relationship between uAPRIL and both renal SLE disease activity and renal SLE laboratory markers. No significant relationship emerged between uAPRIL and histological class of LN or interstitial inflammation. uAPRIL was significantly higher in LN patients with moderate or severe interstitial fibrosis compared to those with mild or less fibrosis.
- 1.7. MCP-1 was detectable in about 45% of SLE patient urine samples, and uMCP-1 was significantly higher in SLE compared to HD.
- 1.8. uMCP-1 was significantly positively related to overall SLE disease activity, but not with flare or organ damage. uMCP-1 was significantly positively related to renal SLE disease activity, and renal SLE laboratory markers. No significant relationship was observed between uMCP-1 with the histological class of LN, interstitial fibrosis, or interstitial inflammation.

2. Introduction

As mentioned in Chapter I, the literature regarding the measurement of BAFF and APRIL as markers of disease state in SLE is a matter of debate. Studies of serum BAFF and APRIL levels and their association with SLE disease activity have generated variable results, with not all studies reporting a relationship with disease activity indices (reviewed in[184]). A potential explanation for the variation of serum BAFF and APRIL levels observed in various studies may be related to their possible loss in the urine in SLE patients with nephritis.[26] Studies of urinary BAFF and APRIL concentrations in the setting of SLE have not previously been published. Urinary concentrations of BAFF have been reported in SLE, being higher in renal SLE, however in a study presented in abstract form only to date.[39] However, in a study undertaken prior to my PhD, I recently reported that serum BAFF concentrations were significantly elevated while serum APRIL were decreased in patients with a history of LN,[185] suggesting the possibility that the BAFF/APRIL system is particularly relevant to this subset of patients. Moreover, intra-renal BAFF and APRIL expression has been reported in LN.[110, 153] The potential of therapeutically targeting BAFF or APRIL in LN, and the utility of measuring BAFF and APRIL family molecules in the management of LN, remain unknown. Urinary MCP-1 has been reported to be associated with LN, correlating with overall and renal disease activity, and particularly with renal flare (reviewed in [69]), and thus urinary MCP-1 could be considered as the current “gold standard” biomarker for LN. Comparing the performance of urinary SLE biomarker candidates with urinary MCP-1 would be of value for further characterization of these candidate biomarkers.

Here, I aimed to determine the presence of BAFF and APRIL in the urine of SLE patients, and the associations of their presence with clinical characteristics of SLE, particularly in relation to LN. I have also evaluated urinary BAFF and APRIL in patients with non-SLE related (IgA) nephropathy as a disease control group. I also characterized the

previously reported urinary biomarker for LN, uMCP-1,[1, 129, 148] to which uBAFF and uAPRIL could be compared.

3. Results

Patient characteristics

One hundred and twenty-three urine samples and 66 matching serum samples from 86 SLE patients were analysed. At baseline, among the 86 patients studied, 44 SLE patients (51.2%) had a matching serum tested. Baseline characteristics of the patients whose data are reported in this Chapter are displayed in **Table 7**. The median age and disease duration were 39 and 7.5 years, respectively. The majority of patients were female, and nearly two thirds were receiving glucocorticoids. More than 20% of the patients had active renal disease, according to the SLEDAI-2k score (**Table 7**). For all analyses, only baseline samples and matching clinical data were used, unless otherwise stated.

Serum and matching urine samples from 41 patients (29.3% female) were also analysed in a control group with IgA nephropathy, with a median age and disease duration of 43 and 6.2 years, respectively. Matching serum was only tested for BAFF. A further healthy control group comprised 36 healthy subjects (58.3% female), with median age 28.5 years (**Table 7**). Only urine samples were analysed in this HD cohort.

Patients with SLE were significantly older than healthy subjects ($p=0.004$), while no significant difference in age was observed between patients with SLE and IgA nephropathy (data not shown; **Table 7**). There were significantly more female patients in the SLE cohort compared to HD and IgA cohorts ($p=0.01$ and $p<0.0001$, respectively; data not shown; **Table 7**). UPCR was significantly higher in the IgA nephropathy patient cohort compared to SLE ($p=0.022$), while no significant difference in renal function was observed.

Table 7. Demographical, clinical and biological characteristics of SLE, IgA nephropathy and HD cohorts at baseline.

Characteristics	HD (N=36)	SLE (N = 86)	IgA nephropathy (N = 41)	<i>p</i>
<i>Demographics</i>				
Age (years)	28.5 [24.4-37.2]	39 [30.1-47.9]	43 [32.5-54]	0.0002
Female, n (%)	21 (58.3)	71 (82.6)	12 (29.3)	<0.0001
<i>Ethnicity</i>				
Caucasian, n (%)	N/A	51 (59.3)	N/A	N/A
Asian, n (%)	N/A	32 (37.2)	N/A	N/A
Hispanic, n (%)	N/A	2 (2.3)	N/A	N/A
<i>Disease</i>				
Disease duration (years)	N/A	7.5 [3.4-14.8]	6.2 [4.3-8.3]	NS
SLEDAI-2k	N/A	4 [2-6]	N/A	N/A
PGA	N/A	0.75 [0-1]	N/A	N/A
SLICC-SDI	N/A	0 [0-1]	N/A	N/A
Active renal disease*, n (%)	N/A	16 (18.6)	N/A	N/A
Biopsy-proven LN	N/A	29 (33.7)	N/A	N/A
<i>Treatment</i>				
Prednisone or equivalent, n (%)	N/A	53 (61.6)	N/A	N/A
Prednisone or equivalent (mg/day)	N/A	5 [0-10]	N/A	N/A
Hydroxychloroquine (HCQ), n (%)	N/A	76 (88.4)	N/A	N/A
HCQ (mg/day)	N/A	400 [200-400]	N/A	N/A
Methotrexate, n (%)	N/A	8 (9.3)	N/A	N/A
Azathioprine, n (%)	N/A	27 (31.4)	N/A	N/A
Mycophenolate mofetil, n (%)	N/A	10 (11.6)	N/A	N/A
Mycophenolic acid, n (%)	N/A	0 (0)	N/A	N/A
Cyclosporine A, n (%)	N/A	0 (0)	N/A	N/A

Characteristics	HD (N=36)	SLE (N = 86)	IgA nephropathy (N = 41)	<i>p</i>
Cyclophosphamide, n (%)	N/A	0 (0)	N/A	N/A
Abatacept	N/A	1 (1.2)	N/A	N/A
<i>Clinical laboratory data</i>				
Serum creatinine (μmol/l)	N/A	62 [53-80]	N/A	N/A
CRP (mg/l)	N/A	1.4 [0.5-3.8]	N/A	N/A
ESR (mm/h)	N/A	14 [7-35]	N/A	N/A
C3 (g/l)	N/A	0.88 [0.67-1.11]	N/A	N/A
C4 (g/l)	N/A	0.15 [0.12-0.22]	N/A	N/A
ANA +, n (%)	N/A	80 (93)	N/A	N/A
Anti-dsDNA Abs (IU) (normal <7)	N/A	17 [5.3-68.3]	N/A	N/A
Anti-Sm Abs +, n (%)	N/A	14 (16.3)	N/A	N/A
Anti-Ro (SSA) Ab +, n (%)	N/A	34 (39.5)	N/A	N/A
Anti-La (SSB) Ab +, n (%)	N/A	26 (30.2)	N/A	N/A
RF +, n (%)	N/A	22 (25.6)	N/A	N/A
UPCR (g/mmol)	N/A	0.02 [0.01-0.03]	0.04 [0.01, 0.08]	0.022
Urine micro WBC (x 10 ⁶ /l)	N/A	5 [0-20]	N/A	N/A
Urine micro RBC (x 10 ⁶ /l)	N/A	5 [0-15]	N/A	N/A
eGFR (ml/min)	N/A	90 [72.8-90]	77.8 [65.5, 91.5]	NS

Data are expressed as median [IQR] or as number (percentage). N: number of individuals in cohort. Percentages (%) are calculated based on N. Mann Whitney and Kruskal-Wallis tests were used to analyse differences between two and more than two variables, respectively. Mann Whitney tests were two-tailed. Group of categorical data were analysed by Chi-square test.

* Assessed by the renal components of the SLEDAI-2k score.

ANA: antinuclear antibody; C3: complement component 3; C4: complement component 4; CRP: C-reactive protein; dsDNA: double-stranded deoxyribonucleic acid; eGFR: estimated glomerular filtration rate; ESR: erythrocyte sedimentation rate; HCQ: hydroxychloroquine; IU: international units; LN: Lupus nephritis; N/A: Not applicable; NS: not significant; PGA: physician global assessment; RBC: red blood cells; RF: rheumatoid factor; SLE: systemic lupus erythematosus; SLEDAI-2k: SLE Disease Activity Index; SLICC-SDI: Systemic Lupus International Collaborating Clinics-SLE Damage Index; Sm: Smith; SS: Sjögren's syndrome; SSA: SS antigen A; SSB: SS antigen B; UPCR: urine protein/creatinine ratio; WBC: white blood cells.

Associations of uBAFF with SLE

I first determined the concentration of BAFF in the urine of SLE patients, as well as of patients with non-SLE nephropathy and healthy subjects as control groups. When applying a sensitivity cutoff using the last standard dilution from the standard curve (62.5 pg/ml), BAFF was detectable in 8.1% (7/86) of SLE patient urine samples, and mean (SEM) uBAFF was 8.3 (4.5) pg/ μ mol at baseline (**Figure 56A**). BAFF was not detected in any healthy control (0/34) or IgA nephropathy patient (0/40) urine sample. No significant difference in uBAFF was noted between SLE and HD or IgA nephropathy cohorts (**Figure 56A**), likely not reaching significance because of the small proportion of SLE patients with detectable uBAFF. Accordingly, uBAFF was not detectable significantly more frequently in SLE compared to HD ($p=0.19$, Fisher's exact test), or to IgA nephropathy ($p=0.1$, Fisher's exact test). However, uBAFF was significantly higher in SLE patients with active disease compared to HD ($p=0.029$; **Figure 56B**). Of note, when using the minimum detectable dose (MDD; 6.44 pg/ml) reported by the ELISA kit manufacturer, mean uBAFF was significantly higher in SLE compared to HD and IgA nephropathy ($p=0.039$, and $p=0.026$, respectively; **Figure 56C**). However, for maximal stringency, the data for uBAFF in the Results section of this Chapter were generated with a sensitivity cutoff of 62.5 pg/ml.

SLE and pSS are heterogeneous systemic AID sharing some common aspects of pathogenesis. As urinary BAFF was not detected in any control groups, I decided to quantify urinary BAFF in pSS patients, as another disease control group. Among the 33 pSS patients enrolled, 87.9% were female, 13.3% (2/15) were of Asian ethnicity, and mean (SD) age and median [IQR] disease duration were 61.9 (7.4) and 19.4 [14.4, 26.6] years, respectively. The median [IQR] ESSDAI was 2 [0, 4]. All pSS patients had a serum sample collected, and 28/33 had a matching urine sample collected. Urinary BAFF was not detected in any pSS patient, while serum BAFF was detected in all samples. It is noteworthy that none of the pSS

patients who had urinary BAFF quantified had active renal pSS, according to the ESSDAI score.

Because uBAFF was detectable in a low proportion of SLE patients, in addition to analyses conducted on uBAFF concentrations as a continuous variable, categorizing uBAFF as binary variable enabled more meaningful statistical analyses. Because none of the subjects among the healthy and disease control groups had detectable levels of uBAFF, the threshold for elevated uBAFF was defined at “0”. Hence, the threshold used to categorize uBAFF was the detection limit of the assay used. One can speculate that the absence of detection may not equal the absence of the protein in the biological sample, as a more sensitive assay might be able to detect this cytokine. However, at the very least this cutoff still reflects high versus lower levels of uBAFF.

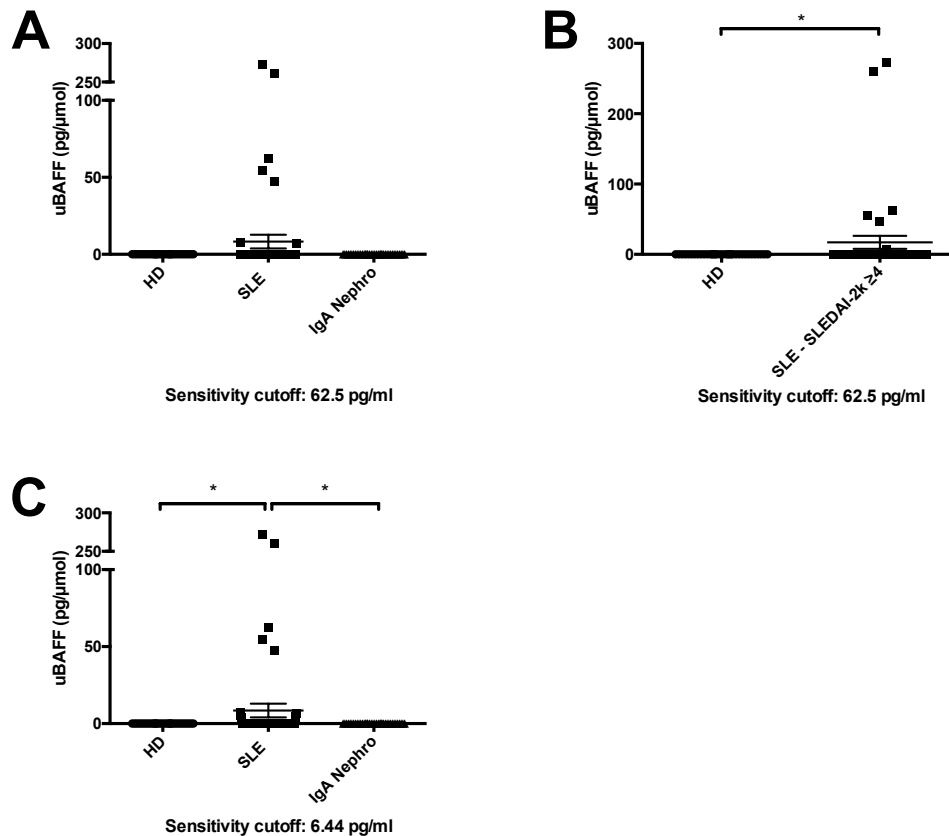


Figure 56. uBAFF in SLE.

Overall SLE disease activity was assessed by the SLEDAI-2k score. (A) uBAFF in SLE compared to HD and IgA nephropathy cohorts ($n=86$, $n=34$, and $n=40$, respectively), using the ELISA sensitivity cutoff of 62.5 pg/ml. (B) uBAFF in SLE patients with active overall SLE disease compared to HD (HD: $n=34$; and SLEDAI-2k ≥ 4 : $n=41$), using the ELISA sensitivity cutoff of 62.5 pg/ml. (C) uBAFF in SLE compared to HD and IgA nephropathy cohorts ($n=86$, $n=34$, and $n=40$, respectively), using the ELISA sensitivity cutoff of 6.44 pg/ml. uBAFF is expressed in pg/μmol.

In panels A-C, horizontal bars indicate the mean with SEM.

* $p < 0.05$

I next compared uBAFF between patients stratified by ethnicity. uBAFF was significantly higher in Asian compared to Caucasian SLE patients ($p=0.009$; **Figure 57**).

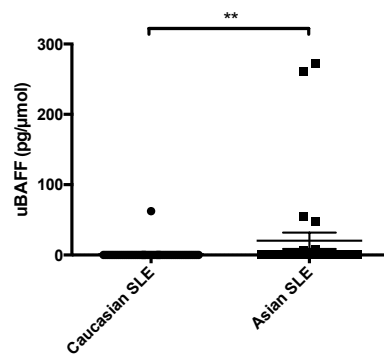


Figure 57. Associations of uBAFF with ethnicity.

uBAFF according to ethnicity [Caucasian (n=51) vs. Asian (n=32)] in SLE.

uBAFF is expressed in pg/ μ mol.

Horizontal bars indicate the mean with SEM.

****** $p < 0.01$.

Associations between serum and urinary BAFF

No significant difference in serum BAFF concentrations was seen between SLE patients with detectable uBAFF and those without (**Figure 58A**). Similarly, no significant difference in uBAFF was seen between SLE patients with high compared to non-elevated serum BAFF (**Figure 58B**). Due to the low number of SLE patients with detectable uBAFF, no correlation analysis between serum BAFF and uBAFF was carried out.

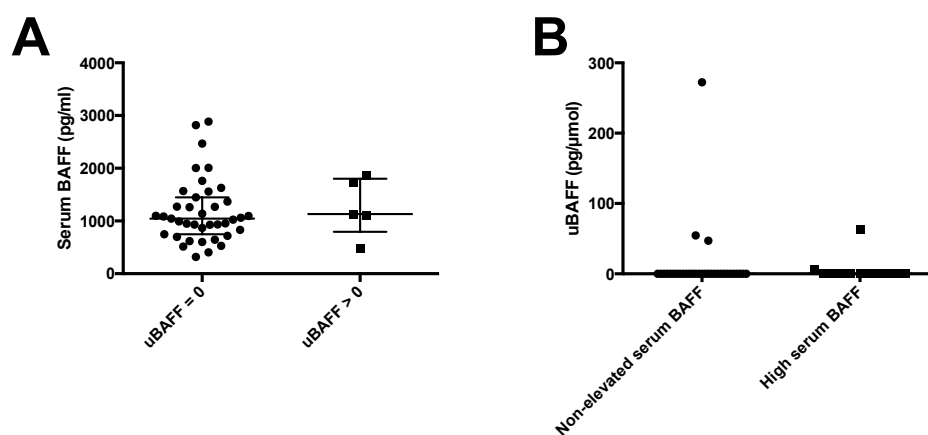


Figure 58. Associations between serum BAFF with uBAFF in SLE.

Serum BAFF concentrations were defined as non-elevated (< 1438.2 pg/ml) or high (≥ 1438.2 pg/ml), as per the Methods Chapter. (A) Serum BAFF concentrations according to the presence of uBAFF in SLE (uBAFF = 0: $n=39$; uBAFF > 0: $n=5$). (B) uBAFF according to the levels of serum BAFF in SLE (non-elevated serum BAFF: $n=32$; high serum BAFF: $n=12$).

Serum BAFF concentrations and uBAFF are expressed in pg/ml and pg/μmol, respectively. In panels A-B, horizontal bars indicate the median with [IQR].

Associations of uBAFF with SLE disease activity

I next investigated whether there was any association between uBAFF and SLE disease activity. Mean uBAFF was significantly higher in SLE patients with active overall disease compared to those without ($p=0.029$; **Figure 59A**). Indeed, uBAFF was only detectable in patients with active SLE, being undetectable in patients with SLEDAI-2k <4 . As previously mentioned, mean uBAFF was also significantly higher in patients with active SLE compared to HD (**Figure 56B**). SLE patients experiencing a flare of disease also had higher uBAFF compared to those without ($p=0.012$; **Figure 59B**). uBAFF was significantly higher in SLE patients with anti-Sm Abs compared to those without ($p=0.007$; **Figure 59C**). No significant relationship was found with anti-dsDNA Abs (**Figure 59D**), C3 (**Figure 59E**), and C4 (**Figure 59F**), or with other ENA or RF (data not shown).

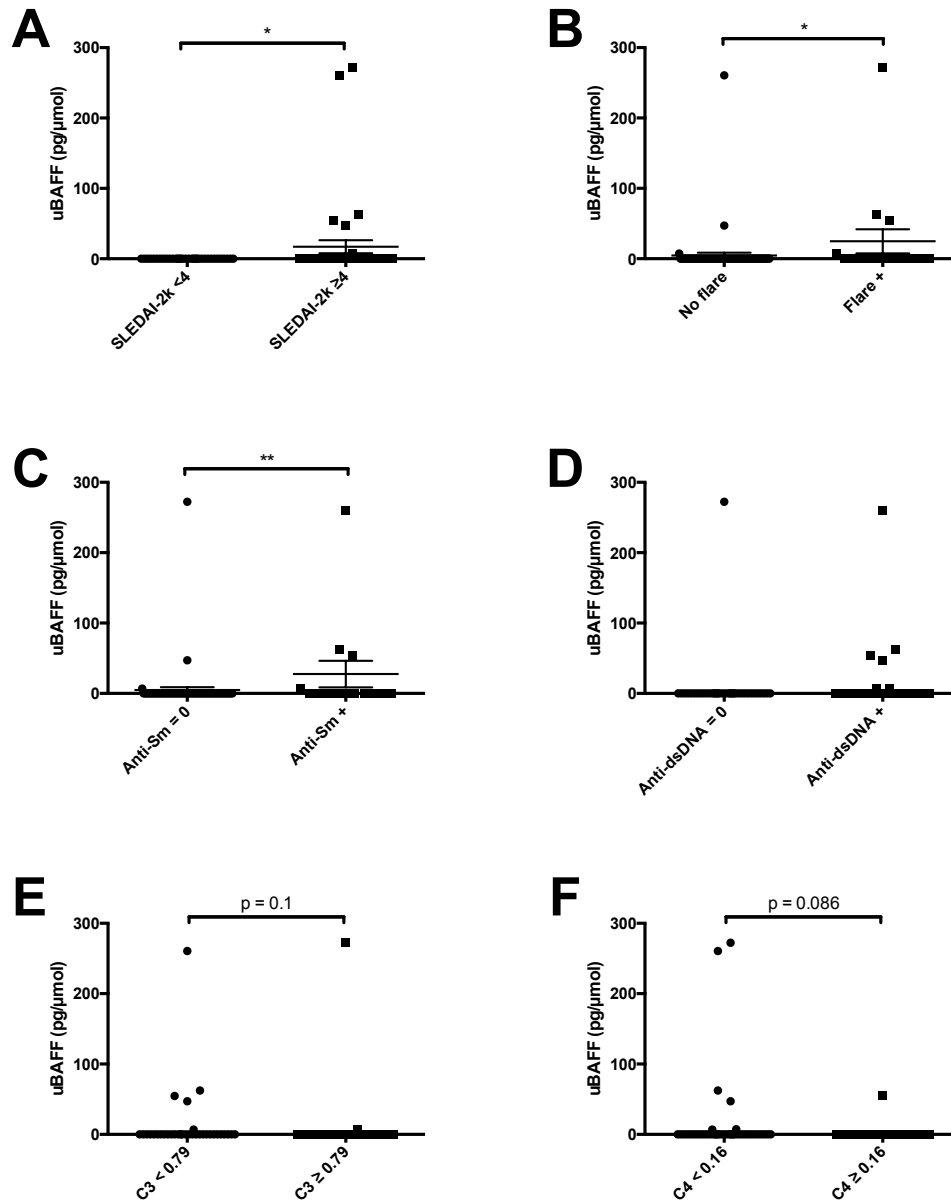


Figure 59. Associations of uBAFF with SLE disease activity.

Overall SLE disease activity was assessed by the SLEDAI-2k score. (A) uBAFF according to overall SLE disease activity (SLEDAI-2k <4: n=34; and SLEDAI-2k ≥4: n=41). (B) uBAFF according to the presence of flare of disease (no flare: n=67; and flare +: n=16). uBAFF according to the presence of (C) anti-Sm (anti-Sm=0: n=67; and anti-Sm+: n=14) and (D) anti-dsDNA Abs (anti-dsDNA=0: n=35; and anti-dsDNA +: n=45). uBAFF according to the levels of (E) C3 (C3 <0.79 g/l: n=34; and C3 ≥0.79 g/l: n=47) and (F) C4 (C4 <0.16 g/l: n=44; and C4 ≥0.16 g/l: n=37).

uBAFF is expressed in pg/μmol. Levels of C3 and C4 are expressed in g/l.

In panels A-C, horizontal bars indicate the mean with SEM. In panels D-F, horizontal bars indicate the median with [IQR].

** p<0.01; * p<0.05

Associations of uBAFF with renal SLE

I then investigated relationships between uBAFF and renal SLE disease activity, including proteinuria, a marker generally regarded as a key indicator of LN activity,[86] as measured by the UPCR. uBAFF was significantly higher in SLE patients with active renal disease according to SLEDAI-2k, compared to those without ($p=0.002$; **Figure 60A**). No significant relationship emerged with UPCR (**Figure 60B**), although mean uBAFF was numerically higher in patients with proteinuria.

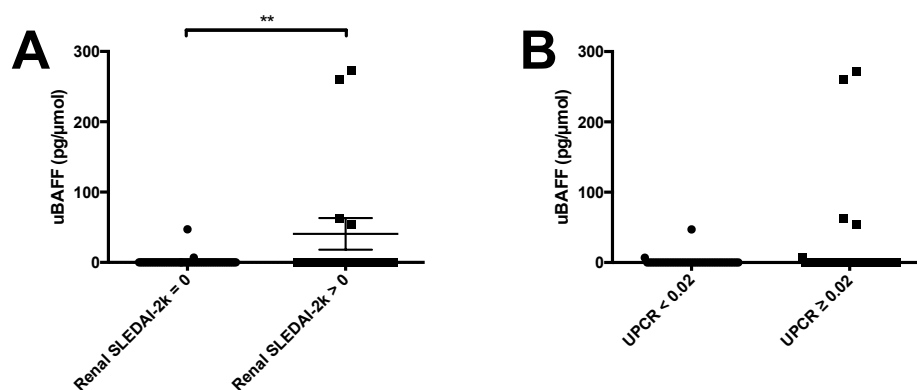


Figure 60. Associations of uBAFF with renal SLE disease activity.

Renal SLE disease activity was assessed by the SLEDAI-2k score. (A) uBAFF according to renal SLE disease activity (renal SLEDAI-2k =0: $n=59$; and renal SLEDAI-2k >0: $n=16$). (B) uBAFF according to UPCR (UPCR < 0.02 g/mmol: $n=40$; UPCR $\geq$ 0.02 g/mmol: $n=43$). uBAFF is expressed in pg/μmol. UPCR is expressed in g/mmol.

In panel A, horizontal bars indicate the mean with SEM. In panel B, horizontal bars indicate the median with [IQR].

** $p<0.01$.

I next investigated the relationship between uBAFF and the histological class of LN in 29 patients from whom renal biopsy data were available. uBAFF was detected in 5/22 (22.7%) of patients with histological class III, IV, or V LN, but in 0/7 patients with lesser forms of LN ($p=0.3$, Fisher's exact test) (**Figure 61A-B**). However, uBAFF was higher in LN patients with moderate interstitial fibrosis compared to those with very mild or mild interstitial fibrosis (**Figure 61C**). Accordingly, uBAFF was significantly higher in grouped LN patients with moderate to severe interstitial fibrosis compared to those with mild or less fibrosis ($p=0.002$) (**Figure 61D**). A table provided in **Appendix 9** displays demographic, clinical and biological characteristics of LN patients according to interstitial fibrosis, comparing patients with moderate to severe interstitial fibrosis ($n=7$) to those with mild or less interstitial fibrosis ($n=22$). As expected, overall and renal disease activity scores, as well as organ damage scores, were significantly higher in LN patients with moderate to severe interstitial fibrosis compared to those with mild or less fibrosis (**Appendix 9**). Also as expected, LN patients with moderate to severe interstitial fibrosis had significantly higher UPCR, higher serum creatinine, lower eGFR and more inflammation (ESR) compared to those with mild or less fibrosis. There was no significant difference in age, gender, ethnicity, treatment used, complement activity or auto-Ab production between LN patients with moderate to severe interstitial fibrosis compared to those with mild or less fibrosis (**Appendix 9**).

Among IgA nephropathy patients with available detailed renal biopsy results, median [IQR] sclerosed glomerular and crescentic glomerular scores were 0.1 [0.04, 0.28] and 0 [0, 0], respectively. In addition, 27 (73%), seven (18.9%) and one (2.7%) IgA nephropathy patients had mild, moderate and severe interstitial fibrosis, while two (5.4%) had no interstitial fibrosis. There was no significant difference in the presence of moderate to severe interstitial fibrosis between LN [24.1% (7/22)] and IgA nephropathy [21.6% (8/37)] patients

($p>0.99$). Since uBAFF was not detected in any IgA nephropathy patient in the present study, although some had features of moderate to severe interstitial fibrosis, this supports the hypothesis that the presence of uBAFF is associated with interstitial fibrosis in the particular setting of LN.

No significant relationship emerged between uBAFF and interstitial inflammation (data not shown). Regarding histopathological activity and chronicity indexes, as this only refers to Class III and IV, the corresponding subset only comprised 15/29 SLE patients [Active (n=9); Chronic (n=2); both Active and Chronic (n=4)]. Hence, uBAFF distribution could not be compared between Active and Active/Chronic subsets.

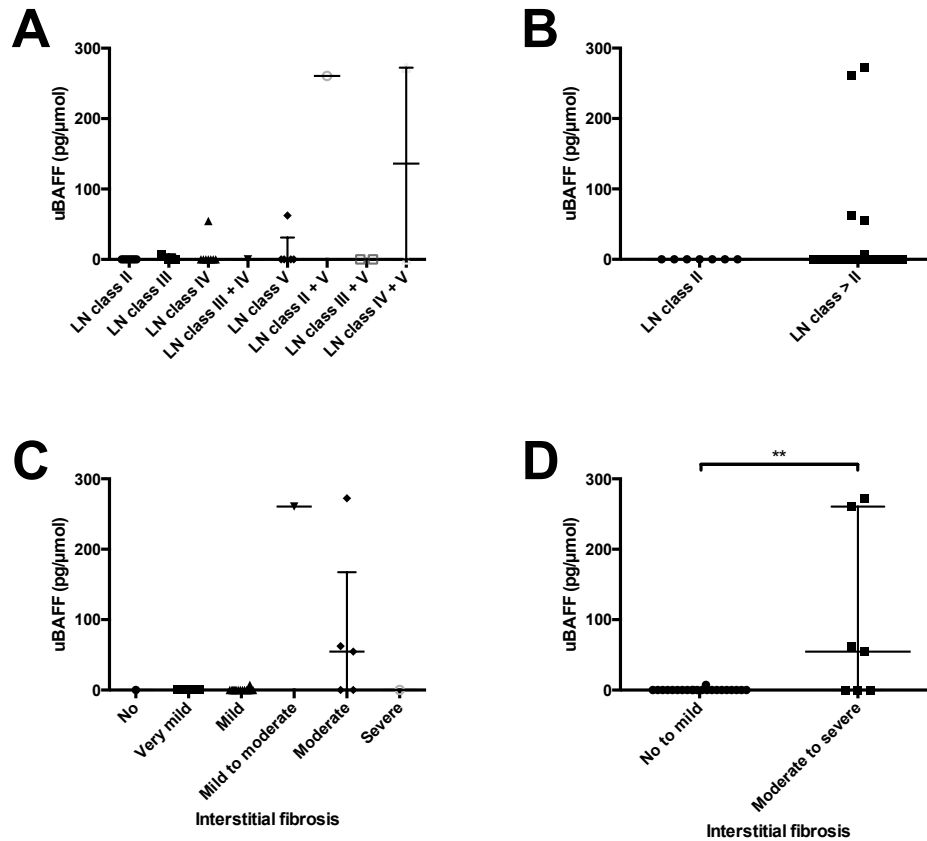


Figure 61. Associations of uBAFF with kidney biopsy LN assessment.

(A) uBAFF according to the histological class of LN (LN class II: $n=7$; LN class III: $n=3$; LN class IV: $n=8$; LN class III+IV: $n=1$; LN class V: $n=5$; LN class II+V: $n=1$; LN class III+V: $n=2$; LN class IV+V: $n=2$). (B) uBAFF between SLE patients with class II LN ($n=7$) compared to those with higher class of LN ($n=22$). (C) uBAFF according to interstitial fibrosis in LN (No: $n=1$; very mild: $n=5$; Mild: $n=16$; mild to moderate: $n=1$; Moderate: $n=5$; severe: $n=1$). (D) uBAFF according to interstitial fibrosis in LN when comparing patients with moderate to severe ($n=7$) to those with mild or less interstitial fibrosis ($n=22$). uBAFF is expressed in $\text{pg}/\mu\text{mol}$.

In panels A-D, horizontal bars indicate the median with [IQR].

** $p < 0.01$

Associations of baseline uBAFF with subsequent SLE disease activity

I next investigated the relationship between uBAFF determined at the commencement of the study (designated baseline) and disease activity during a subsequent 24 months follow up. Patients studied are part of the Monash Lupus Clinic, where validated measurement of disease activity is recorded longitudinally. Disease activity integrating all subsequent visits over one year following baseline assessment was calculated in 73 SLE patients using the time adjusted mean SLEDAI-2k (AMS). AMS (1 year) was significantly higher in SLE patients with detectable baseline uBAFF compared to those without ($p=0.011$; **Figure 62A**).

Fifty-five SLE patients who had baseline uBAFF were clinically assessed at 18 months, with a median [IQR] time interval between clinical visits of 18.2 [17.9-18.9] months. Fifty-three SLE patients were also clinically assessed at 24 months, with a median [IQR] time interval between clinical visits of 24.3 [23.3-25.1] months. SLEDAI-2k at 18 months was significantly higher in SLE patients with detectable uBAFF at baseline compared to those with no uBAFF detectable ($p=0.005$; **Figure 62B**). C3 and C4 levels at 18 months were significantly lower in SLE patients with detectable uBAFF at baseline compared to those with no uBAFF detectable ($p=0.008$ and $p=0.022$, respectively; **Figure 62C-D**). No significant relationship was seen between baseline uBAFF and disease activity measured by PGA, or with anti-dsDNA, at 18 months (data not shown). No significant relationship was observed between baseline uBAFF and overall disease activity (SLEDAI-2k or PGA) or C4 levels recorded at 24 months (data not shown). However, C3 levels at 24 months were significantly lower in SLE patients with detectable uBAFF at baseline compared to those with no uBAFF detectable ($p=0.015$; **Figure 62E**). There was also a non-significant trend toward higher anti-dsDNA Abs levels at 24 months in SLE patients with detectable uBAFF at baseline compared to those with no uBAFF detectable ($p=0.052$; data not shown).

I also investigated change over time in uBAFF among 25 SLE patients who had a subsequent urine sample collection, with a median [IQR] time interval between clinical visits of 3.5 [1.9-18] months. I evaluated correlation between between-visits changes in uBAFF with between-visits changes in overall disease activity and in laboratory SLE markers. No significant association was noted (data not shown).

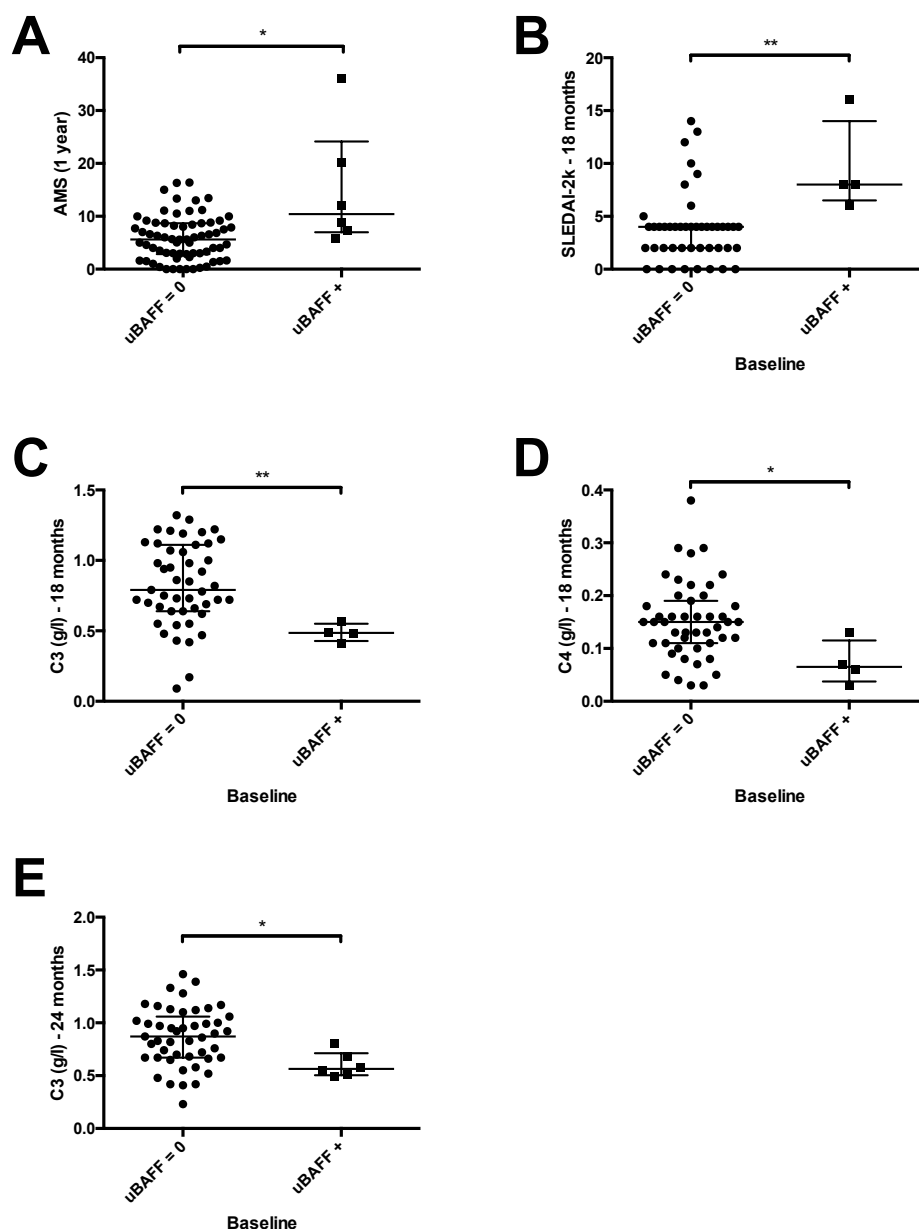


Figure 62. Association of baseline uBAFF with SLE disease activity over time.

Overall SLE disease activity was assessed by the SLEDAI-2k score. (A) AMS integrating all subsequent visits over one year following baseline assessment according to baseline uBAFF (uBAFF = 0: n=67; and uBAFF +: n=6). (B) Overall SLE disease activity recorded at 18 months according to baseline uBAFF (uBAFF = 0: n=45; and uBAFF +: n=4). C3 (C) and C4 (D) levels recorded at 18 months according to baseline uBAFF (uBAFF = 0: n=47; and uBAFF +: n=4). (E) C3 levels recorded at 24 months according to baseline uBAFF (uBAFF = 0: n=47; and uBAFF +: n=6).

uBAFF is expressed in pg/ μ mol. C3 and C4 levels are expressed in g/l.

In panels A-E, horizontal bars indicate the median with [IQR].

** $p < 0.01$; * $p < 0.05$

Associations of baseline uBAFF with subsequent renal SLE disease activity

To complement the above observation, I then investigated whether baseline uBAFF was predictive of subsequent renal disease activity. There was no significant difference in renal SLEDAI-2k or UPCR recorded at 18 months between SLE patients with or without detectable uBAFF at baseline (**Figure 63A-B**). However, baseline uBAFF was significantly higher in SLE patients with an active renal disease at 18 months compared to those without ($p=0.04$; **Figure 63C**). No significant relationship emerged between baseline uBAFF and renal SLE disease activity assessed at 24 months (data not shown). When patients with longitudinal uBAFF measurements were assessed, no significant correlation was noted between change in uBAFF with change in renal SLEDAI-2k, or with change in creatinine, eGFR, or UPCR (data not shown).

Regarding the potential effects of outliers on uBAFF associations with SLE, Appendix 11 displays the difference in the results of analysis with or without these outliers included. Collectively, exclusion of outliers for uBAFF did not lead to major change in the results, except for revealing a lack of significance for the association of baseline uBAFF with subsequent renal SLEDAI-2k recorded at 18-months.

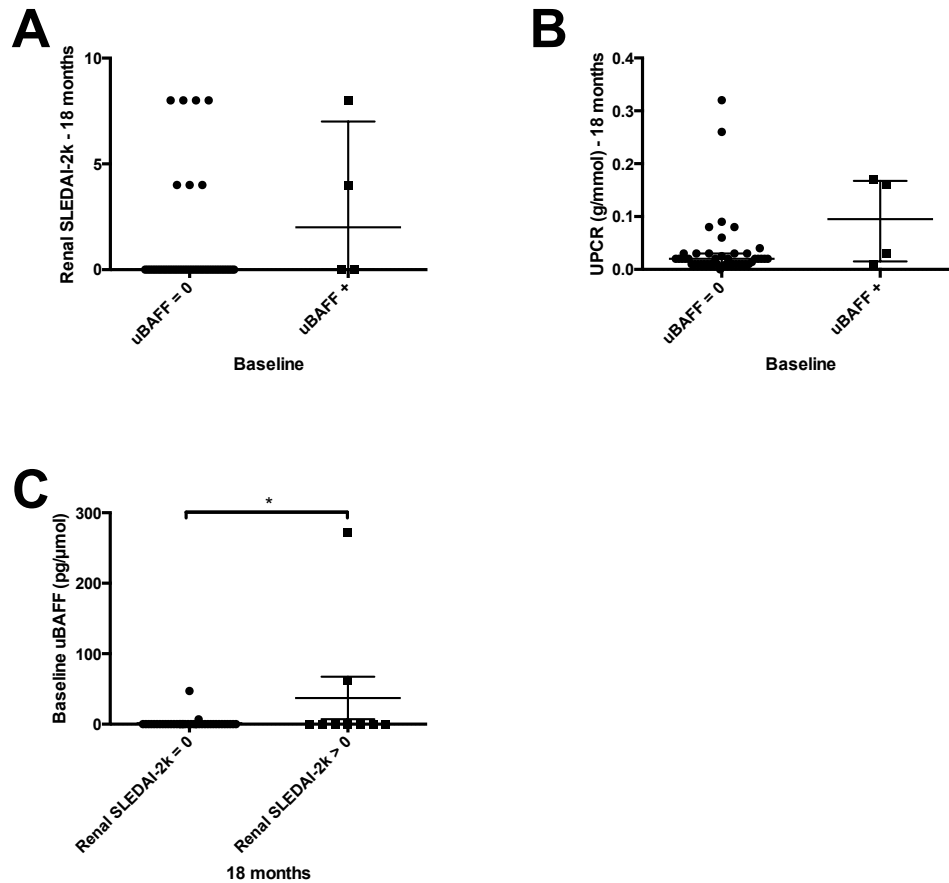


Figure 63. Association between baseline uBAFF with renal SLE disease activity over time. Renal SLE disease activity was assessed by the SLEDAI-2k score. Renal SLE disease activity recorded at 18 months according to baseline uBAFF (uBAFF = 0: n=44; and uBAFF +: n=4). (B) UPCr recorded at 18 months according to baseline uBAFF (uBAFF = 0: n=45; and uBAFF +: n=4). (C) Baseline uBAFF according to the renal SLE disease activity recorded at 18 months (Renal SLEDAI-2k = 0: n=39; Renal SLEDAI-2k > 0: n=9). uBAFF is expressed in pg/ μ mol. UPCr is expressed in g/mmol. In panels A-C, horizontal bars indicate the mean with SEM. * $p < 0.05$

Associations of uAPRIL in SLE

Using the sensitivity cutoff of the last standard dilution from the standard curve (0.78125 ng/ml), APRIL was detectable in 32.1% (25/78) of SLE patient urine samples at baseline (**Figure 64A**). The mean (SEM) uAPRIL was 0.23 (0.08) ng/ μ mol. APRIL was detectable in 37.1% (13/35) of healthy control urine samples, and the mean (SEM) uAPRIL was 0.25 (0.13) ng/ μ mol (**Figure 64A**). No significant difference emerged in detection of uAPRIL between SLE and HD ($p=0.67$, Fisher's exact test). APRIL was detectable in 58.8% (10/17) of patients with IgA nephropathy, and the mean (SEM) uAPRIL was 0.22 (0.05) ng/ μ mol (**Figure 64A**). There was no significant difference in uAPRIL concentrations between SLE and HD or IgA nephropathy cohorts (**Figure 64A**). The same was true when using the limit of detection provided by the ELISA manufacturer (0.4 ng/ml) (**Figure 64B**). Using this limit of detection led to inclusion of uAPRIL only in two HD. The data for uAPRIL in the Results section of this Chapter were generated with a sensitivity cutoff of 0.78125 ng/ml.

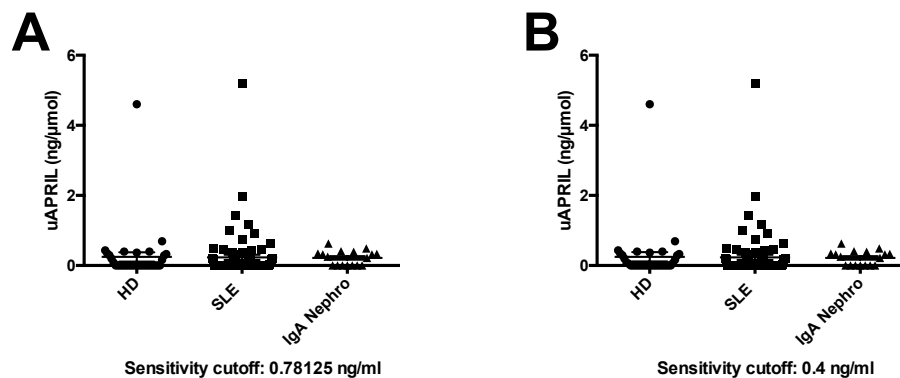


Figure 64. uAPRIL in SLE.

uAPRIL in SLE compared to HD and IgA nephropathy cohorts, using the sensitivity cutoff of (A) 0.78125 ng/ml, or (B) 0.4 ng/ml ($n=78$, $n=35$, and $n=17$, respectively).

uAPRIL is expressed in ng/ μ mol.

In panels A-B, horizontal bars indicate the mean with SEM.

I next analysed ethnic differences in uAPRIL. No significant difference was observed in uAPRIL between Caucasian and Asian SLE patients (**Figure 65**).

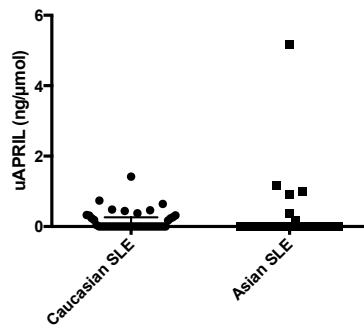


Figure 65. Association of uAPRIL with ethnicity.
*uAPRIL according to ethnicity [(Caucasian (n=44) vs. Asian (n=31)] in SLE.
uAPRIL is expressed in ng/μmol.
Horizontal bars indicate the median with [IQR].*

Associations between serum and urinary APRIL

No significant difference in serum APRIL concentrations was seen between SLE patients with detectable uAPRIL and those without (**Figure 66A**). Similarly, no significant difference in uAPRIL was seen between SLE patients with detectable serum APRIL and those without (**Figure 66B**). Accordingly, no significant correlation was observed between serum APRIL concentrations and uAPRIL (**Figure 66C**). However, there was a non-significant trend toward a positive correlation between uAPRIL and serum APRIL concentrations when restricting analysis to the subset of SLE patients with detectable uAPRIL ($r=0.47$, $p=0.068$; **Figure 66D**).

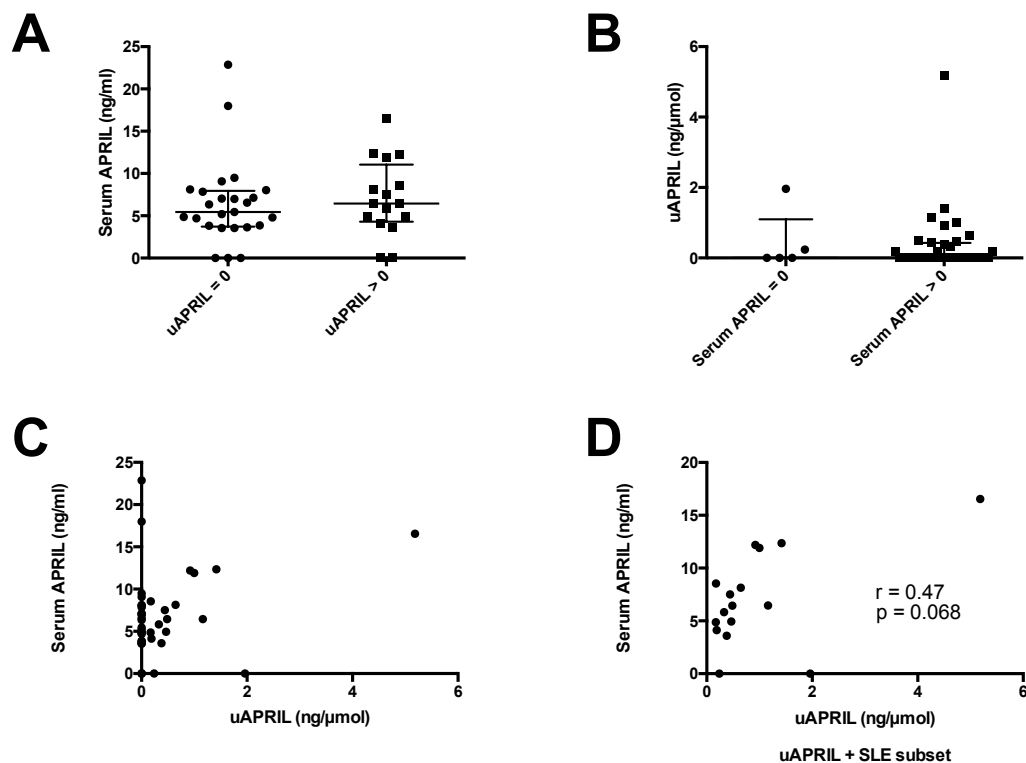


Figure 66. Associations between serum APRIL with uAPRIL in SLE.

(A) Serum APRIL concentrations according to the presence of uAPRIL in SLE (uAPRIL = 0: $n=25$; uAPRIL > 0: $n=16$). (B) uAPRIL according to the presence of serum APRIL in SLE (Serum APRIL = 0: $n=5$; Serum APRIL > 0: $n=36$). Correlation between uAPRIL and serum APRIL concentrations (C) in SLE ($n=41$), and (D) in the subset of SLE patients with detectable uAPRIL ($n=16$).

Serum APRIL concentration and uAPRIL are expressed in ng/ml and ng/μmol, respectively. In panels A-B, horizontal bars indicate the median with [IQR].

Associations of uAPRIL with SLE disease activity

There was no significant difference in uAPRIL between SLE patients with active overall disease compared to those without using a SLEDAI-2k cutoff of 4 (**Figure 67A**). However, uAPRIL was weakly but significantly positively correlated with SLEDAI-2k ($r=0.27$, $p=0.028$; **Figure 67B**). There was also a non-significant trend toward a correlation between uAPRIL and disease activity measured using the PGA ($r=0.21$, $p=0.067$; **Figure 67C**). No significant association with flare of disease was observed (data not shown). There was also a non-significant trend toward a correlation between uAPRIL and permanent damage measured by SLICC ($r=0.21$, $p=0.063$; **Figure 67D**). Moreover, uAPRIL was significantly correlated with serum CRP ($r=0.24$, $p=0.039$; **Figure 67E**), and haemoglobin (Hb) ($r=-0.38$, $p=0.0009$; **Figure 67F**). There was no significant relationship between uAPRIL and anti-dsDNA Abs, anti-ENA Abs, RF, C3 and C4, and ESR (data not shown).

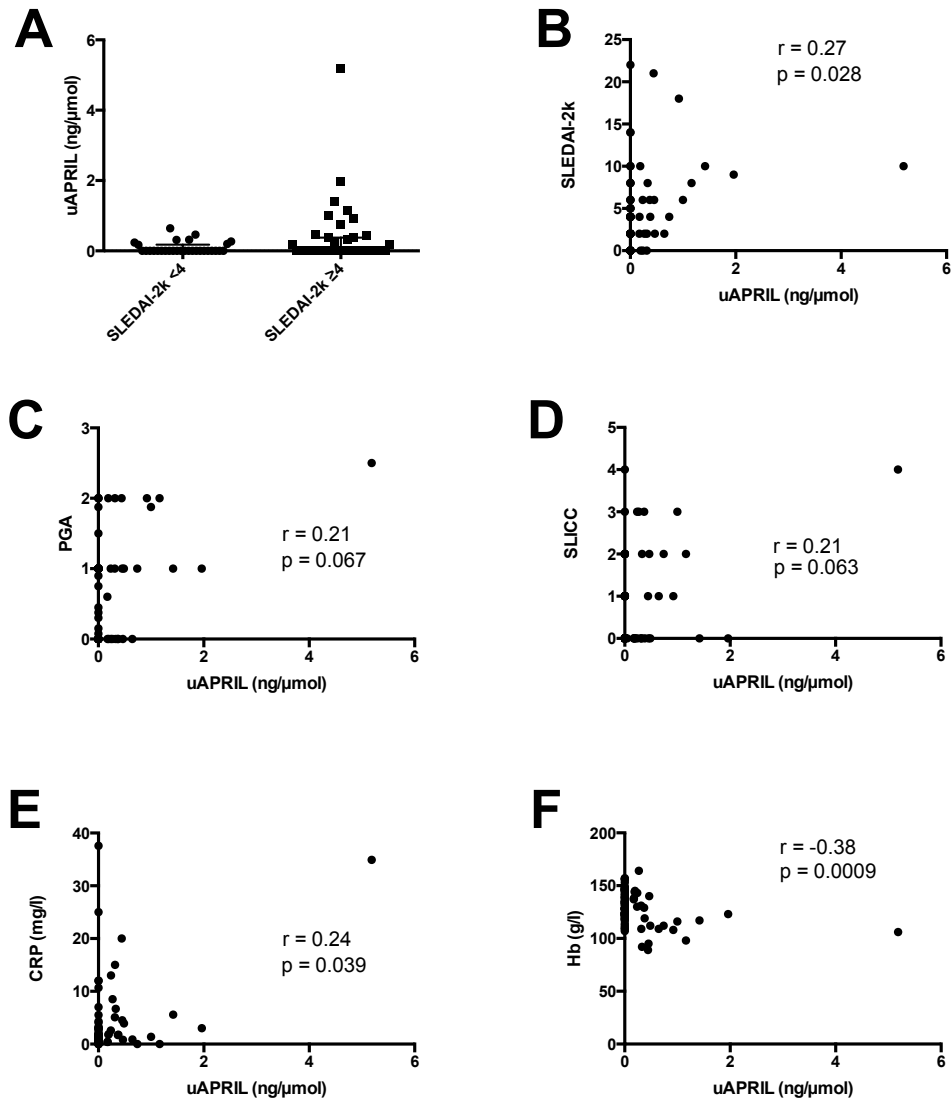


Figure 67. Associations of uAPRIL with SLE disease activity.

Overall SLE disease activity was assessed by the SLEDAI-2k score. (A) uAPRIL according to overall SLE disease activity (SLEDAI-2k <4: n=30; SLEDAI-2k ≥4: n=39). (B) Correlation between uAPRIL and SLEDAI-2k (n=69). (C) Correlation between uAPRIL and PGA in SLE (n=75). (D) Correlation between uAPRIL and SLICC (n=78). Correlation between uAPRIL and (E) CRP (n=74) and (F) Hb (n=75) in SLE.

uAPRIL is expressed in ng/μmol. Levels of CRP and Hb are expressed in mg/l and g/l, respectively.

In panel A, horizontal bars indicate the median with [IQR].

Associations of uAPRIL with renal SLE

uAPRIL was significantly higher in SLE patients with active renal disease compared to those without ($p=0.006$; **Figure 68A**). uAPRIL was also significantly correlated with renal SLEDAI-2k ($r=0.33$, $p=0.005$; **Figure 68B**). Of note, uAPRIL was not significantly correlated with SLEDAI-2k modified to exclude renal components (data not shown), suggesting that the renal component drove the association of uAPRIL with overall disease activity. SLE patients with $eGFR < 90$ had significantly higher uAPRIL compared to those with $eGFR \geq 90$ ($p=0.002$; **Figure 68C**). There was a non-significant trend toward a correlation between uAPRIL and UPCR ($r=0.2$, $p=0.086$; **Figure 68D**), however no significant difference in uAPRIL was noted according to UPCR when the cohort was stratified as having, or not having, proteinuria using upper limit of normal range (0.02 g/mmol) (**Figure 68E**). uAPRIL was significantly correlated with urine micro WBC and RBC ($r=0.37$, $p=0.001$; **Figure 68F**; and $r=0.38$, $p=0.0008$; **Figure 68G**, respectively).

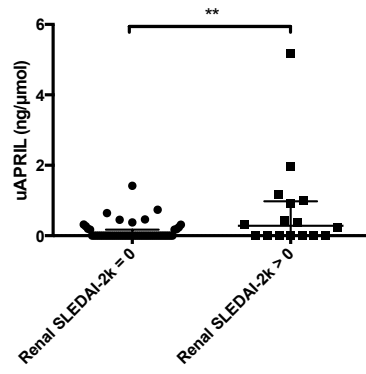
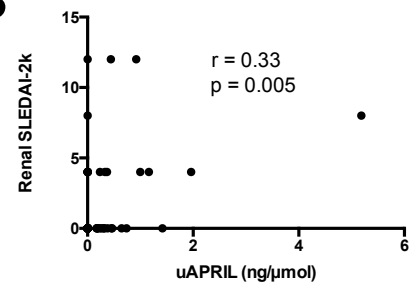
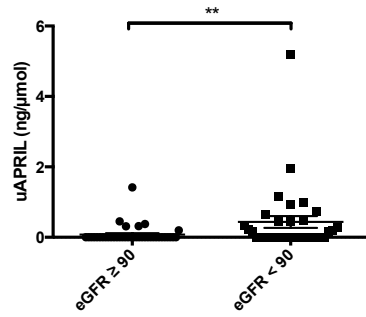
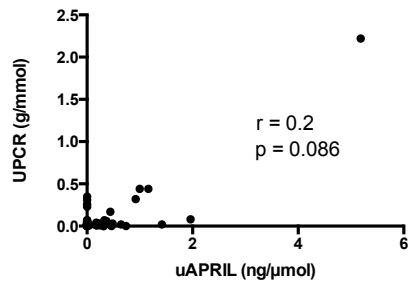
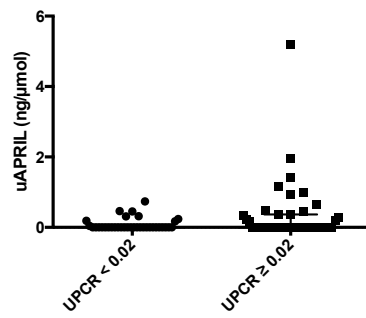
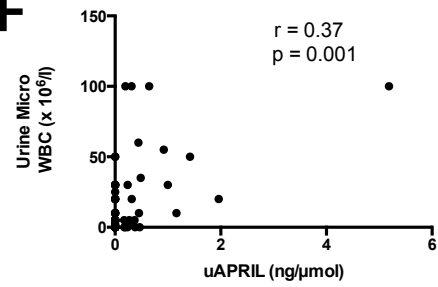
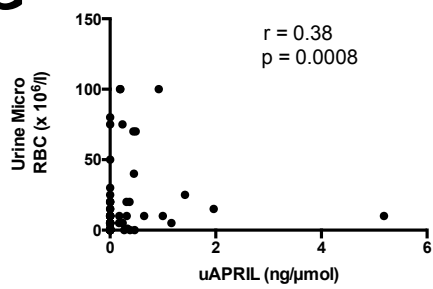
A**B****C****D****E****F****G**

Figure 68. Associations of uAPRIL with renal SLE disease activity.

Renal SLE disease activity was assessed by the SLEDAI-2k score. (A) uAPRIL according to renal SLE disease activity (Renal SLEDAI-2k = 0: n=53; Renal SLEDAI-2k > 0: n=16). (B) Correlation between uAPRIL and renal SLEDAI-2k (n=69). (C) uAPRIL according to eGFR in SLE (eGFR ≥ 90: n=39; eGFR < 90: n=33). (D) Correlation between uAPRIL and UPCR in SLE (n=77). (E) uAPRIL according to UPCR in SLE (UPCR < 0.02: n=35; UPCR ≥ 0.02: n=42). Correlation between uAPRIL and (F) urine micro WBC and (G) urine micro RBC in SLE (n=74).

uAPRIL is expressed in ng/μmol. UPCR and eGFR are expressed in g/mmol and ml/min, respectively. urine micro WBC and RBC are expressed in $\times 10^6/l$.

In panels A and E, horizontal bars indicate the median with [IQR]. In panel C, horizontal bars indicate the mean with SEM.

****** $p < 0.01$.

I next investigated the relationship between uAPRIL and the histological class of LN. uAPRIL was detected in 8/19 (42.1%) of patients with histological class III, IV, or V LN, compared to 2/7 (28.6%) patients with lesser forms of LN ($p=0.67$, Fisher's exact test). No significant relationship was observed between uAPRIL and histological class of LN (**Figure 69A-B**). There appeared to be higher uAPRIL in LN patients with moderate compared to mild or very mild interstitial fibrosis (**Figure 69C**). Accordingly, uAPRIL was significantly higher in LN patients with moderate to severe interstitial fibrosis compared to those with mild or less interstitial fibrosis ($p=0.009$; **Figure 69D**). No significant relationship was observed between uAPRIL and interstitial inflammation (data not shown).

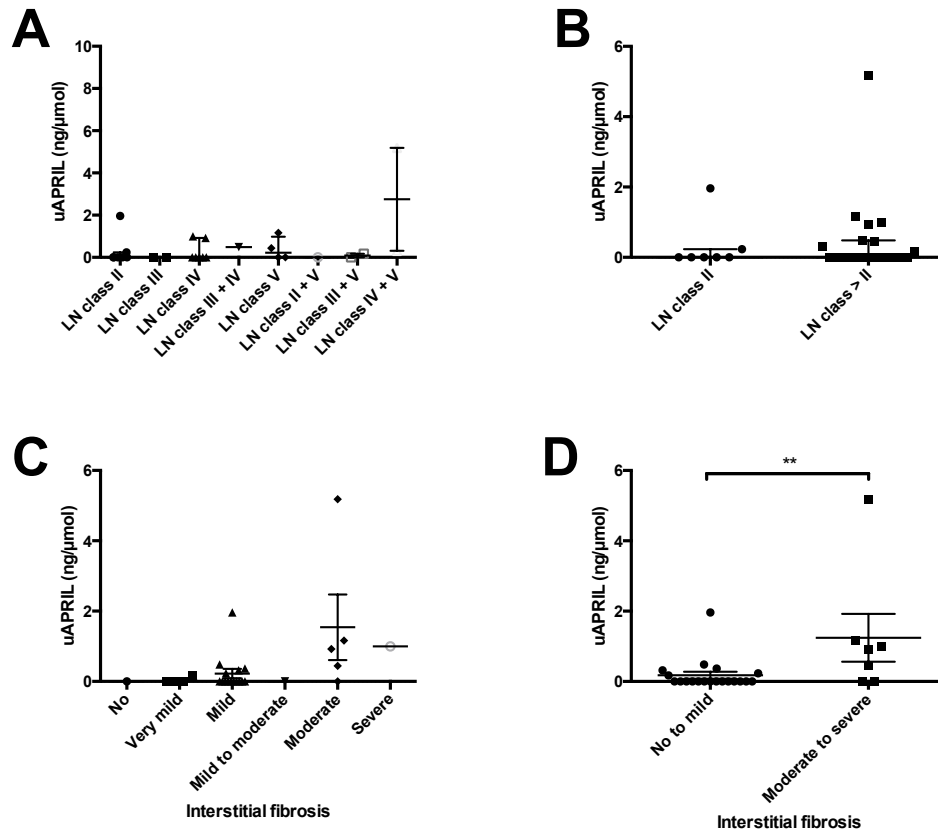


Figure 69. Associations of uAPRIL with kidney biopsy LN assessment.

(A) uAPRIL according to the histological class of LN (LN class II: n=7; LN class III: n=2; LN class IV: n=7; LN class III+IV: n=1; LN class V: n=4; LN class II+V: n=1; LN class III+V: n=2; and LN class IV+V: n=2). (B) uAPRIL between SLE patients with class II LN (n=7) compared to those with higher class of LN (n=19). (C) uAPRIL according to interstitial fibrosis in LN (No=1; very mild: n=4; mild: n=15; mild to moderate: n=1; moderate: n=5; severe: n=1). (D) uAPRIL according to interstitial fibrosis in LN when comparing patients with moderate to severe (n=7) to those with mild or less interstitial fibrosis (n=20).

uAPRIL is expressed in ng/μmol.

In panels A-B, horizontal bars indicate the median with [IQR]. In panels C-D, horizontal bars indicate the mean with SEM.

** $p < 0.01$.

Associations of baseline uAPRIL with subsequent SLE disease activity

No significant correlation was noted between baseline uAPRIL and AMS integrating all subsequent visits over one year following baseline assessment (data not shown). No significant relationship was seen between baseline uAPRIL and overall disease activity measured using the SLEDAI-2k or PGA, or anti-dsDNA, C3 and C4 assessed 18 months from baseline assessment (data not shown). No significant relationship was found between baseline uAPRIL and SLEDAI-2k (**Figure 70A-B**), or anti-dsDNA, C3, and C4 (data not shown) assessed at 24 months. However, PGA at 24 months was significantly higher in SLE patients with detectable baseline uAPRIL compared to those without ($p=0.038$; **Figure 70C**). When analysing 46 samples measured serially in 23 patients, no significant relationship was observed between change in uAPRIL and change in SLE disease activity (data not shown).

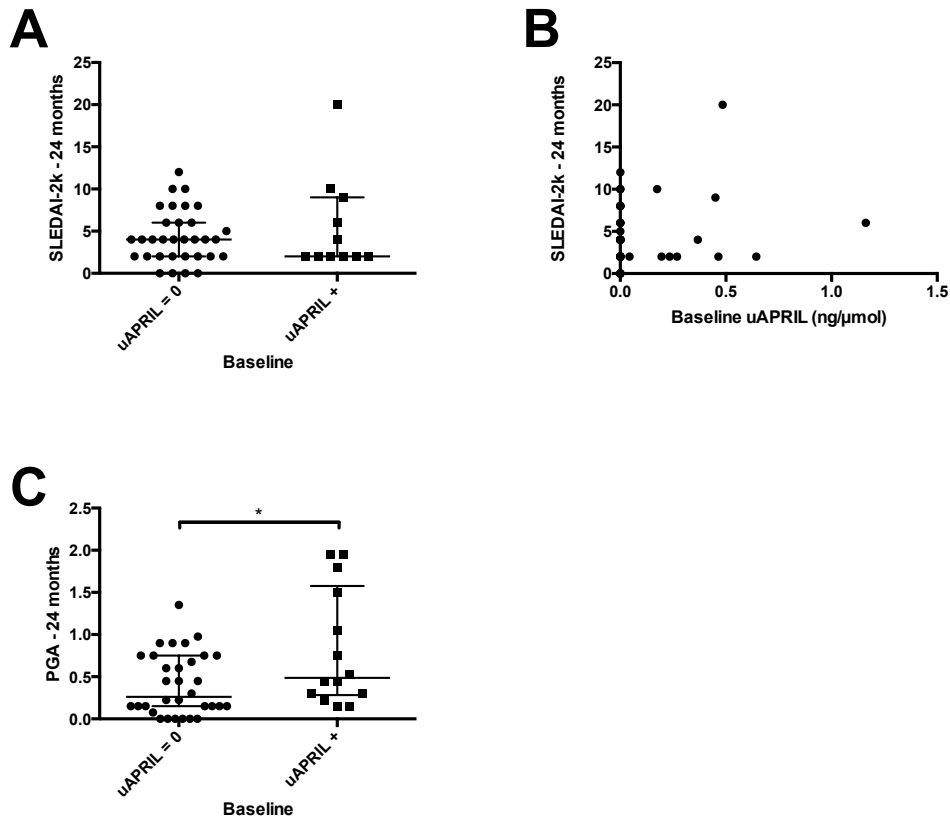


Figure 70. Association between baseline uAPRIL with SLE disease activity over time. Overall SLE disease activity was assessed by the SLEDAI-2k score. (A) Overall SLE disease activity recorded at 24 months according to baseline uAPRIL (uAPRIL = 0: n=32; uAPRIL +: n=11). (B) Correlation between baseline uAPRIL with SLEDAI-2k recorded at 24 months (n=43). (C) Disease activity measured using the PGA recorded at 24 months according to baseline uAPRIL detectability (uAPRIL = 0: n=32; uAPRIL +: n=14). uAPRIL is expressed in ng/μmol. In panels A and C, horizontal bars indicate the median with [IQR].

Associations of baseline uAPRIL with subsequent renal SLE disease activity

No significant relationship emerged between baseline uAPRIL and renal disease activity (**Figure 71A**), or proteinuria (**Figure 71B**) recorded at 18 months, or at 24 months (data not shown) from baseline assessment. In patients in whom serial measures were available, change in uAPRIL was not significantly associated with change in renal SLEDAI-2k or change in renal function markers (creatinine, eGFR, and UPCR) (data not shown).

Regarding the potential effects of one outlier on uAPRIL associations with SLE, Appendix 12 displays the difference in the results of analysis with or without this outlier included. Exclusion of this outlier did not lead to major change in the results.

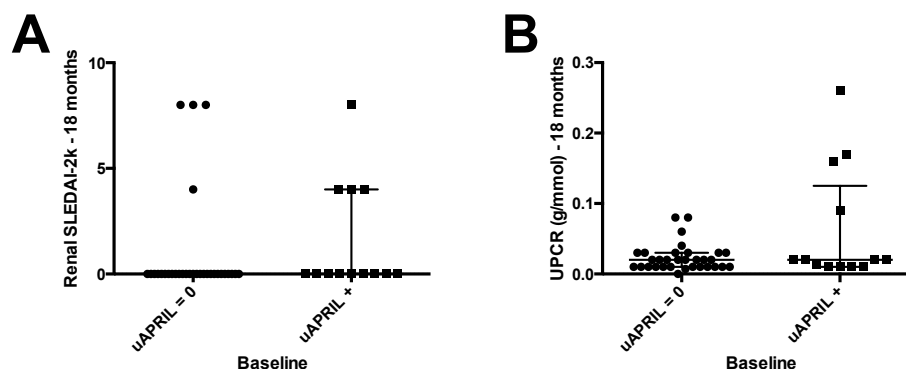


Figure 71. Association between baseline uAPRIL with renal SLE disease activity over time. Renal SLE disease activity was assessed by the SLEDAI-2k score. (A) Renal SLE disease activity recorded at 18 months according to baseline uAPRIL (uAPRIL = 0: n=31; uAPRIL +: n=13). (B) UPCR recorded at 18 months according to baseline uAPRIL (uAPRIL = 0: n=32; uAPRIL +: n=13).

uAPRIL is expressed in ng/ μ mol. UPCR is expressed in g/mmol.
In panels A-B, horizontal bars indicate the median with [IQR].

Associations of uMCP-1 in SLE

I next characterized uMCP-1 in SLE, a previously established LN urinary biomarker. Using the last standard dilution from the standard curve (15.625 pg/ml) as a sensitivity cutoff, uMCP-1 was detectable in 45.8% (22/48) of baseline SLE patients samples (**Figure 72A**). Mean (SEM) uMCP-1 was 29.53 (12.6) pg/ μ mol in SLE. uMCP-1 was detectable in 10% (1/10) of HD, and mean (SEM) uMCP-1 was 0.22 (0.22) pg/ μ mol (**Figure 72A**). uMCP-1 was significantly higher in SLE compared to HD ($p=0.022$; **Figure 72A**). Urinary MCP-1 was not quantified in patients with IgA nephropathy.

uMCP-1 was significantly correlated with uAPRIL ($r=0.48$, $p=0.0006$; **Figure 72B**). Due to the low number of positive samples for uBAFF, no correlation analysis was carried out between uMCP-1 and uBAFF.

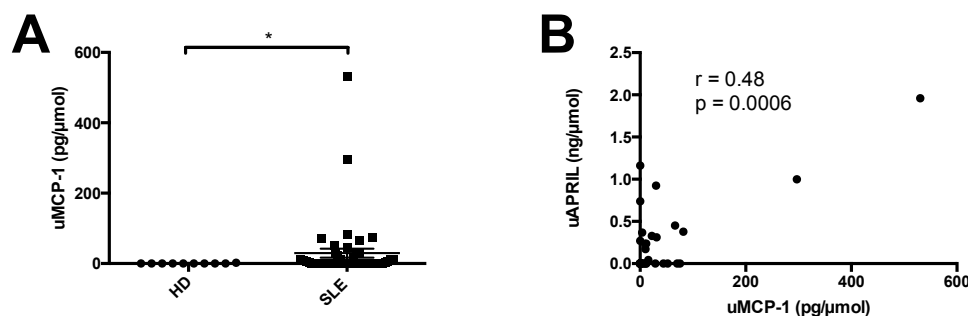


Figure 72. uMCP-1 in SLE.

(A) uMCP-1 in SLE ($n=48$) compared to HD ($n=10$). (B) Correlation between uMCP-1 and uAPRIL in SLE ($n=48$).

uMCP-1 and uAPRIL are expressed in pg/ μ mol and ng/ μ mol, respectively.

In panel A, horizontal bars indicate the mean with SEM.

* $p < 0.05$

I next compared uMCP-1 between patients stratified by ethnicity. There was no significant difference in uMCP-1 between Caucasian and Asian SLE patients (**Figure 73**).

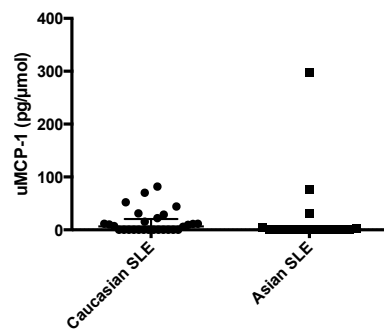


Figure 73. Association of uMCP-1 with ethnicity.
uMCP-1 according to ethnicity [Caucasian (n=28) vs. Asian (n=18)] in SLE.
uMCP-1 is expressed in pg/μmol.
Horizontal bars indicate the median with [IQR].

Associations between serum and urinary MCP-1

Insufficient patients had completion of serum MCP-1 measurement to allow characterisation of relationships between urine and serum MCP-1.

Associations of uMCP-1 with SLE disease activity

uMCP-1 was significantly higher in SLE patients with active disease compared to those without as assessed by the SLEDAI-2k ($p=0.007$; **Figure 74A**). Accordingly, uMCP-1 was significantly correlated with SLEDAI-2k ($r=0.44$, $p=0.003$; **Figure 74B**). No significant relationship was noted with disease activity assessed by PGA, with flare of disease, or with SLICC (data not shown). There was a non-significant trend toward higher uMCP-1 in SLE patients with anti-dsDNA Abs compared to those without ($p=0.057$; **Figure 74C**). No significant relationship was observed of uMCP-1 with inflammation markers CRP and ESR, C3 and C4, anti-ENA Abs and RF (data not shown).

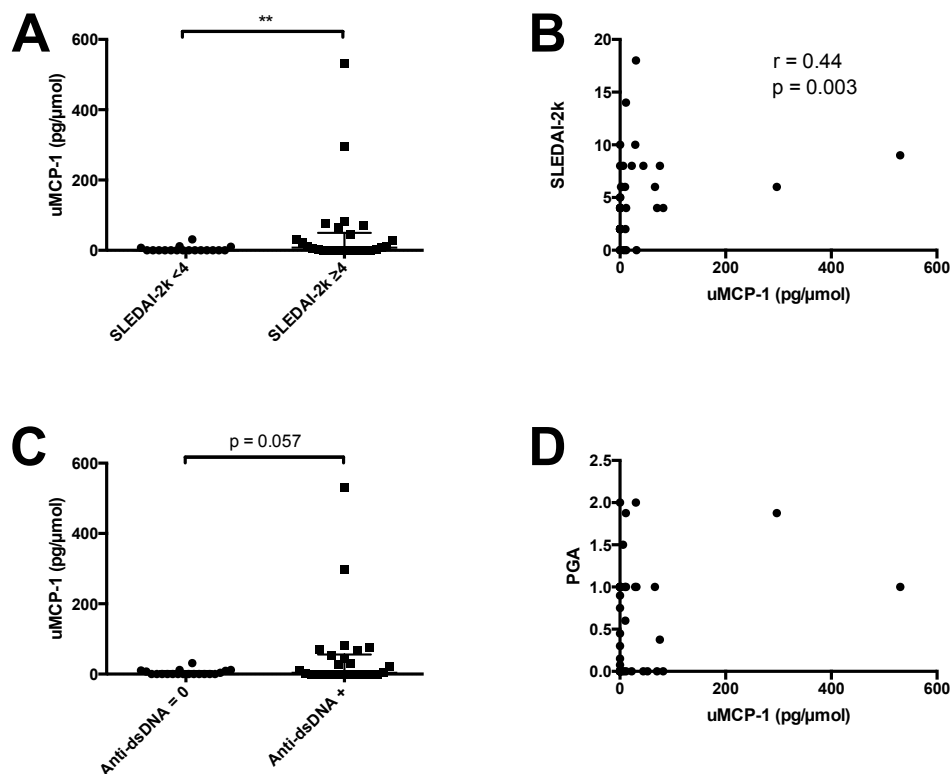


Figure 74. Associations of uMCP-1 with SLE disease activity.

Overall SLE disease activity was assessed by the SLEDAI-2k score. (A) uMCP-1 according to overall SLE disease activity (SLEDAI-2k <4: $n=18$; SLEDAI-2k ≥ 4 : $n=26$). (B) Correlation between uMCP-1 and SLEDAI-2k ($n=44$). (C) uMCP-1 according to the presence of anti-dsDNA Abs in SLE (Anti-dsDNA = 0: $n=20$; Anti-dsDNA +: $n=26$). (D) Correlation between uMCP-1 and PGA ($n=46$).

uMCP-1 is expressed in pg/μmol. Levels of anti-dsDNA Abs are expressed in IU/ml.

In panels A and C, horizontal bars indicate the median with [IQR].

** $p < 0.01$.

Associations of uMCP-1 with renal SLE

uMCP-1 was significantly higher in SLE patients with active renal disease compared to those without ($p=0.0003$; **Figure 75A**). Only three possible values applied to renal SLEDAI-2k among urine samples that were tested for uMCP-1, precluding correlation analysis. Of note, no significant correlation was observed for uMCP-1 with SLEDAI-2k modified to exclude the renal component of the SLEDAI-2k score (data not shown), suggesting that the renal component drove the association observed with overall SLE disease activity. uMCP-1 was significantly correlated with UPCR ($r=0.35$, $p=0.014$; **Figure 75B**), and there was a non-significant trend toward higher uMCP-1 in SLE patients with $UPCR \geq 0.02$ compared to those with $UPCR < 0.02$ ($p=0.07$; **Figure 75C**). uMCP-1 was significantly correlated with urine micro WBC ($r=0.33$, $p=0.027$; **Figure 75D**) and urine micro RBC ($r=0.39$, $p=0.009$; **Figure 75E**). No significant relationship emerged with serum creatinine or eGFR (data not shown).

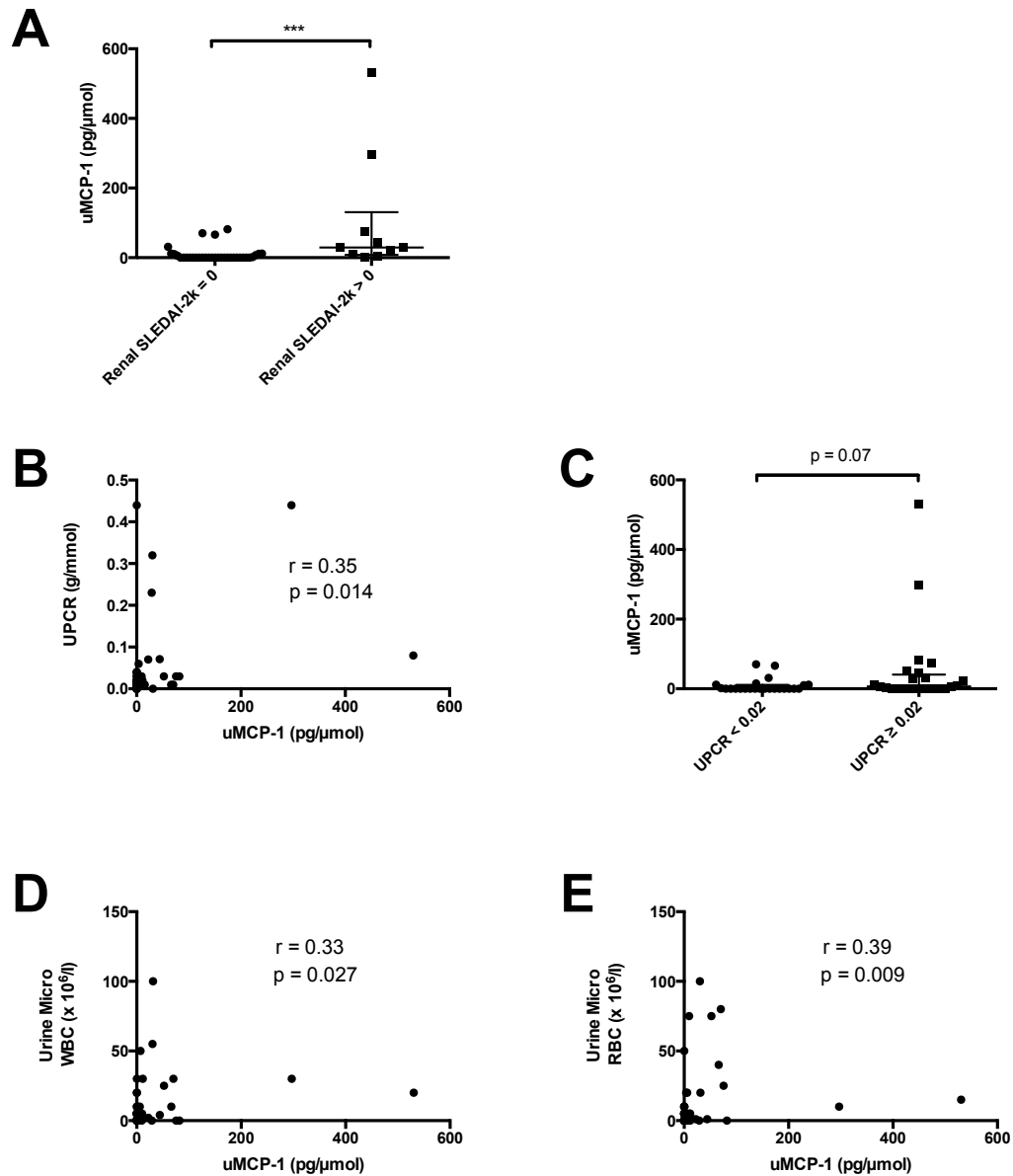


Figure 75. Association of uMCP-1 with renal SLE disease activity.

Renal SLE disease activity was assessed by the SLEDAI-2k score. (A) uMCP-1 according to renal SLE disease activity (Renal SLEDAI-2k = 0: n=34; Renal SLEDAI-2k > 0: n=10). (B) Correlation between uMCP-1 and UPCR in SLE (n=48). (C) uMCP-1 according to UPCR in SLE (UPCR < 0.02 g/mmol: n=24; UPCR ≥ 0.02 g/mmol: n=24). Correlation between uMCP-1 and (D) urine micro WBC and (E) urine micro RBC in SLE (n=45).

uMCP-1 is expressed in pg/μmol. UPCR is expressed in g/mmol. Urine micro WBC and RBC are expressed in x 10⁶/l.

In panels A and C, horizontal bars indicate the median with [IQR].

*** $p < 0.001$.

I next investigated the relationship between uMCP-1 and the histological class of LN. uMCP-1 was detectable in 4/8 (50%) of patients with histological class III, IV, or V LN, and in 3/6 (50%) patients with lesser forms of LN ($p=1$, Fisher's exact test). No significant relationship was observed between uMCP-1 with histological class of LN (**Figure 76A-B**), interstitial fibrosis (**Figure 76C-D**), or interstitial inflammation (data not shown).

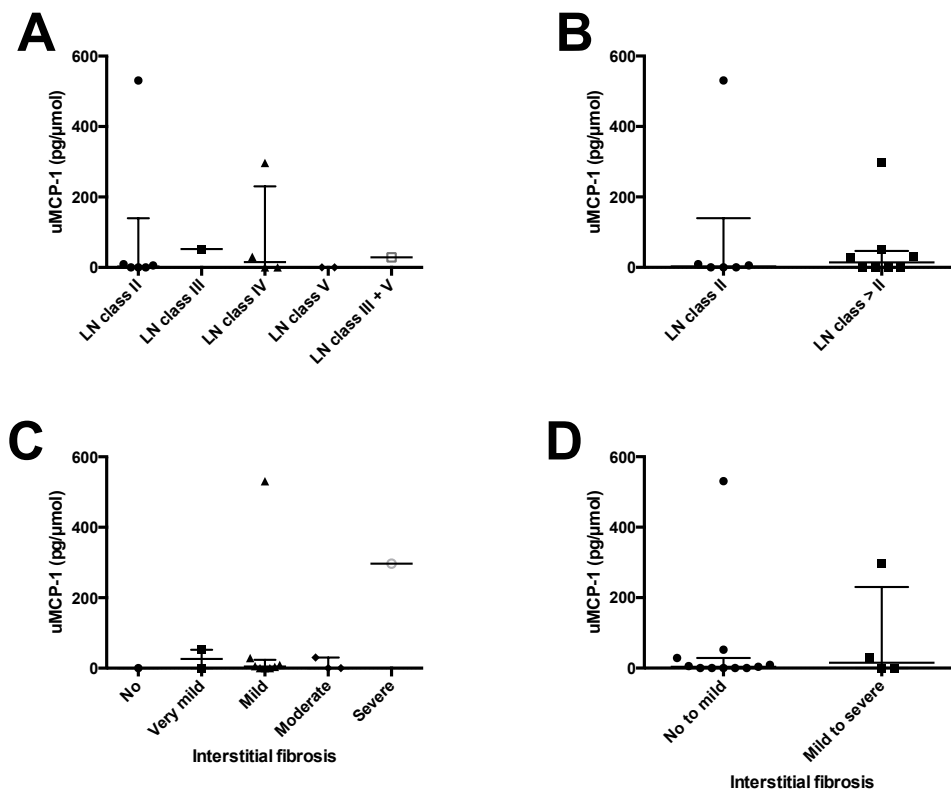


Figure 76. Associations of uMCP-1 with kidney biopsy LN assessment.

(A) uMCP-1 according to the histological class of LN (LN class II: $n=6$; LN class III: $n=1$; LN class IV: $n=4$; LN class V: $n=2$; LN class III+V: $n=1$). (B) uMCP-1 between SLE patients with class II LN ($n=6$) compared to those with higher class of LN ($n=8$). (C) uMCP-1 according to interstitial fibrosis in LN (no: $n=1$; very mild: $n=2$; mild: $n=8$; moderate: $n=3$; severe: $n=1$). (D) uMCP-1 according to interstitial fibrosis in LN when comparing patients with more than mild ($n=4$) to those with mild or less interstitial fibrosis ($n=11$). uMCP-1 is expressed in $\text{pg}/\mu\text{mol}$.

In panels A-D, horizontal bars indicate the median with [IQR].

Associations of baseline uMCP-1 with subsequent SLE disease activity

There was a non-significant trend toward a correlation between baseline uMCP-1 and disease activity integrated over the following year (AMS) (1 year) ($r=0.29$, $p=0.066$; **Figure 77A**). No significant relationship was observed between baseline uMCP-1 and overall SLE disease activity assessed by the SLEDAI-2k or PGA, or anti-dsDNA, C3 and C4 assessed at 18 months from baseline (data not shown). No significant relationship was observed between baseline uMCP-1 and SLEDAI-2k (**Figure 77B-C**), or anti-dsDNA, C3, and C4 (data not shown) recorded at 24 months from baseline. However, baseline uMCP-1 was significantly correlated with PGA recorded at 24 months ($r=0.54$, $p=0.012$; **Figure 77D**). In patients in whom serial samples were available, no relationship was seen between change in uMCP-1 and change in overall SLE disease activity (data not shown).

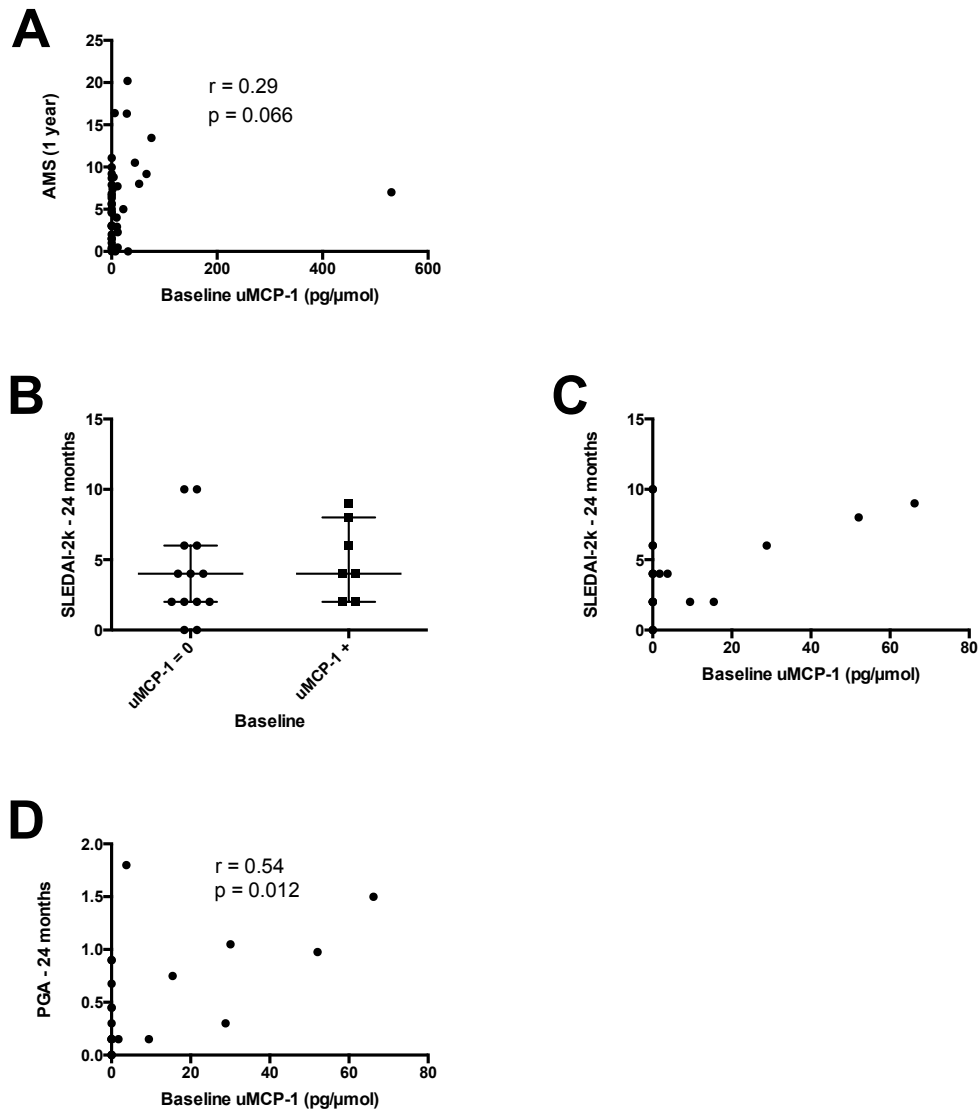


Figure 77. Association between baseline uMCP-1 with SLE disease activity over time.

Overall SLE disease activity was assessed by the SLEDAI-2k score. (A) Correlation between baseline uMCP-1 and AMS integrating all subsequent visits over one year following baseline assessment ($n=41$). (B) Overall SLE disease activity recorded at 24 months according to baseline uMCP-1 (uMCP-1 = 0: $n=13$; uMCP-1 +: $n=7$). Correlation between baseline uMCP-1 with (C) SLEDAI-2k ($n=20$) and (D) PGA ($n=21$) recorded at 24 months. uMCP-1 is expressed in pg/μmol.

In panel B, horizontal bars indicate the median with [IQR].

Associations of baseline uMCP-1 with subsequent renal SLE disease activity

No significant relationship was observed between baseline uMCP-1 and renal SLE disease activity (**Figure 78A**) or proteinuria (**Figure 78B**) recorded at 18 months from baseline assessment, or at 24 months (data not shown). Assessing serial samples, change in uMCP-1 was not significantly associated with change in renal SLEDAI-2k, or in renal function tests (data not shown).

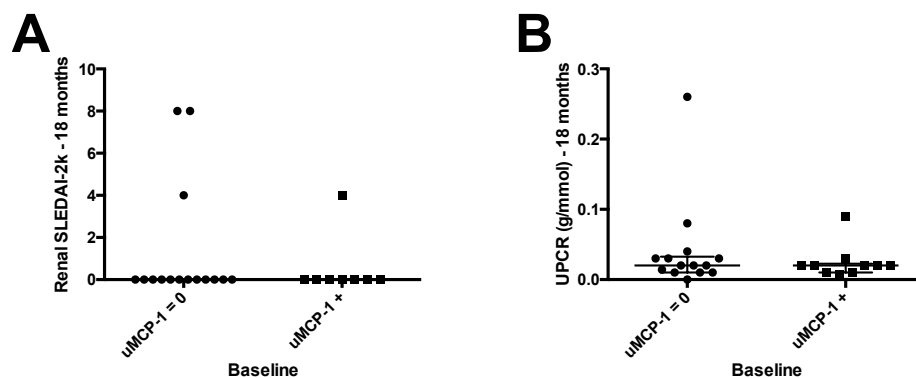


Figure 78. Association between baseline uMCP-1 with renal SLE disease activity over time.

Renal SLE disease activity was assessed by the SLEDAI-2k score. (A) Renal SLE disease activity recorded at 18 months according to baseline uMCP-1 (uMCP-1 = 0; n=15; uMCP-1 +; n=8). (B) UPCR activity recorded at 18 months according to baseline uMCP-1 (uMCP-1 = 0; n=14; uMCP-1 +; n=10).

uMCP-1 is expressed in pg/ μ mol. UPCR is expressed in g/mmol.

In panels A-B, horizontal bars indicate the median with [IQR].

4. Discussion

In the studies presented in this Chapter, I aimed to evaluate the presence of BAFF and APRIL in the urine of SLE patients, and to characterize clinical associations with their detection, particularly in LN. Another aim was to determine whether the presence of these cytokines in the urine of SLE patients may predict overall and/or renal SLE disease activity. uBAFF and uAPRIL were compared to uMCP-1, the previously best characterised urinary biomarker in SLE.

I report here for the first time associations of uAPRIL with SLE, paired with analysis of uBAFF and uMCP-1. uBAFF, but not uAPRIL, was significantly higher in SLE patients than in healthy controls. Increased serum levels of BAFF and APRIL have been reported in SLE and other AID, such as RA and pSS (reviewed in[187]). However, their clinical relevance as SLE biomarkers, and to the pathogenesis of SLE, is still being debated, including in studies presented elsewhere in this thesis (reviewed in[184]). Association between serum BAFF or APRIL levels and disease activity score is variable across studies. No published peer-reviewed study has investigated the presence of BAFF and APRIL in urine of SLE patients. Davis *et al.* reported the presence of BAFF in the urine of all tested SLE patients but not in healthy subjects, however in a study presented in abstract form only to date.[39] The authors reported urinary BAFF levels being higher in SLE patients with renal manifestations, correlating with proteinuria, and characterised by a non-significant trend toward an association with SLEDAI-2k. However, this study was of small sample size, quantifying BAFF, without APRIL, by ELISA in the urine of only 18 SLE patients and 10 healthy subjects. Moreover, the authors did not correct for effect on urinary BAFF concentrations due to dilution by normalising against urine creatinine concentrations.

In the current study, BAFF was detectable in the urine of less than 10% of SLE patients, but concentrations were significantly higher than in healthy subjects or IgA

nephropathy patients. The presence of BAFF in the urine can be explained as the result of one or more processes. Firstly, urinary BAFF may reflect excretion of serum BAFF, however in my study no relationship was found between BAFF concentrations in the urine and in the serum. Secondly, urinary BAFF may reflect non-specific losses due to glomerular damage and proteinuria.[26] However, arguing against this, among the 18 patients with IgA nephropathy who had both urine and serum tested for BAFF, mean (SEM) serum BAFF concentrations was 1001 (77.5) pg/ml, while uBAFF was not detectable. Finally, urinary BAFF may represent *in situ* BAFF production by infiltrating leukocytes and/or renal epithelial cells,[110, 153] in line with the reported production of BAFF by salivary gland epithelial cells in pSS.[79] Collectively, the current data suggest that the presence of BAFF in the urine of SLE patients may be the consequence of pathological mechanisms operative in LN rather than simply a reflection of systemic BAFF production.

The present study evaluated the presence of BAFF in the urine of pSS patients, as a control group for SLE. None of the pSS patients from this cohort had BAFF detected in their urine sample, even when serum BAFF was detectable. One could speculate that uBAFF may be specific to SLE, and particularly LN. However, since none of the pSS patients had an active renal disease, it would be of value to quantify BAFF in the urine samples of pSS patients with active renal disease. This may help to define whether uBAFF is specific to LN or to active renal disease in the setting of autoimmunity. One of the challenges to performing such study would be the rarity of renal disease in pSS patients, estimated to be less than 10%.[51]

In cross sectional analysis, uBAFF was significantly positively associated with overall disease activity, as measured by the SLEDAI-2k, and with flare of disease. A significant positive relationship was observed between uBAFF and active LN measured using the renal SLEDAI-2k, albeit not with individual laboratory measures such as proteinuria. While

uBAFF was not related to the histological class of LN or interstitial inflammation, uBAFF was significantly positively associated with interstitial fibrosis in LN patients. Moreover, longitudinal analysis demonstrated an association of baseline uBAFF with subsequent renal disease activity, suggesting that urinary BAFF measurement may predict future SLE renal disease activity. However, as urinary BAFF was detectable in only a few samples in our cohort, caution should be taken when interpreting these data. Of note, exclusion of two outliers for uBAFF led to a change in significance for the association of baseline uBAFF with subsequent renal SLEDAI-2k recorded at 18-months, to non-significant. Further research is needed to confirm these findings, in a larger SLE cohort, in light of the low rate of detectable uBAFF. Alternatively, future assays with lower detection limits may reveal stronger associations than those able to be demonstrated here.

Building on original observations linking anti-dsDNA autoimmunity and BAFF overexpression in mice,[167] some human SLE studies reported a correlation between serum BAFF and/or APRIL concentrations and disease activity or anti-dsDNA antibody concentrations,[122] whereas others did not (reviewed in[184]). Compared to a previous study investigating correlations of serum BAFF with disease activity,[122] the current cohort is characterised by more severe disease activity and higher proportion of patients with Asian ethnicity. Neither uBAFF nor uAPRIL was correlated with anti-dsDNA antibodies levels in our study. However, uBAFF was significantly higher in SLE patients with anti-Sm autoantibodies, and thus uBAFF may for some reason associate with disease associated with specific SLE antibodies. The lack of correlation of uBAFF with anti-dsDNA titres requires confirmation in other cohorts.

uBAFF was significantly higher in Asian compared to Caucasian SLE patients. This may be related to the higher rate of renal SLE in Asians [35.7% (10/28)] compared to Caucasians ([11.4% (5/44)] in the present study. This may also be explained by difference in

disease severity between these ethnic groups (reviewed in[182, 184]). Further research is needed to determine whether uBAFF may be a biomarker of particular utility in Asian SLE patients suffering from LN.

uAPRIL and uMCP-1 were detectable in nearly one third and about 45% of SLE patients, respectively, hence in a higher proportion of SLE patients than uBAFF. As opposed to uMCP-1, uAPRIL was not significantly higher in SLE compared to HD. A non-significant trend toward a positive correlation between serum and urine concentrations was observed, however only in SLE patients with detectable uAPRIL. Interestingly, uAPRIL was significantly correlated with uMCP-1. These findings are in line with previous studies showing that APRIL is detectable more frequently than BAFF in the serum of patients with SLE,[185] and suggest that urinary APRIL may be a surrogate for systemic APRIL levels.

uAPRIL was significantly related to overall SLE disease activity, as assessed by the SLEDAI-2k score. uAPRIL was also characterized by a non-significant trend toward correlations with PGA and SLICC indices. One of the most important findings of the current study is that uAPRIL was significantly associated with renal lupus. uAPRIL was significantly associated with renal SLEDAI-2k, and, in contrast to uBAFF, with renal SLE laboratory markers including eGFR, and a non-significant trend with UPCR. In addition, a significant relationship with interstitial fibrosis was also observed. Exclusion of renal variables from the SLEDAI-2k abolished associations of uAPRIL with overall disease activity, suggesting that uAPRIL may be a renal-centric biomarker in SLE. No predictive value emerged for uAPRIL regarding renal SLE. Nevertheless, as APRIL was frequently present in the urine of healthy individuals, its value as a marker of overall or renal disease activity in SLE requires further confirmation.

uMCP-1 has previously been reported as a marker of LN in several studies, compared to which the present study is of modest size.[1, 129, 148] Here, I found that the presence of

uMCP-1 had stronger statistical association with the presence of active nephritis than did either uBAFF or uAPRIL, as assessed by the renal SLEDAI-2k and UPCR. Although no significant association was found between uMCP-1 and subsequent renal disease activity, there was a non-significant trend toward a correlation with AMS integrated all subsequent visits over one year, suggesting also potential predictive value as a SLE biomarker, as previously reported.[148]

Table 8 summarises findings on relationships between both uBAFF and uAPRIL compared to uMCP-1, with renal SLE disease activity.

Table 8. Heat map of *p* values regarding relationships between uBAFF, uAPRIL, and uMCP-1 with renal SLE disease at baseline.

	uBAFF	uAPRIL	uMCP-1
Renal SLEDAI-2k			
<i>Association</i>	0.002	0.006	0.0003
<i>Correlation</i>	ND	0.005	ND
UPCR	NS	0.086	0.014
eGFR	NS	0.002	NS
Interstitial fibrosis	0.002	0.009	NS

*A 2-Colour scale has been applied, with red as the lowest *p* value, and white as *p* equal or above to 0.05. Presence of an active renal SLE disease was assessed using the SLEDAI-2k score. All *p* values are from Mann Whitney test, unless otherwise specified. *p* values displayed for renal SLEDAI-2k (Correlation), and for UPCR are from Spearman correlation test.*

APRIL: a proliferation-inducing ligand; BAFF: B cell-activating factor from the tumour necrosis factor family; eGFR: estimated glomerular filtration rate; MCP-1: monocyte chemoattractant protein 1; ND: not determined; NS: not significant; SLE: Systemic lupus erythematosus; SLEDAI: SLE disease activity index; SLEDAI-2K: SLEDAI 2000; UPCR: urine protein/creatinine ratio.

The potential mechanistic association of these molecules with LN requires consideration. In BAFF-Tg mice, BAFF overexpression led to a SLE/SS-like syndrome, characterized by glomerulonephritis with Ig deposits.[96] As mentioned earlier, intra-renal expression of both BAFF and APRIL was reported in LN.[110, 153] As previously discussed, the presence of BAFF in the urine of some SLE patients in the present study may result, at least in part, from *in situ* production, potentially by infiltrated autoreactive immune cells and/or renal resident cells. Moreover, BAFF has been reported to play a role in mesangial cell proliferation via signalling through BAFF-R.[198] Tubulointerstitial mRNA levels of BCMA and TACI have also been reported higher in LN.[110] Collectively, the presence of BAFF in renal tissue reflected in the urine of SLE patients, may play a role in LN pathogenesis, potentially via the BAFF-BAFF-R axis in mesangial cells. However, the role of BAFF and/or APRIL signalling via tubulointerstitial TACI and/or BCMA has yet to be explored. The role of both BAFF and APRIL in LN remained to be delineated, particularly in light of anti-BAFF and atacicept trials in LN.

As discussed in Chapter VI, SLE patients with acute LN were excluded from the two belimumab phase III clinical trials (BLISS-52 and BLISS-76) which have shown efficacy in SLE patients primarily with mucocutaneous and musculoskeletal manifestations;[54, 109] hence data on anti-BAFF therapy efficiency in LN are currently lacking. However, *post-hoc* pooled analyses of these two trials suggest potential interest for belimumab in renal SLE (reviewed in[184]). Among SLE patients without organ domain involved at baseline, rates of patients with worsening of the renal domain, as assessed by the SELENA-SLEDAI, were significantly lower when treated with belimumab compared to placebo at 52 weeks.[98] Among the 267 SLE patients with renal involvement at baseline, authors report numerically higher rates of patients with renal SELENA-SLEDAI improvement when treated with belimumab compared to placebo.[43] Among the patients with proteinuria ≥ 1 g/24h at

baseline, numerically higher renal remission rates were observed with belimumab compared to placebo.[43] Numerically less renal flare rates were observed in the pooled study cohort when treated with belimumab. Of particular interest was the observation that patients with renal involvement receiving MMF at baseline were more likely to benefit from combination therapy with belimumab, when compared to placebo.[43] Collectively, these data show potential benefits for belimumab therapy in LN. However, the BLISS-52 and BLISS-76 trials were not designed to investigate the effect of belimumab at the organ level. Further studies are warranted to assess the efficacy of BAFF-targeting therapies in LN. As mentioned in Chapter VI, trials of belimumab, and of another anti-BAFF therapy (blisibimod), to evaluate their respective efficiency in LN are currently registered (ClinicalTrials.gov identifiers: NCT01639339; NCT02260934; NCT02284984; NCT02514967). My findings suggest the potential utility of uBAFF measurement as a surrogate endpoint in such studies.

Atacicept, a recombinant form of TACI which can bind both BAFF and APRIL, has also been studied in SLE (reviewed in[187]). A phase II/III clinical trial studying atacicept in combination with MMF and corticosteroids in active LN was terminated after the observation of increased infection rates and decreased serum IgG levels, after enrolling only six patients amongst whom four received atacicept.[57] While drop in serum IgG levels was actually observed before atacicept exposure, and may thus incriminate MMF as a potential cause, these data raise the question of the potential benefit of blocking both BAFF and APRIL in LN. Another trial was conducted using atacicept in patients with moderate-to-severe SLE, excluding moderate-to-severe glomerulonephritis and severe CNS disease, and reported no difference in flare rate or time to first flare between atacicept 75mg and placebo.[76] Data on atacicept 150mg suggested benefits of higher dosage versus placebo, however this arm was terminated due to the occurrence of two deaths (infection).[76] Of particular interest is the recently published *post-hoc* analyses of this trial showing that elevated baseline of both

serum BAFF and APRIL levels were associated with greater treatment response to atacicept, suggesting serum BAFF and APRIL as potential biomarker to stratify SLE patients for atacicept therapy.[64] A phase IIb clinical trial on atacicept in SLE and its long-term follow-up study are currently ongoing (ClinicalTrials.gov identifiers: NCT01972568 and NCT02070978). However, no atacicept trial is currently registered to study its efficiency in LN. In the present study, uBAFF and uAPRIL were both significantly associated with renal SLE manifestations, suggesting that both BAFF and APRIL may play a role in LN, and that patients with LN may benefit from BAFF +/- APRIL-targeting therapy. Whether uBAFF and/or uAPRIL may help to stratify SLE patients who are more likely to respond to atacicept and/or belimumab, particularly in LN, remains to be determined.

A number of caveats apply to the interpretation of the findings of this study. Firstly, the SLE patients were from a single centre. However, in this centre, longitudinal data collection and matched sample handling are well defined;[185, 186] nonetheless confirmation in other well-characterised SLE cohorts would be desirable. Patients with renal disease in this study, for the most part, had established renal disease; analysis of urinary BAFF and APRIL in SLE patients without LN to determine the association of urinary BAFF and APRIL with subsequent development of LN, for example in a multi-centre inception cohort, would be of value. In the current study, the sample size is larger than some of previous studies of urinary biomarkers in SLE,[45, 113, 189] and has the advantage of longitudinal disease activity data being available over a period subsequent to baseline, but a still larger study, ideally with longitudinal analysis of changes in uBAFF and uAPRIL in LN patients undergoing therapy would be of interest. The serum samples studied were from a longitudinal cohort and not timed to the renal biopsy. Analysis of associations of urinary BAFF and APRIL with matching histological class of LN in a larger SLE cohort are required, in light of the small sample of patients with such available data in our cohort. This might determine whether

uBAFF and/or uAPRIL measurement could guide the need for renal biopsy in SLE. The healthy control group was of modest sample size. Finally, in light of the low number of patients with detectable uBAFF in our SLE cohort, caution should be taken when interpreting these results, while awaiting confirmation by larger studies.

In conclusion, urinary APRIL and BAFF appear to be biomarkers for renal SLE disease activity. The association of a single measurement of urinary BAFF with current and subsequent renal disease activity is a novel observation, as is the association between urinary APRIL and renal SLE. These findings bring new understanding of the role of the BAFF/APRIL system in SLE, suggesting that both BAFF and APRIL play a role in LN. Urinary BAFF and APRIL may be useful tools for the detection and monitoring of LN. Demonstration of a functional role for BAFF and APRIL in the pathogenesis of LN requires further study. The exclusion of patients with active nephritis from clinical trials of belimumab means that information on the potential of anti-BAFF therapies in the treatment of this complication of SLE is lacking.[54, 109] The current findings suggest that such studies may be justified, and moreover, suggest the inclusion of urinary BAFF and APRIL measurement as a surrogate outcome measure in such trials.

CHAPTER IX: Concluding Discussion

In the studies presented in this thesis, I have tested the overarching hypothesis that serum and urine biomarkers can be used to stratify subsets of SLE patients in relation to clinical phenotype. I aimed to increase understanding of the role of BAFF and related proteins in SLE and pSS, by undertaking composite biomarker studies including measurement of components of the Fas/FasL and the Type II IFN systems, as well as considering potential roles of soluble BAFF receptors, and urinary BAFF and APRIL. The present work focused on the analysis of clinically defined SLE subsets, stratified by clinical phenotype and by ethnicity.

In this thesis, I have tested the sub-hypothesis that SLE patient stratification by clinical phenotype in analysis of associations of serum biomarkers may characterize subsets of patients with pathway-dependent SLE manifestations. I have investigated relationships of serum soluble components of the BAFF/APRIL, Fas/FasL and Type II IFN systems with SLE. In the studies presented in Chapter III, I found that serum sFas was related to overall SLE disease activity, and specifically to renal disease. In contrast, sFasL was not related to overall SLE disease activity as assessed by the SLEDAI-2k score. However, sFasL was related to anti-dsDNA and anti-Sm Abs. While bearing in mind the small subset of SLE patients with neurological manifestations in the studied cohort, and therefore the constraints on interpretation, both sFas and sFasL were found related to neurological phenotype. In the studies presented in Chapter V, I showed that serum BAFF was related to overall SLE disease activity, and particularly to renal disease activity, and to a lesser extent to musculoskeletal SLE manifestations. Only a non-significant trend toward an association was found between serum BAFF and neurological SLE. I found no relationship between serum IFN- γ and SLE disease activity, particularly no relationship with renal SLE, in contrast to previously published studies. In the studies presented in Chapter VI, I investigated

relationships of soluble forms of BAFF receptors with SLE. I first reported the potential confounding effect of anti-BAFF and B cell-depleting agents when measuring these soluble receptors. I found that serum sTACI was negatively correlated with overall SLE disease activity, but not associated with any clinical phenotype. In contrast to sTACI, but similar to sFasL, no relationship was observed between sBCMA and overall disease activity as assessed by the SLEDAI-2k score. However, an association with anti-dsDNA Abs was found. Also similar to sFasL, an association between serum sBCMA and neurological SLE was also observed, with the caveat as above that numbers of patients thus affected were small. Finally, no relationship emerged between sBAFF-R and overall SLE disease activity. Only a non-significant trend toward an association between sBAFF-R and immunological SLEDAI-2k was seen.

Collectively, the BAFF/APRIL and Fas/FasL systems appear to play a role in SLE, whereby sFas, BAFF and sTACI may be serum biomarkers of interest. At the organ level, BAFF and sFas appear to be related to renal manifestations, while only BAFF was found to be related to musculoskeletal SLE. Further research is needed to define whether these potential biomarkers, particularly those related to clinical phenotypes, are redundant or complementary to defined pathway-dependent SLE manifestations. For instance, several different biomarkers for renal SLE may characterise the same patient subsets where several pathways are simultaneously activated in disease pathogenesis. In this scenario, the potential for combination therapy targeting the affected pathways may be of value, although of course this requires future study. Alternatively, one pathway may be activated downstream of another; hence selecting the optimal ‘upstream’ target within these pathways may provide an alternative targeted therapy approach. In another potential scenario, different biomarkers for renal SLE may characterise different LN patient subsets where distinct pathways are individually activated in disease pathogenesis, defining biologically distinct subsets of renal

SLE. In this scenario, distinct targeted therapy may be defined according to each patient subset. Such approach would pave the way to a personalised medicine approach to treating aspects of SLE in individual patients stratified using biomarkers.

In my thesis I have tested the sub-hypothesis that SLE patients can be stratified using combinations of the biomarkers measured. I have also tested the sub-hypothesis that biomarkers measured in combination may better reflect immunological pathways involved in SLE, compared to individual markers. In SLE, sFasL/sFas ratio was related to overall disease activity, and particularly LN. The association between sFasL/sFas ratio and overall disease activity was similar to the one observed between sFas and disease activity. At the organ level, while associations between sFas and sFasL/sFas ratio with UPCR were similar, association between renal SLEDAI-2k and sFas was stronger than that with sFasL/sFas ratio. These novel findings require confirmation in independent cohorts, but are among the first studies to test ways in which to combine multiple biomarkers to better inform patient stratification.

In a further exploration of the utility of biomarker combinations, I focussed on patients with and without elevated BAFF. I chose this target as it is the only currently targetable cytokine in SLE in clinical practice, and as it is known that only a subset of SLE patients responds to BAFF blockade, the possibility of a BAFF-independent subset of SLE is raised. My studies have identified a SLE patient subset, defined by non-elevated serum BAFF levels, wherein serum sFasL/sFas ratios were significantly associated with mucocutaneous SLE, while such a relationship was absent in SLE patients with high serum BAFF levels. Of note, neither sFas nor sFasL were individually related to mucocutaneous manifestation in the non-elevated BAFF subset. Conversely, the relationship between both sFas and sFasL/sFas ratio and renal SLE was only observed in patients with high BAFF. If one speculates that SLE patients with non-elevated serum BAFF levels may have non-BAFF-mediated disease, then sFasL might emerge as a target for therapeutic intervention in non-BAFF-mediated SLE,

where biologics such as belimumab may be ineffective. Conversely, as efficacy of anti-BAFF therapy has not been demonstrated in renal SLE, it may be that targeting sFasL in combination with BAFF would be of value in LN. My findings suggest that patients potentially amenable to such an approach could be identified using serum biomarkers. This underlines potential pathway-dependent immunological SLE phenotypes, for which therapeutic strategies might be considered accordingly. These data also revealed a potential composite biomarker strategy involving measurement of BAFF and sFas with or without sFasL to assess disease activity in LN. Further research is needed to validate such composite biomarker approaches, or therapeutic targeting strategies, in LN.

Of particular interest after having shown a positive relationship between serum BAFF and IFN- γ in SLE, I found that the relationship between serum BAFF and renal SLE was only observed in patients with detectable serum IFN- γ . As mentioned above, using logistic regression, the association between BAFF and renal SLE was significant in univariable analysis. However, in multivariable analysis, while IFN- γ was not found as a confounder for this association, only a non-significant trend toward an association was observed after adjusting for serosal SLEDAI-2k domain. These data suggest that the interplay between the BAFF/APRIL and the Type II IFN systems may be important to consider in patients with LN, which is particularly relevant when defining therapeutic targets for intervention. This composite biomarker approach may help to define a subset of patients with LN who may be more likely to respond to anti-BAFF therapy. This might also help to identify patients with LN who may benefit from a potential combination therapy including BAFF-targeted and IFN- γ -targeted therapies. Finally, this composite biomarker approach may help to assess LN disease activity and potentially guide the need for kidney biopsy. As noted, further research is needed to support clinical translation of these ideas, but my findings provide the first evidence that could underpin such an approach.

I also investigated the clinical associations of the soluble BAFF receptors in relation with BAFF in SLE. I first studied interference between BAFF and its soluble receptors, potentially defining a technical strategy when using a multiplex approach for their measurement, suggesting that BAFF should be quantified separately. These outcomes could be used as potential guidelines for further studies quantifying components of the BAFF/APRIL system, and particularly the cytokine BAFF. Serum BAFF/sTACI ratio was significantly associated with overall SLE disease activity, characterised by a stronger association compared to sTACI alone. At the organ level, a potential relationship between BAFF/sTACI and BAFF/sBAFF-R ratios with musculoskeletal disease was observed, which requires further investigation. It is noteworthy that such relationship with musculoskeletal manifestations was not seen with serum sTACI and sBAFF-R alone, and to a less extent with BAFF alone, again pointing to the possible additional information obtained by measuring biomarkers in combination.

Collectively, therefore, my thesis provides evidence that combination of biomarkers from the same pathway may offer advantages compared to individual markers. BAFF/sTACI and BAFF/sBAFF-R ratios might be of interest as biomarkers for musculoskeletal disease compared to the individual markers. Combining biomarkers from the same and different pathways may also offer advantages, by identifying SLE patient subsets with phenotypic manifestations not evident when using individual biomarkers, such as the relationship between serum sFasL/sFas ratio and mucocutaneous SLE disease activity in the subset of patients with non-elevated BAFF levels. Also of interest was the observation of a relationship between BAFF combined with sFas, sFasL/sFas ratio or IFN- γ , with renal SLE.

In the studies presented in Chapter VIII, I have tested the sub-hypothesis that measurement of BAFF and APRIL in the urine of SLE patients may be clinically relevant, particularly in lupus nephritis. Both BAFF and APRIL were detectable in the urine of some SLE patients. As mentioned previously, reports are inconsistent across studies investigating serum levels of these two cytokines in AID and their relationship with disease activity. The presence of BAFF and APRIL in the urine of some SLE patients provide some potential explanations regarding these discrepancies, with cytokine loss in the urine as one of the mechanisms potentially explaining their presence in the urine of patients. However, the reason for these discrepancies may be multifactorial including also differences in studied populations, assay sensitivity, presence of soluble BAFF receptors, etc.

Both urinary BAFF and APRIL were associated with overall SLE disease activity, and particularly with LN. These data suggest uBAFF and uAPRIL as potential renal-centric SLE biomarkers. Measurement of uBAFF and/or uAPRIL may help to assess LN, potentially improving detection of LN, and guiding the need for kidney biopsy. These data also emphasize the urgent unmet need to evaluate the BAFF-targeting therapies in patients with LN, in light of the exclusion of patients with acute LN from the two phase III clinical trials of belimumab. Some data also suggest studying uBAFF as a potential long-term predictive LN biomarker.

Collectively, both uBAFF and uAPRIL were associated with LN, suggesting their potential as SLE biomarkers. Future studies of anti-BAFF interventions in LN, trials of which are underway, could benefit from analysis of stratification of response according to uBAFF/uAPRIL concentrations, and if proven this approach could be helpful in selecting patients for therapy.

In conclusion, the studies presented in this thesis relating cytokines to overall SLE disease activity reveal that several cytokines, soluble receptors, and ratios of these were significantly related to overall SLE disease activity. Several potential biomarkers, including composite measures, emerged in association with renal, mucocutaneous and potentially musculoskeletal and neurological SLE phenotypes. These data may identify SLE patient subsets for pathway-dependent biological therapeutic intervention. This may particularly help to fine-tune the use of the anti-BAFF drug belimumab, as well as encouraging clinical trials targeting the BAFF/APRIL system in patients suffering from renal SLE. **Table 9** summarizes the relationships of all these analytes with SLE disease manifestations. Future studies should seek to confirm these findings in independent cohorts, and ideally in relation to targeted interventions. Future studies may also take advantage of emerging technologies allowing more ‘unbiased’ analysis of dozens or hundreds of protein analytes in biological specimens.

Table 9. Heat map of association p values between serum/urinary analytes of the BAFF/APRIL, Fas/FasL and Type II IFN systems, with overall and organ-specific SLE disease.

	SLEDAI-2k	Renal	UPCR	Musculoskeletal	Immunological
sFas	0.02	0.001	0.029	NS	NS
sFasL	NS	NS	NS	NS	NS
sFasL/sFas	0.03	0.021	0.017	NS	NS
IFN-γ	NS	NS	NS	NS	NS
BAFF	0.002	0.035*	†	0.044	NS
sTACI	0.048	NS	NS	NS	NS
sBCMA	NS	NS	NS	NS	NS
sBAFF-R	NS	NS	NS	NS	0.039**
BAFF/sTACI	0.003	0.026†	NS	0.009	NS
BAFF/sBCMA	NS	NS	NS	NS	NS
BAFF/sBAFF-R	NS	NS	NS	0.016	NS
uBAFF	0.029	0.002	NS	NS	NS
uAPRIL	§	0.006	0.086	NS	NS
uMCP-1	0.007	0.0003	0.014	NS	NS

A 2-Colour scale has been applied, with red as the lowest p value, and white as p equal or above to 0.05. p values were from Mann-Whitney test, unless otherwise specified. Correlation with UPCr were from Spearman correlation tests. One-tailed p value is shown in italic. Overall, renal, musculoskeletal and immunological SLE disease activity were assessed using the SLEDAI-2k score.

[†] A significant correlation emerged with UPCr, only in SLE subset with detectable IFN- γ .

[§] A significant correlation emerged when using Spearman correlation test ($p=0.028$).

^{} Association only found in univariable analysis using logistic regression.*

*^{**} Only a non-significant trend toward an association was observed between sBAFF-R with active immunological disease in univariable analysis using logistic regression.*

[¶] This association was not confirmed using logistic regression model.

APRIL: a proliferation-inducing ligand; BAFF: B cell-activating factor from the tumour necrosis factor family; BAFF-R: BAFF receptor; BCMA: B cell maturation antigen; FasL: Fas ligand; IFN- γ : Interferon-gamma; MCP-1: monocyte chemoattractant protein 1; NS: not significant; sBAFF-R: soluble BAFF-R; sBCMA: soluble BCMA; sFas: soluble Fas; sFasL: soluble FasL; SLE: Systemic lupus erythematosus; sTACI: soluble TACI; TACI: Transmembrane activator and cyclophilin ligand interactor; UPCr: urine protein/creatinine ratio.

A further potential implication from this work may be the emergence of new non-invasive tools to assess renal SLE disease activity, for which the kidney biopsy is currently the gold standard. In **Table 9** I summarize observed associations of the measured cytokines with renal SLEDAI-2k and the renal disease activity marker UPCr. uMCP-1 and serum sFas showed the strongest associations with renal SLE disease activity. As mentioned above, of particular interest was the observation that the relationship between serum sFas or sFasL/sFas ratio with renal SLE was only present in patients with high BAFF. Hence, stratifying SLE patients according to serum BAFF levels may help to fine-tune the use of serum sFas and sFasL as LN disease activity biomarkers, or suggest patients for whom targeting the Fas/FasL system could be efficient. I also observed that the relationship between serum BAFF and renal SLE disease activity was only seen in SLE patients with detectable IFN- γ . However, while IFN- γ was not a confounder, only a non-significant trend toward an association was observed after multivariable analysis. Finally, I identified associations between both uBAFF and uAPRIL with renal SLE disease activity. Altogether, my findings underline that complex

immunological pathways are involved in LN pathogenesis, involving an interplay between the BAFF/APRIL, Fas/FasL and Type II IFN systems. Identifying primary pathway(s) involved in each individual patient may help to better assess LN activity and potentially guide therapeutic intervention. A combination therapeutic strategy potentially targeting these systems might also be valuable in light of these data, although once again as noted further confirmatory studies are needed. Finally, in the context of clinical trials of biologics targeting the BAFF/APRIL system in LN, associations between urinary BAFF and APRIL and renal SLE disease activity prompt further investigation of the use of these measurements as guides for therapeutic intervention, in patients with LN who might be eligible to receive anti-BAFF therapy.

This area of translational research is complex, even with the finite number of analytes measured in my studies. In the future, algorithms based on multiple analyte measurement could emerge to guide patient stratification and/or personalise medicine. In **Figures 79 and 80**, I depict tree algorithms whereby SLE patients could be stratified using the biomarkers identified in my work for overall and renal SLE disease activity. Whether some or all of these biomarker strategies are redundant, or could be combined into a more complex matrix, remains to be determined. These algorithmic diagrams are presented here not as definitive recommendations for SLE management, but as an example of the type of approach that could emerge from studies of the type I have presented in my thesis.

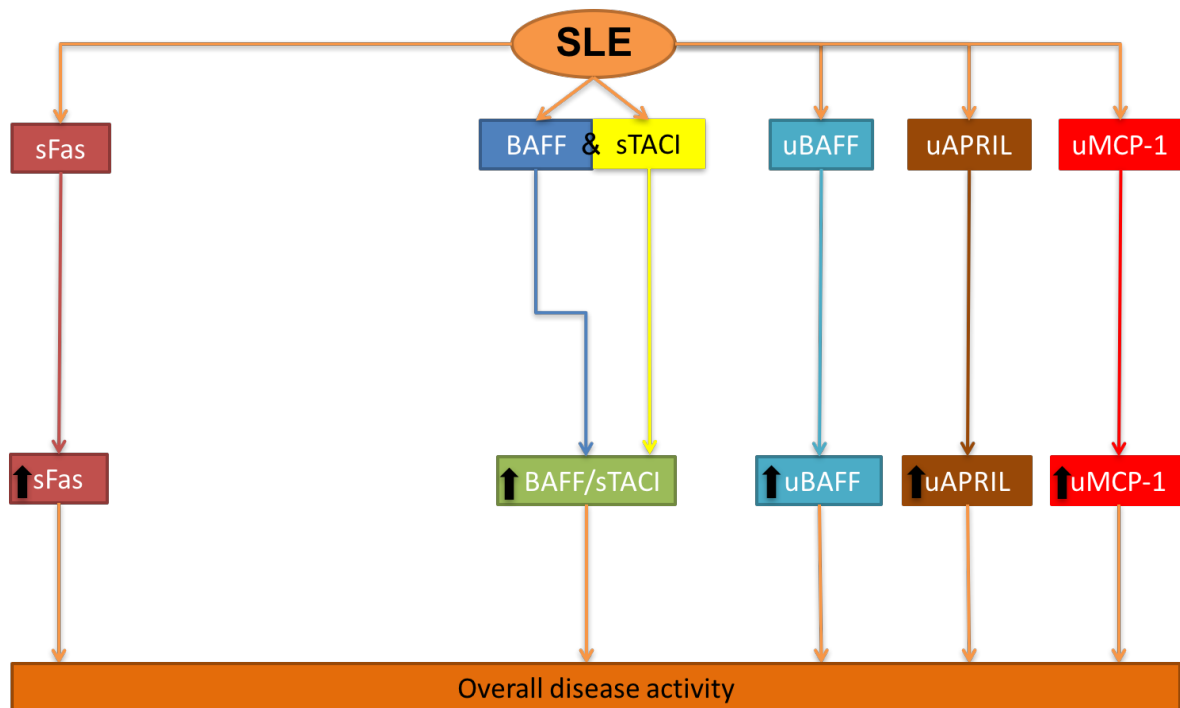


Figure 79. Tree algorithm suggesting SLE patient stratification by serum or urine components of the BAFF/APRIL and/or Fas/FasL systems as potential biomarkers for overall disease activity.

APRIL: a proliferation-inducing ligand; BAFF: B cell-activating factor from the tumour necrosis factor family; MCP-1: monocyte chemoattractant protein 1; s: soluble; SLE: Systemic lupus erythematosus; TACI: transmembrane activator and cyclophilin ligand interactor.

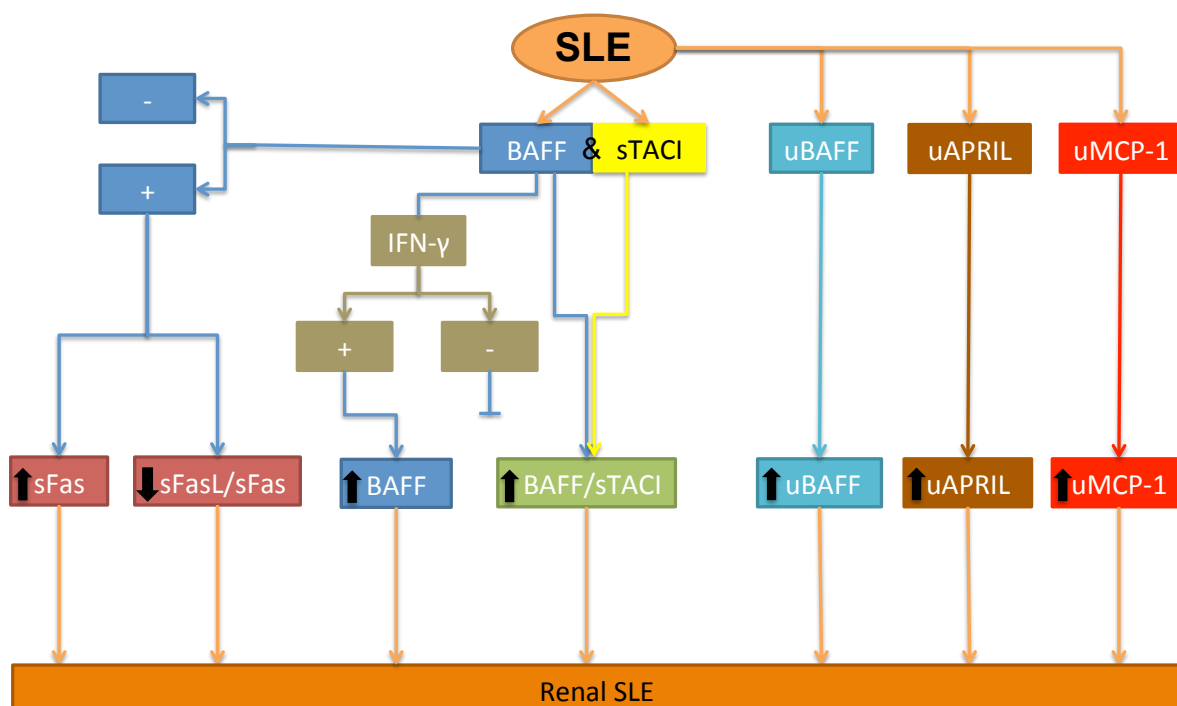


Figure 80. Tree algorithm suggesting SLE patient stratification by serum or urine components of the BAFF/APRIL, Fas/FasL and/or Type II IFN systems as potential renal-centric biomarkers.

The symbols + and – refer, in the examples of BAFF and IFN- γ , to elevated/detectable vs the converse.

APRIL: a proliferation-inducing ligand; BAFF: B cell-activating factor from the tumour necrosis factor family; FasL: Fas ligand; IFN: interferon; MCP-1: monocyte chemoattractant protein 1; s: soluble; SLE: Systemic lupus erythematosus; TACI: transmembrane activator and cyclophilin ligand interactor.

In addition, I have also tested the sub-hypothesis that ethnicity may influence serum biomarker levels and their relationships with SLE. SLE is a multifactorial disease, where genetic and environmental factors play a role in disease pathogenesis. Ethnicity is acknowledged to be associated with both risk and phenotype of the disease. Compared to Caucasians, SLE prevalence is reported to be higher among Asian SLE patients, who are also acknowledged to suffer from more severe disease (reviewed in[184]). Asians SLE patients have a younger onset of the disease, and present with a particular phenotype with increased

renal manifestations and decreased photosensitivity, as well as increased frequency of anti-dsDNA and anti-Sm autoAbs, and hypocomplementaemia.[60]

In the studies presented in this thesis, I found significant variations in serum and/or urine concentrations of components of the BAFF/APRIL and Fas/FasL systems according to SLE patient ethnicity, which underline potential differences in immunological pathways involved in SLE pathogenesis, particularly between Asians and Caucasians. Whether the observed significant increase in serum sFasL and sBCMA, and uBAFF, as well as the non-significant trend toward lower serum sTACI and higher sBAFF-R concentrations, in Asian compared to Caucasian SLE patients plays a role in the higher prevalence and severity of the disease reported in Asian patients should be pursued in larger cohorts. Further research is also warranted to determine whether uBAFF may be of particular interest as a biomarker for Asian SLE patients with LN.

I have also shown that some relationships between sFas and/or sFasL and SLE disease activity were dependent on ethnicity, emphasizing differences in immunological pathways involved in SLE pathogenesis between ethnic groups. Of particular interest, as reported in Chapter III, the relationships observed between renal SLE disease activity and either serum sFas or sFasL/sFas ratio were significant in Asian, but not in Caucasian patients. In the present studies, some of the studied analytes were particularly associated with complement activity, but not with anti-dsDNA Abs, in the Asian SLE subset. In Asian patients, serum sTACI was significantly positively associated with C3 levels, and there was a non-significant trend toward a positive correlation between serum sTACI with C4. BAFF/sTACI ratio was also significantly negatively associated with C3 levels. In contrast, in the subset of Caucasian SLE patients, anti-dsDNA was significantly positively associated with serum BAFF, BAFF/sTACI ratio and IFN- γ levels, and accordingly complement C3 was significantly negatively associated with serum BAFF, BAFF/sTACI ratio and also BAFF/BAFF-R ratio.

Complement C4 was significantly negatively associated with serum sBAFF-R, and a non-significant trend toward a positive association between C4 and sFasL/sFas ratio was observed (data not shown). Collectively, serum sTACI appears to be particularly associated with complements C3 and C4 levels in Asian SLE patients, while serum BAFF, sBAFF-R and BAFF/sTACI ratio appear to be related to anti-dsDNA levels in Caucasians. Whilst these findings support the role of the BAFF/APRIL system in both Asian and Caucasian SLE patients, they also underline potential subtle differences in the BAFF/APRIL system according to ethnicity. Conceivably, therapies targeting the cytokine BAFF or one of its cell surface receptors, such as mTACI, may lead to different response in Asian versus Caucasian SLE patients. Few Asian patients were included in clinical trials of belimumab, and my findings suggest the need for new studies of BAFF pathway therapies in these patients.

Finally, I tested the sub-hypothesis that pathway-dependent clinical phenotypes, composite biomarker strategies, and the clinical relevance of urinary measurement of BAFF, may be specific to SLE, by studying pSS in comparison. While no difference emerged in serum BAFF concentrations, serum sTACI was significantly lower, while serum sBCMA was significantly higher, in SLE patients compared to pSS. I did find a significant negative relationship between serum sTACI and overall pSS disease activity, matching what I observed in SLE. In contrast, the absence of a relationship between pSS disease activity and serum BAFF suggests the possibility that BAFF might not be the optimal therapeutic target from the BAFF/APRIL system in pSS. These differences between SLE and pSS may reflect different regulation of the BAFF/APRIL system by their endogenous cognate soluble decoy receptors, which is of relevance when considering therapeutic strategies targeting components of the BAFF/APRIL system. This also suggests that, although these diseases might be BAFF-mediated in some patients, the optimal target of the BAFF/APRIL system for therapeutic intervention may differ between pSS and SLE patients. Further research is needed

to confirm this hypothesis; particularly since the BAFF-targeting therapy belimumab is approved in SLE, and is in clinical trials in pSS, while other biologics targeting the BAFF/APRIL system are also being evaluated in SLE.

In pSS, in contrast to findings in SLE, no relationship was seen between soluble components of the Fas/FasL system and overall disease activity. Collectively, these data suggest that, while the Fas/FasL system may play a role in both SLE and pSS, the involvement of this pathway may differ between SLE and pSS.

I also found a significant relationship between serum BAFF and sFas in both SLE and pSS, which appeared, furthermore, specific to musculoskeletal manifestations of both these conditions. This suggests that, rather than being specific to one particular AID, the interplay between the BAFF/APRIL and Fas/FasL systems may be of particular importance in the musculoskeletal phenotype of these AID. In contrast, when I quantified BAFF in urine samples of both pSS and SLE patients, uBAFF was detectable in SLE, and was associated with renal disease, but no pSS patients had detectable uBAFF. This again highlights the nuances of understanding the relationships between biomarkers, and the potential therapeutic targets they represent, in the context of specific clinical manifestations of AID. In keeping with this, the positive relationship between serum BAFF and sFas was only significant in Caucasian SLE patients; missing ethnicity data in pSS cohort precluded similar analysis in pSS. Hence, BAFF-targeting therapeutic approaches in SLE and pSS, for instance with belimumab, should also consider the potential interplay with the Fas/FasL system, particularly in Caucasian SLE patients, and in patients harbouring musculoskeletal manifestations.

Among the caveats to interpretation of my findings, listed in the Chapters of this thesis, the use and the dosage of corticosteroids and immunosuppressants potentially have complex impact on the data. However, it is important to note that such limitations apply not

only to the present studies, but to all studies focused on biomarker approach in human disease, and particularly in SLE where the broad and variable use of immunosuppressants makes it difficult to control such factors. Much larger studies would be required to account statistically for these potential confounders. However, as anti-cytokine treatments will generally be used in patients with active disease despite standard therapy, understanding the relationship of biomarkers with disease in the context of therapy remains important. In the studies presented in this thesis, some SLE and pSS patients subsets were small or very small, such as neurological or musculoskeletal SLE subsets. Hence, caution is required when interpreting these data, while awaiting future validation studies in independent cohorts. The healthy control groups used in my studies were also small. Longitudinal data analysis in the questions examined in Chapters III to Chapter VII would be of interest, exploring potential predictive value for these biomarkers. While Chapter VIII included longitudinal data analysis, only 25 SLE patients (29%) had a followup urine sample collected matching with clinical data. Further research is needed, on larger longitudinal SLE cohorts, to explore the potential for urinary BAFF and APRIL as predictors of SLE disease activity, particularly renal manifestations.

In conclusion, in this thesis, I have characterised the clinical associations of multiple biomarkers in a large and well-defined SLE cohort. I report for the first time the presence and clinical associations of sBAFF-R in human sera in SLE, as well as for sTACI and sBCMA in sera from patients with pSS. I have also described for the first time the clinical associations at the phenotypic level of serum sBCMA in SLE. I have described the clinical associations at the phenotypic level of sTACI in a large and well-defined SLE cohort. This work is also the first to describe the presence and clinical associations of APRIL in the urine of patients with SLE, and to evaluate the presence of both BAFF and APRIL in the urine of SLE patients followed longitudinally. I report for the first time the clinical associations of the ratio

between sFasL and sFas in pSS, and I have evaluated for the first time the clinical associations of soluble ligand and receptor using the recently validated ESSDAI scoring system. This work is the first to evaluate the presence uBAFF in pSS.

I have tested the overarching hypothesis that serum and urine biomarkers can be used to stratify subsets of SLE patients according to clinical phenotype. The novel findings of this thesis are listed in Appendix 13. By quantifying components of the BAFF/APRIL, Fas/FasL and the Type II IFN systems, I have identified immunological profiles related to disease activity in SLE and pSS, with some particular differences between these diseases. My data show that some immunological profiles were related to particular phenotypic manifestations and ethnicity. I have also further characterized the interplay between the BAFF/APRIL, Fas/FasL and Type II IFN systems in human SLE and pSS. I have reported the potential benefit of quantifying some components of these systems in combination, potentially yielding a composite biomarker strategy to better assess disease activity and guide therapeutic intervention. This work gives potential new explanations regarding the discrepancies across studies investigating serum BAFF and APRIL in SLE, by considering the presence of these two cytokines in the urine of patients, as well as considering the presence of the three BAFF receptors in their soluble form.

This work adds to understanding of the pathogenesis of both SLE and pSS, by identifying immunological pathways potentially mediating particular phenotypic manifestations. This may lead to the identification of new therapeutic strategies, for example in non-BAFF-mediated SLE and in pSS, as well as to better direct the use of BAFF/APRIL targeting therapies. This work underscores the need to stratify SLE into subsets characterized by specific immunological pathways operative in defined phenotypic subsets, particularly in the era of targeted biologic therapies for SLE and pSS.

More research is needed on the role of the BAFF/APRIL system in SLE pathogenesis, and particularly in LN. Whether BAFF, APRIL and/or other members of this system such as TACI are potential therapeutic targets in LN remains to be determined. Future prospective and longitudinal studies in larger multicentre and multiethnic SLE and pSS cohorts are warranted, particularly to validate the suggested tree algorithms model for SLE patient stratification. Such studies focused on clinical phenotype would require at least 30 patients per clinical phenotype, as well as a broad range of overall disease activity with a minimum of 30 patients with each of inactive, active and highly active disease.

Using a combination of biomarkers may assist in patient assessment, particularly in LN, potentially guiding the need for kidney biopsy. This approach might also lead to the ability to optimize patient selection for therapeutic intervention. Investigating the effect of such biological stratification for patient selection in future clinical trials would be required for such an application to be validated. Integrating the potential biomarkers presented in this thesis with additional analytes from biological systems acknowledged to play a role in disease pathogenesis into a more complex multidimensional matrix may lead to a more precise individual patient immunological profile, that may serve as a foundation for personalised medicine.

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Appendix 1

Emails from R & D Systems regarding analysis of interference of sBCMA and sTACI with serum BAFF detection by Luminex

2014-01-30 Melissa O'Donnell [REDACTED]

Hello again,

I was not sure if the lab would have more information for me today but as soon as I sent my email to you both today, I received an update from the lab.

Fabien - If you are still interested, the 6-plex can be built and shipped early next week. The new kit will provide comparable results with the kits shipped earlier (lot # 1344262). With our most recent testing, a shift in the curve shape for BAFF in the presence of BCAM and TACI is observed with an impact on higher abundance samples. What did you observe with the most recent multiplex? Are you looking at relative values or do you require more accurate results? If you are only looking at relative values, this should not be a problem but if you require more accurate results, the lab recommends splitting the panel. Please let me know how you would like to proceed and if you have any questions.

Sincerely,

Melissa O'Donnell

Technical Service Representative

R & D Systems
[REDACTED]

[REDACTED]

[REDACTED]

--

On 7 February 2014 at 02:24, Melissa O'Donnell [REDACTED]

wrote:

Dear Fabien,

Interference by BCMA and TACI causes a drop in the MFIs for the BAFF standard curve resulting in increased sample values for BAFF. Values for BMCA and TACI are not affected. If you want accurate values and not just relative values, we recommend splitting the 6-plex into a 2-plex with BAFF and Fas Ligand and a 4-plex with BCMA, TACI, FAS and IFN gamma. If you decide to go the route of splitting the analytes, I have been approved to replace all 3 of the 6-plexes with the 2-plex and 4-plex kits. It will not be a problem to wait until March to make a decision.

I hope this answers your questions, if not let me know. In the meantime, I will cancel the order for the 6-plex. I will enter a new order when you decide how you would like to proceed.

Sincerely,

Melissa O'Donnell

Technical Service Representative

R & D Systems

[REDACTED]

Fax: [REDACTED]

Phone [REDACTED]

Appendix 2

Associations of uBAFF with SLE in the whole cohort and in the subset comprising only urine samples with PIC.

	SLE cohort (N=86)	SLE subset where PIC was added to urine samples (N=68)	Difference
	P value	P value	
Associations of uBAFF with SLE			
SLE vs. HD vs. IgAN (cutoff 62.5)	NS	0.019*	Y
SLE vs. HD detectability (cutoff 62.5)	NS	NS	
SLE vs. IgAN detectability (cutoff 62.5)	NS	0.045	Y
SLE vs. HD vs. IgAN (cutoff 6.44)	0.007 [#]	0.002 [†]	
uBAFF according to ethnicity	0.009	0.006	
Associations between serum and urinary BAFF			
Serum BAFF according to uBAFF detectability	NS	NS	

	SLE cohort (N=86)	SLE subset where PIC was added to urine samples (N=68)	Difference
	P value	P value	
Associations of uBAFF with SLE disease activity			
uBAFF according to overall SLEDAI-2k	0.029	0.028	
uBAFF according to flare	0.012	0.006	
uBAFF according to anti-Sm	0.007	0.008	
uBAFF according to anti-dsDNA	NS	NS	
Associations of uBAFF with renal SLE			
uBAFF according to renal SLEDAI-2k	0.002	0.003	
uBAFF according to UPCR	NS	NS	
uBAFF according to kidney biopsy histological class	NS	NS	
uBAFF according to kidney biopsy interstitial fibrosis	0.007 [§]	0.01 [¶]	
uBAFF according to kidney biopsy interstitial inflammation	NS	NS	

	SLE cohort (N=86)	SLE subset where PIC was added to urine samples (N=68)	Difference
	P value	P value	
Associations of baseline uBAFF with subsequent SLE disease activity			
AMS (1 year) according to uBAFF detectability	0.011	0.009	
SLEDAI-2k at 18-months according to uBAFF detectability	0.005	0.006	
C3 at 18-months according to uBAFF detectability	0.008	0.01	
C4 at 18-months according to uBAFF detectability	0.022	0.019	
C3 at 24-months according to uBAFF detectability	0.015	0.015	
Associations of baseline uBAFF with subsequent renal SLE disease activity			
Renal SLEDAI-2k at 18-months according to uBAFF detectability	NS	NS	
UPCR at 18-months according to uBAFF detectability	NS	NS	
uBAFF according to renal SLEDAI-2k at 18-months	0.04	0.044	

Mann Whitney and Kruskal-Wallis tests (followed by Dunn's multiple comparisons test) were used to analyse differences between two and more than two variables, respectively.

* Kruskal-Wallis test is shown. Dunn's test: SLE higher than HD ($p=0.072$); SLE higher than IgAN ($p=0.052$).

Kruskal-Wallis test is shown. Dunn's test: SLE higher than HD ($p=0.039$); SLE higher than IgAN ($p=0.026$).

† Kruskal-Wallis test is shown. Dunn's test: SLE higher than HD ($p=0.012$); SLE higher than IgAN ($p=0.008$).

§ Kruskal-Wallis test is shown. Dunn's test: very mild vs. moderate: $p=0.023$; mild vs. moderate: $p=0.008$.

¶ Kruskal-Wallis test is shown. Dunn's test: very mild vs. moderate: $p=0.045$; mild vs. moderate: $p=0.012$.

AMS: Adjusted mean SLEDAI-2k; BAFF: B cell-activating factor from the tumour necrosis factor family; C3: complement component 3; C4: complement component 4; dsDNA, double-stranded deoxyribonucleic acid; HD: healthy donor; IgAN: immunoglobulin A nephropathy; NS: Not significant; PIC: Protease Inhibitor Cocktail; SLE: Systemic lupus erythematosus; SLEDAI: SLE disease activity index; SLEDAI-2K: SLEDAI 2000; SLICC: systemic lupus international collaborating clinics; Sm: Smith; UPCR: urine protein/creatinine ratio.

Appendix 3

Associations of uAPRIL with SLE in the whole cohort and in the subset comprising only urine samples with PIC.

	SLE cohort (N=86)	SLE subset where PIC was added to urine samples (N=68)	Difference
	P value	P value	
Associations of uAPRIL in SLE			
SLE vs. HD detectability (cutoff 0.78125)	NS	NS	
SLE vs. HD vs. IgAN (cutoff 0.78125)	NS	NS	
SLE vs. HD vs. IgAN (cutoff 0.4)	NS	NS	
uAPRIL according to ethnicity	NS	NS	
Associations between serum and urinary APRIL			
Serum APRIL according to uAPRIL detectability	NS	NS	
Serum APRIL vs. uAPRIL	NS	NS	

	SLE cohort (N=86)	SLE subset where PIC was added to urine samples (N=68)	Difference
	P value	P value	
Serum APRIL vs. uAPRIL in subset of uAPRIL detectable samples	NS*	NS	
Associations of uAPRIL with SLE disease activity			
uAPRIL according to overall SLEDAI-2k	NS	NS	
uAPRIL vs. overall SLEDAI-2k	0.028 (r=0.27)	0.026 (r=0.31)	
uAPRIL vs. PGA	NS [#]	0.039 (r=0.27)	Y
uAPRIL according to flare	NS	NS	
uAPRIL vs. SLICC	NS [†]	NS	
Associations of uAPRIL with renal SLE			
uAPRIL according to renal SLEDAI-2k	0.006	0.038	
uAPRIL vs. renal SLEDAI-2k	0.005 (r=0.33)	0.031 (r=0.3)	
uAPRIL vs. SLEDAI-2k minus renal SLEDAI-2k	NS	NS	

	SLE cohort (N=86)	SLE subset where PIC was added to urine samples (N=68)	Difference
	P value	P value	
uAPRIL according to eGFR	0.002	0.008	
uAPRIL vs. UPCR	NS [§]	NS	
uAPRIL according to UPCR	NS	NS [¶]	
uAPRIL according vs. urine micro WBC	0.001 (r=0.37)	0.001 (r=0.42)	
uAPRIL according vs. urine micro RBC	0.0008 (r=0.38)	0.0002 (r=0.48)	
uAPRIL according to kidney biopsy histological class	NS	NS	
uAPRIL according to kidney biopsy interstitial fibrosis	0.009	NS [¥]	Y
uAPRIL according to kidney biopsy interstitial inflammation	NS	NS	
Associations of baseline uAPRIL with subsequent SLE disease activity			
uAPRIL vs. AMS (1 year)	NS	NS	
SLEDAI-2k at 24-months according to uAPRIL detectability	NS	NS	

	SLE cohort (N=86)	SLE subset where PIC was added to urine samples (N=68)	Difference
	P value	P value	
uAPRIL vs. SLEDAI-2k at 24-months	NS	NS	
PGA at 24-months according to uAPRIL detectability	0.038	0.038	
Associations of baseline uAPRIL with subsequent renal SLE disease activity			
Renal SLEDAI-2k at 18-months according to uAPRIL detectability	NS	NS	
UPCR at 18-months according to uAPRIL detectability	NS	NS	

Mann Whitney was used to analyse differences between two variables. Spearman's test was used to test correlations between variables.

* $p=0.068$; $r=0.47$

$p=0.067$; $r=0.21$

† $p=0.063$; $r=0.21$

§ $p=0.086$; $r=0.2$

¶ $p=0.073$

¥ $p=0.053$

AMS: Adjusted mean SLEDAI-2k; APRIL: a proliferation-inducing ligand; HD: healthy donor; eGFR, estimated glomerular filtration rate; IgAN: immunoglobulin A nephropathy; NS: Not significant; PGA: physician's global assessment; PIC: Protease Inhibitor Cocktail; RBC, red blood cells; SLE: Systemic lupus erythematosus; SLEDAI: SLE disease activity index; SLEDAI-2K: SLEDAI 2000; SLICC: systemic lupus international collaborating clinics; UPCr: urine protein/creatinine ratio; WBC, white blood cells.

Appendix 4

Associations of uMCP-1 with SLE in the whole cohort and in the subset comprising only urine samples with PIC.

	SLE cohort (N=86)	SLE subset where PIC was added to urine samples (N=68)	Difference
	P value	P value	
Associations of uMCP-1 in SLE			
SLE vs. HD	0.022	0.038	
uMCP-1 vs. uAPRIL	0.0006 (r=0.48)	0.028 (r=0.4)	
uMCP-1 according to ethnicity	NS	NS	
Associations of uMCP-1 with SLE disease activity			
uMCP-1 according to overall SLEDAI-2k	0.007	NS	Y
uMCP-1 vs. overall SLEDAI-2k	0.003 (r=0.44)	0.026 (r=0.43)	
uMCP-1 according to flare	NS	NS	
uMCP-1 vs. PGA	NS	NS	

	SLE cohort (N=86)	SLE subset where PIC was added to urine samples (N=68)	Difference
	P value	P value	
uMCP-1 vs. SLICC	NS	NS	
Associations of uMCP-1 with renal SLE			
uMCP-1 according to renal SLEDAI-2k	0.0003	0.003	
uMCP-1 vs. SLEDAI-2k minus renal SLEDAI-2k	NS	NS	
uMCP-1 vs. UPCR	0.014 (r=0.35)	0.0396 (r=0.38)	
uMCP-1 according to UPCR	NS*	0.041	Y
uMCP-1 vs. urine micro WBC	0.027 (r=0.33)	0.022 (r=0.44)	
uMCP-1 vs. urine micro RBC	0.009 (r=0.39)	0.049 (r=0.38)	
uMCP-1 according to kidney biopsy histological class	NS	NS	
uMCP-1 according to kidney biopsy interstitial fibrosis	NS	NS	
uMCP-1 according to kidney biopsy interstitial inflammation	NS	NS	

	SLE cohort (N=86)	SLE subset where PIC was added to urine samples (N=68)	Difference
	P value	P value	
Associations of baseline uMCP-1 with subsequent SLE disease activity			
uMCP-1 vs. AMS (1 year)	NS [#]	NS	
SLEDAI-2k at 24-months according to uMCP-1 detectability	NS	NS	
uMCP-1 vs. SLEDAI-2k at 24-months	NS	NS	
uMCP-1 vs. PGA at 24-months	0.012 (r=0.54)	NS	Y
Associations of baseline uMCP-1 with subsequent renal SLE disease activity			
Renal SLEDAI-2k at 18-months according to uMCP-1 detectability	NS	NS	
UPCR at 18-months according to uMCP-1 detectability	NS	NS	

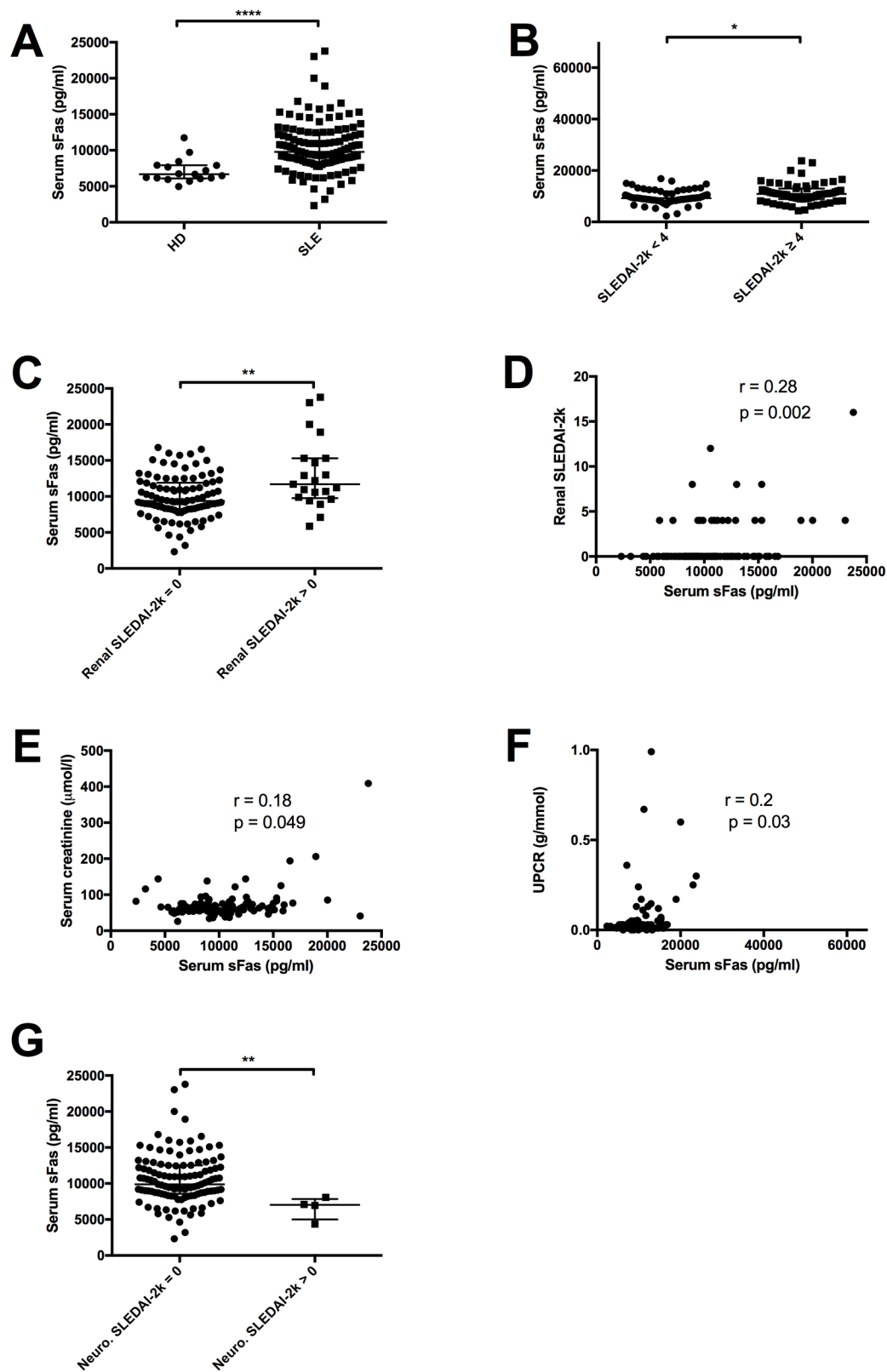
Mann Whitney was used to analyse differences between two variables. Spearman's test was used to test correlations between variables.

* $p=0.07$

$p=0.066$; $r=0.29$

AMS: Adjusted mean SLEDAI-2k; APRIL: a proliferation-inducing ligand; HD: healthy donor; MCP-1: monocyte chemoattractant protein 1; NS: Not significant; PGA: physician's global assessment; PIC: Protease Inhibitor Cocktail; RBC, red blood cells; SLE: Systemic lupus erythematosus; SLEDAI: SLE disease activity index; SLEDAI-2K: SLEDAI 2000; SLICC: systemic lupus international collaborating clinics; UPCR: urine protein/creatinine ratio; WBC, white blood cells.

Appendix 5



Appendix 5. Associations between serum sFas with SLE disease activity.

Overall and renal SLE disease activity was assessed by the SLEDAI-2k score. (A) Serum sFas in SLE patients (n=119) compared to HD (n=17). (B) Serum sFas concentrations according to overall SLE disease activity (SLEDAI-2k < 4: n=59; SLEDAI-2k ≥ 4: n=60). (C) Serum sFas concentrations according to renal SLE disease activity (Renal SLEDAI-2k = 0: n=98; Renal SLEDAI-2k > 0: n=21). (D) Correlation between serum sFas and renal SLEDAI-2k (n=119). (E) Correlation between serum sFas and serum creatinine (n=117). (F) Correlation between serum sFas and UPCR (n=114). (G) Serum sFas concentrations according to neurological SLE disease activity (Neuro. SLEDAI-2k = 0: n=115; Neuro. SLEDAI-2k > 0: n=4).

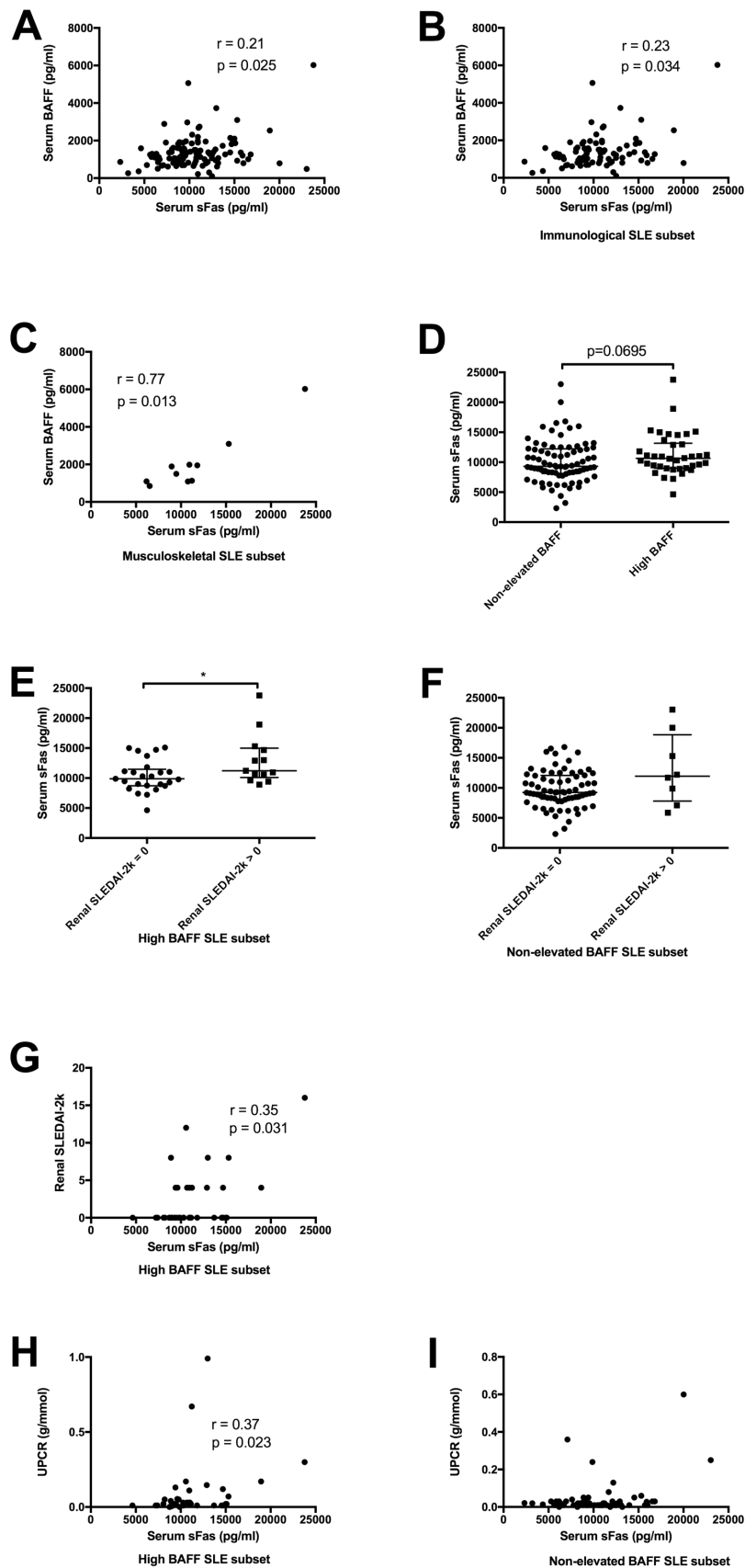
One outlier value for serum sFas in SLE (63591.65 pg/ml) has been excluding.

Serum sFas concentrations are expressed in pg/ml. Serum creatinine and UPCR are expressed in μmol/l and g/mmol, respectively. Neuro. SLEDAI-2k stands for neurological SLEDAI-2k.

In panels A-C and G, horizontal bars indicate the median with [IQR].

**** $p < 0.0001$; ** $p < 0.01$; * $p < 0.05$

Appendix 6



Appendix 6. Relationship between serum sFas and BAFF in SLE.

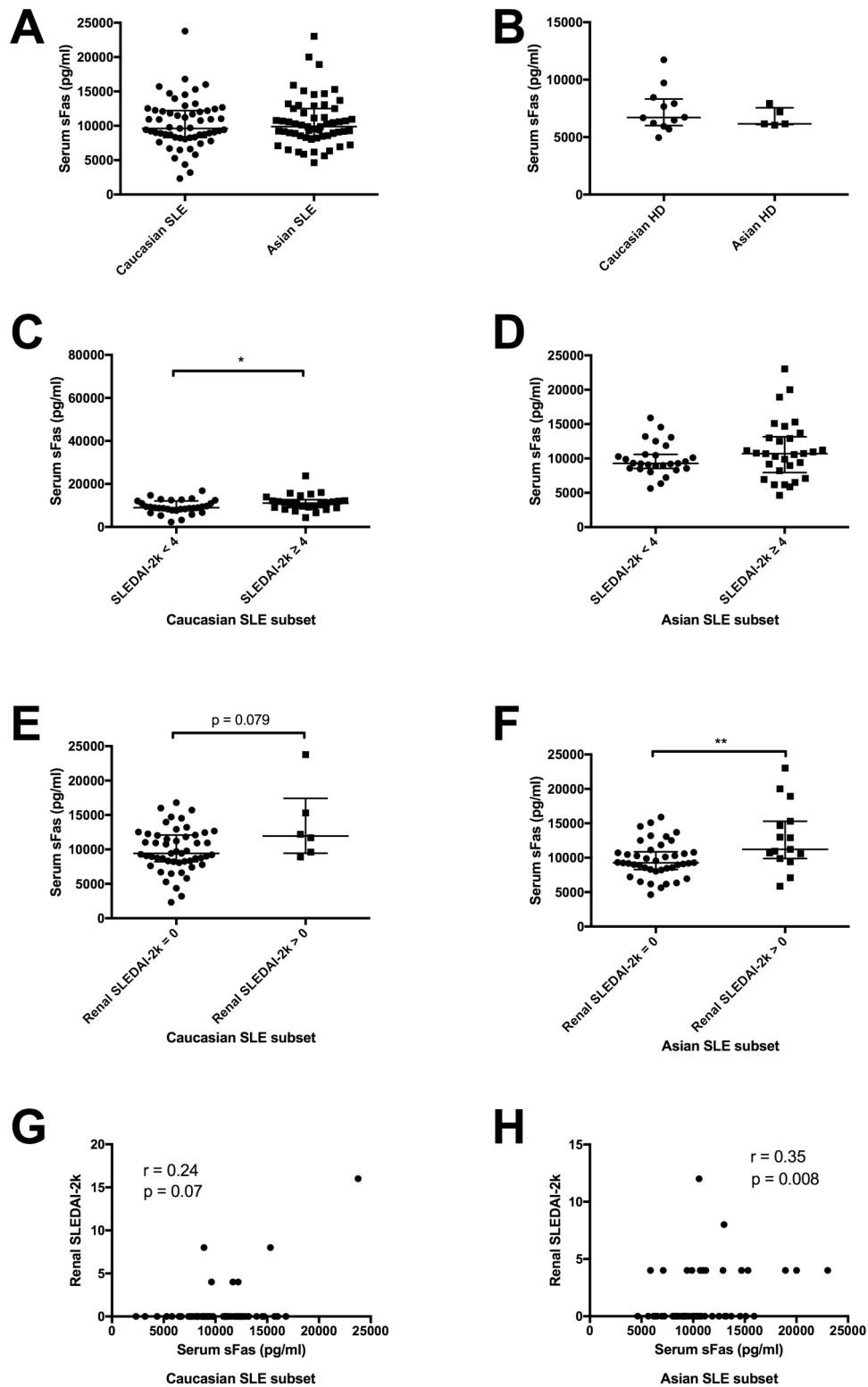
Serum BAFF concentrations were defined as non-elevated (< 1438.2 pg/ml) or high (≥ 1438.2 pg/ml), as per the Methods Chapter. Renal SLE disease activity was assessed by the SLEDAI-2k score. (A) Correlation between serum sFas and BAFF concentrations in SLE ($n=119$). Correlation between serum sFas and BAFF concentrations in (B) immunological ($n=85$) and (C) musculoskeletal SLE subsets ($n=10$). (D) Serum sFas concentrations according to serum BAFF concentrations in SLE (Non-elevated BAFF: $n=81$; High BAFF: $n=38$). Serum sFas concentrations according to renal SLE disease activity in the (E) high (Renal SLEDAI-2k = 0: $n=25$; Renal SLEDAI-2k > 0 : $n=13$) and (F) non-elevated serum BAFF SLE subsets (Renal SLEDAI-2k = 0: $n=73$; Renal SLEDAI-2k > 0 : $n=8$). (G) Correlation between serum sFas concentrations and renal SLEDAI-2k in the high serum BAFF SLE subset ($n=38$). Correlation between serum sFas concentrations and UPCR in the (H) high ($n=37$) and (I) non-elevated serum BAFF SLE subsets ($n=77$).

One outlier value for serum sFas in SLE (63591.65 pg/ml) has been excluding.

Serum sFas and BAFF concentrations are expressed in pg/ml. UPCR is expressed in g/mmol. In panels D-F, horizontal bars indicate the median with [IQR].

* $p<0.05$

Appendix 7



Appendix 7. Influence of ethnicity on the association between serum sFas with SLE.

Overall, renal and musculoskeletal SLE disease activity was assessed by the SLEDAI-2k score. (A) Serum sFas according to ethnicity [Caucasian (n=57) vs. Asian (n=57)] in SLE. (B) Serum sFas according to ethnicity [Caucasian (n=12) vs. Asian (n=5)] in HD. Serum sFas concentrations according to overall disease activity in (C) Caucasian (SLEDAI-2k < 4: n=31; SLEDAI-2k ≥ 4: n=26) and (D) Asian SLE subsets (SLEDAI-2k < 4: n=27; SLEDAI-2k ≥ 4: n=30). Serum sFas concentrations between patients with inactive and active renal SLE disease in (E) Caucasian (Renal SLEDAI-2k = 0: n=51; Renal SLEDAI-2k > 0: n=6) and (F) Asian SLE subsets (Renal SLEDAI-2k = 0: n=42; Renal SLEDAI-2k > 0: n=15). Correlation between serum sFas concentrations and renal SLEDAI-2k in (G) Caucasian (n=57) and (H) Asian SLE subsets (n=57).

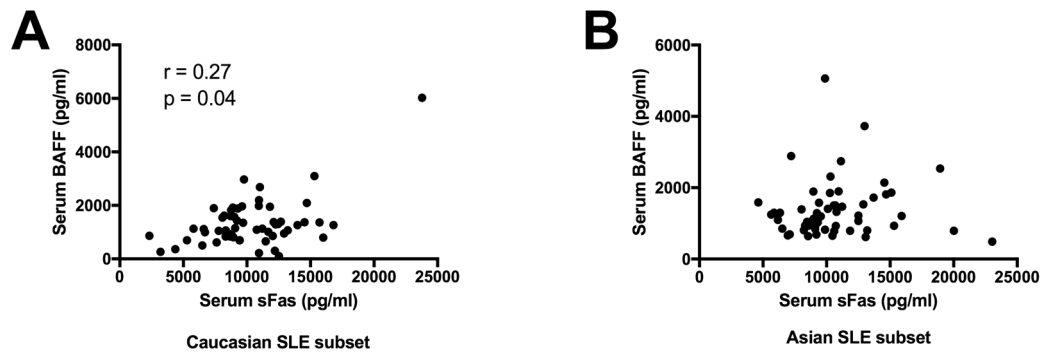
One outlier value for serum sFas in SLE (63591.65 pg/ml) has been excluding.

Serum sFas concentrations are expressed in pg/ml. Musculo. SLEDAI-2k stands for musculoskeletal SLEDAI-2k.

In panels A-F, horizontal bars indicate the median with [IQR].

**p<0.01; *p<0.05

Appendix 8



Appendix 8. Influence of ethnicity on the relationship between sFas and BAFF in SLE.

Serum BAFF concentrations were defined as non-elevated (< 1438.2 pg/ml) or high (≥ 1438.2 pg/ml), as per the Methods Chapter. Correlation between serum sFas and BAFF concentrations in (A) Caucasian ($n=57$) and (B) Asian patients ($n=57$).

One outlier value for serum sFas in SLE (63591.65 pg/ml) has been excluding.

Serum sFas and BAFF concentrations are expressed in pg/ml.

Appendix 9

Demographic, clinical and biological characteristics of patients with LN according to interstitial fibrosis severity.

Characteristics	LN with moderate to severe interstitial fibrosis (n=7)	LN with mild or less interstitial fibrosis (n=22)	p
Demographics			
Age (years)	36.7 [28.7, 40.5]	35.5 [29.5, 44]	0.91
Female, n (%)	6 (85.7)	16 (72.7)	0.65
Ethnicity			0.22
Non-Asian, n (%)	2 (28.6)	13 (59.1)	
Asian, n (%)	5 (71.4)	9 (40.9)	
Disease			
Disease duration (years)	12.8 [10.2, 19.5]	5.3 [3.3, 9.6]	0.004
SLEDAI-2k	8 [6, 18]	4 [2, 7]	0.03
PGA	2 [1, 2]	1 [0, 1.1]	0.04
SLICC-SDI	2 [1, 3]	0 [0, 0]	0.001
Active renal disease [*] , n (%)	6 (85.7)	5 (29.4)	0.02
Treatment			
Prednisone or equivalent, n (%)	7 (100)	15 (68.2)	0.15
Prednisone or equivalent (mg/day)	20 [5, 25]	5 [0, 14.4]	0.09

Characteristics	LN with moderate to severe interstitial fibrosis (n=7)	LN with mild or less interstitial fibrosis (n=22)	p
Hydroxychloroquine (HCQ), n (%)	6 (85.7)	21 (95.5)	0.43
HCQ (mg/day)	400 [200, 400]	400 [200, 400]	0.82
Methotrexate, n (%)	0 (0)	2 (9.1)	>0.99
Azathioprine, n (%)	2 (28.6)	10 (45.5)	0.67
Mycophenolate mofetil, n (%)	3 (42.9)	5 (22.7)	0.36
Mycophenolic acid, n (%)	0 (0)	0 (0)	>0.99
Cyclosporine A, n (%)	0 (0)	0 (0)	>0.99
Cyclophosphamide, n (%)	0 (0)	0 (0)	>0.99
Abatacept	0 (0)	0 (0)	>0.99
Clinical laboratory data			
Serum creatinine (μmol/l)	160 [80, 191]	66 [52.5, 87]	0.007
CRP (mg/l)	1.45 [0, 23.73]	1.8 [0.95, 4.9]	0.96
ESR (mm/h)	57 [17.25, 97.75]	14 [6.3, 42.3]	0.019
C3 (g/l)	0.69 [0.59, 0.78]	0.9 [0.64, 1.06]	0.09
C4 (g/l)	0.14 [0.11, 0.28]	0.13 [0.12, 0.2]	0.99
ANA +, n (%)	6 (100)	21 (95.5)	>0.99
Anti-dsDNA Abs (IU)	28 [16, 72]	21 [11.5, 91.5]	0.91
Anti-Sm Abs +, n (%)	3 (50)	6 (30)	0.63
Anti-Ro (SSA) Ab +, n (%)	3 (50)	10 (50)	>0.99

Characteristics	LN with moderate to severe interstitial fibrosis (n=7)	LN with mild or less interstitial fibrosis (n=22)	p
Anti-La (SSB) Ab +, n (%)	3 (50)	7 (35)	0.64
RF +, n (%)	1 (33.3)	3 (21.4)	>0.99
UPCR (g/mmol)	0.35 [0.17, 0.44]	0.03 [0.01, 0.05]	0.001
Urine micro WBC (x 10 ⁶ /l)	30 [5, 60]	5 [0, 22.5]	0.056
Urine micro RBC (x 10 ⁶ /l)	10 [5, 70]	10 [0, 20]	0.44
eGFR (ml/min)	31 [26, 69]	90 [74, 90]	0.003

n: number of individuals in each subset. Data are expressed as median [IQR] or as number (percentage). Mann Whitney test was used to analyse differences between two variables. Group of categorical data were analysed by Fisher's exact test.

* Assessed by the renal components of the SLEDAI-2k score.

ANA: antinuclear antibody; C3: complement component 3; C4: complement component 4; CRP: C-reactive protein; dsDNA: double-stranded deoxyribonucleic acid; eGFR: estimated glomerular filtration rate; ESR: erythrocyte sedimentation rate; HCQ: hydroxychloroquine; IU: international units; PGA: physician global assessment; RBC: red blood cells; RF: rheumatoid factor; SLE: systemic lupus erythematosus; SLEDAI-2k: SLE Disease Activity Index; SLICC-SDI: Systemic Lupus International Collaborating Clinics-SLE Damage Index; Sm: Smith; SS: Sjögren's syndrome; SSA: SS antigen A; SSB: SS antigen B; UPCR: urine protein/creatinine ratio; WBC: white blood cells.

Appendix 10

Demographic, clinical and biological characteristics of SLE patients categorised by ethnicity.

Characteristics	Asian SLE (N = 58)	Caucasian SLE (N=59)	p
Age (years)	39.9 [32.4-50.1]	47.5 [34.6-60.4]	0.02
Female, n (%)	52 (89.7)	47 (79.7)	NS
Disease duration (years)	7.2 [4-14.1]	9 [4.6-17.7]	NS
Overall disease activity			
SLEDAI-2k	4 [2-6]	2 [0-4]	NS
PGA	0.5 [0.2-0.9]	0.3 [0.2-0.6]	0.045
Treatment			
Prednisone or equivalent, n (%)	36 (78.3)	30 (61.2)	NS
Prednisone or equivalent (mg/day)	5 [1.4-10]	2.5 [0-7.5]	NS
Hydroxychloroquine (HCQ), n (%)	52 (96.3)	51 (94.4)	NS
HCQ (mg/day)	350 [200-400]	400 [200-400]	NS
Methotrexate, n (%)	4 (18.2)	7 (21.2)	NS
Azathioprine, n (%)	15 (48.4)	12 (38.7)	NS
Mycophenolate mofetil, n (%)	9 (50)	7 (21.9)	NS
Mycophenolic acid, n (%)	2 (11.8)	1 (3.5)	NS
Cyclosporine A, n (%)	0 (0)	0 (0)	NS
Cyclophosphamide, n (%)	0 (0)	0 (0)	NS
Clinical laboratory data			
Hb (g/l)	129 [120-139]	135 [126-142]	NS
Platelets (x 10⁹/l)	224 [189.8-	241 [189-296]	NS

Characteristics	Asian SLE (N = 58)	Caucasian SLE (N=59)	p
	274.5]		
CRP (mg/l)	1 [0.5-2.7]	1.9 [1.4-4.1]	0.003
ESR (mm/h)	17 [9.8-33.5]	11 [5-24]	0.02
C3 (g/l)	0.9 [0.6-1]	1 [0.8-1.1]	NS
C4 (g/l)	0.16 [0.12-0.24]	0.15 [0.11-0.2]	NS
Anti-dsDNA Ab (IU/ml)	34 [11.8-169]	16 [4-138]	NS
Anti-dsDNA Ab +, n (%)	32 (55.2)	28 (47.5)	NS
ANA +, n (%)	57 (98.3)	56 (100)	NS
Anti-Sm Ab +, n (%)	19 (33.9)	4 (7)	0.0004
Anti-Ro (SSA) Ab +, n (%)	34 (60.7)	16 (28.1)	0.0006
Anti-La (SSB) Ab +, n (%)	16 (28.6)	12 (21.1)	NS
UPCR (g/mmol)	0.02 [0.01-0.05]	0.02 [0.01-0.03]	NS
eGFR (ml/min)	90 [90-90]	90 [72.5-90]	0.002

N: number of individuals in each SLE subset. Data are expressed as median [IQR] or as number (percentage). Percentages (%) are calculated based on (n) available data. Mann Whitney test was used to analyse differences between two variables. Mann Whitney tests were two-tailed. Group of categorical data were analysed by Fisher's exact test.

Ab: antibody; ANA: anti-nuclear antibody; C3: complement component 3; C4: complement component 4; CRP: C-reactive protein; dsDNA: double-stranded deoxyribonucleic acid; eGFR: estimated glomerular filtration rate; ESR: erythrocyte sedimentation rate; Hb: haemoglobin; HCQ: hydroxychloroquine; IU, international units; NS: not significant; PGA: Physician's Global Assessment; SLE: systemic lupus erythematosus; SLEDAI-2k: SLE Disease Activity Index 2000; Sm: Smith; SSA: Sjögren's syndrome antigen A; SSB: Sjögren's syndrome antigen B; UPCR: Urine protein/creatinine ratio.

Appendix 11

Associations of uBAFF with SLE in the whole cohort and in the subset excluding two outliers.

	SLE cohort	SLE cohort excluding outliers	Difference
	(N=86)	(N=84)	
	P value	P value	
Associations of uBAFF with SLE			
SLE vs. HD vs. IgAN (cutoff 62.5)	NS	NS	
SLE vs. HD detectability (cutoff 62.5)	NS	NS	
SLE vs. IgAN detectability (cutoff 62.5)	NS	NS	
SLE vs. HD vs. IgAN (cutoff 6.44)	0.007 [#]	0.0154 [¥]	
uBAFF according to ethnicity	0.009	NS [¥]	Y
Associations between serum and urinary BAFF			
Serum BAFF according to uBAFF detectability	NS	NS	

	SLE cohort	SLE cohort excluding outliers	Difference
	(N=86)	(N=84)	
	P value	P value	
Associations of uBAFF with SLE disease activity			
uBAFF according to overall SLEDAI-2k	0.029	NS	Y
uBAFF according to flare	0.012	0.019	
uBAFF according to anti-Sm	0.007	0.008	
uBAFF according to anti-dsDNA	NS	NS	
Associations of uBAFF with renal SLE			
uBAFF according to renal SLEDAI-2k	0.002	0.045	
uBAFF according to UPCR	NS	NS	
uBAFF according to kidney biopsy histological class	NS	NS	
uBAFF according to kidney biopsy interstitial fibrosis	0.007 [§]	0.03 [*]	
uBAFF according to kidney biopsy interstitial inflammation	NS	NS	

	SLE cohort	SLE cohort excluding outliers	Difference
	(N=86)	(N=84)	
	P value	P value	
Associations of baseline uBAFF with subsequent SLE disease activity			
AMS (1 year) according to uBAFF detectability	0.011	NS [†]	Y
SLEDAI-2k at 18-months according to uBAFF detectability	0.005	0.008	
C3 at 18-months according to uBAFF detectability	0.008	0.023	
C4 at 18-months according to uBAFF detectability	0.022	0.009	
C3 at 24-months according to uBAFF detectability	0.015	0.038	
Associations of baseline uBAFF with subsequent renal SLE disease activity			
Renal SLEDAI-2k at 18-months according to uBAFF detectability	NS	NS	
UPCR at 18-months according to uBAFF detectability	NS	NS	
uBAFF according to renal SLEDAI-2k at 18-months	0.04	NS	Y

Mann Whitney and Kruskal-Wallis tests (followed by Dunn's multiple comparisons test) were used to analyse differences between two and more than two variables, respectively. Two outliers have been excluded from data analyses in the SLE cohort (uBAFF=272.2885 pg/μmol, and uBAFF=260.5708 pg/μmol).

[#] *Kruskal-Wallis test is shown. Dunn's test: SLE higher than HD ($p=0.039$); SLE higher than IgAN ($p=0.026$).*

[§] *Kruskal-Wallis test is shown. Dunn's test: very mild vs. moderate: $p=0.023$; mild vs. moderate: $p=0.008$.*

[‡] *Kruskal-Wallis test is shown. Dunn's test: non-significant trend toward SLE higher than HD ($p=0.07$); SLE higher than IgAN ($p=0.0495$).*

^{*} *Kruskal-Wallis test is shown. Dunn's test: very mild vs. moderate: $p=0.059$; mild vs. moderate: $p=0.039$.*

^ψ $p=0.06$

[¶] $p=0.074$

AMS: Adjusted mean SLEDAI-2k; BAFF: B cell-activating factor from the tumour necrosis factor family; C3: complement component 3; C4: complement component 4; dsDNA, double-stranded deoxyribonucleic acid; HD: healthy donor; IgAN: immunoglobulin A nephropathy; NS: Not significant; PIC: Protease Inhibitor Cocktail; SLE: Systemic lupus erythematosus; SLEDAI: SLE disease activity index; SLEDAI-2K: SLEDAI 2000; SLICC: systemic lupus international collaborating clinics; Sm: Smith; UPCR: urine protein/creatinine ratio.

Appendix 12

Associations of uAPRIL with SLE in the whole cohort and in the subset excluding one outlier.

	SLE cohort	SLE cohort excluding outlier	Difference
	(N=86)	(N=85)	
	P value	P value	
Associations of uAPRIL in SLE			
SLE vs. HD detectability (cutoff 0.78125)	NS	NS	
SLE vs. HD vs. IgAN (cutoff 0.78125)	NS	NS	
SLE vs. HD vs. IgAN (cutoff 0.4)	NS	NS	
uAPRIL according to ethnicity	NS	NS	
Associations between serum and urinary APRIL			
Serum APRIL according to uAPRIL detectability	NS	NS	
Serum APRIL vs. uAPRIL	NS	NS	
Serum APRIL vs. uAPRIL in subset of uAPRIL detectable samples	NS*	NS	

	SLE cohort (N=86) P value	SLE cohort excluding outlier (N=85) P value	Difference
Associations of uAPRIL with SLE disease activity			
uAPRIL according to overall SLEDAI-2k	NS	NS	
uAPRIL vs. overall SLEDAI-2k	0.028 (r=0.27)	NS [¶]	Y
uAPRIL vs. PGA	NS [#]	NS	
uAPRIL according to flare	NS	NS	
uAPRIL vs. SLICC	NS [†]	NS	
Associations of uAPRIL with renal SLE			
uAPRIL according to renal SLEDAI-2k	0.006	0.017	
uAPRIL vs. renal SLEDAI-2k	0.005 (r=0.33)	0.017 (r=0.29)	
uAPRIL vs. SLEDAI-2k minus renal SLEDAI-2k	NS	NS	
uAPRIL according to eGFR	0.002	0.004	

	SLE cohort (N=86) P value	SLE cohort excluding outlier (N=85) P value	Difference
uAPRIL vs. UPCR	NS [§]	NS	
uAPRIL according to UPCR	NS	NS	
uAPRIL according vs. urine micro WBC	0.001 (r=0.37)	0.003 (r=0.34)	
uAPRIL according vs. urine micro RBC	0.0008 (r=0.38)	0.0009 (r=0.38)	
uAPRIL according to kidney biopsy histological class	NS	NS	
uAPRIL according to kidney biopsy interstitial fibrosis	0.009	NS [‡]	Y
uAPRIL according to kidney biopsy interstitial inflammation	NS	0.046 ^ψ	Y
Associations of baseline uAPRIL with subsequent SLE disease activity			
uAPRIL vs. AMS (1 year)	NS	NS	
SLEDAI-2k at 24-months according to uAPRIL detectability	NS	NS	
uAPRIL vs. SLEDAI-2k at 24-months	NS	NS	

	SLE cohort	SLE cohort excluding outlier	Difference
	(N=86)	(N=85)	
	P value	P value	
PGA at 24-months according to uAPRIL detectability	0.038	0.038	
Associations of baseline uAPRIL with subsequent renal SLE disease activity			
Renal SLEDAI-2k at 18-months according to uAPRIL detectability	NS	NS	
UPCR at 18-months according to uAPRIL detectability	NS	NS	

Mann Whitney and Kruskal-Wallis tests were used to analyse differences between two and more than two variables, respectively. Spearman's test was used to test correlations between variables. One outlier has been excluded from data analyses in the SLE cohort (uAPRIL= 5.184744 ng/μmol), and one in the HD cohort (uAPRIL= 4.604797 ng/μmol).

** $p=0.068$; $r=0.47$*

$p=0.067$; $r=0.21$

† $p=0.063$; $r=0.21$

§ $p=0.086$; $r=0.2$

¶ $p=0.055$; $r=0.24$

‡ $p=0.061$

ψ Kruskal-Wallis test is shown. No statistically significant difference between subsets (very mild vs. mild vs. moderate vs. no interstitial inflammation) using Dunn's multiple comparisons test.

AMS: Adjusted mean SLEDAI-2k; APRIL: a proliferation-inducing ligand; HD: healthy donor; eGFR, estimated glomerular filtration rate; IgAN: immunoglobulin A nephropathy; NS: Not significant; PGA: physician's global assessment; PIC: Protease Inhibitor Cocktail; RBC, red blood cells; SLE: Systemic lupus erythematosus; SLEDAI: SLE disease activity index; SLEDAI-2K: SLEDAI 2000; SLICC: systemic lupus international collaborating clinics; UPCR: urine protein/creatinine ratio; WBC, white blood cells.

Appendix 13

Novel findings presented in this thesis.

Chapter	Findings	Comments
III	Serum sFasL/sFas ratio was related to overall SLE disease activity and renal SLE disease activity	1
	A significant relationship between serum BAFF and sFas was noted in SLE. This relationship was specific for patients with immunological and musculoskeletal manifestations of disease	
	The relationship between serum sFas and renal SLE was only seen in patients with high serum BAFF	
	The relationship between the sFasL/sFas ratio and mucocutaneous SLE disease activity was seen only in patients with non-elevated serum BAFF	
	The relationship between the sFasL/sFas ratio and renal SLE was restricted to patients with high serum BAFF	
	All data presented in section "Influence of ethnicity on serum sFas and sFasL and their relationships with SLE disease activity"	
IV	First study of these biomarkers using the pSS scoring system ESSDAI, and to evaluate the relationship between serum soluble components from the Fas/FasL system and organ-specific pSS disease	2

Chapter	Findings	Comments
	Serum sFas was negatively associated with the biological ESSDAI domain (in univariable analysis only)	
	There was a non-significant trend toward a relationship between serum sFasL and pSS lymphadenopathy in univariable analysis	
	No significant difference emerged in sFasL/sFas ratio between pSS and HD. There was no relationship between sFasL/sFas ratio and overall and organ-specific pSS disease activity	
	There was a positive relationship between serum BAFF and sFas in pSS, particularly in patients with articular phenotype	
	No significant relationship emerged between serum sFas and pSS disease activity when stratified according to serum BAFF levels	
	No significant relationship emerged between serum sFasL or sFasL/sFas ratio and BAFF in pSS	
V	The relationship between serum BAFF and overall SLE disease activity was independent of ethnicity	
	I report in SLE a significant positive relationship between serum BAFF and IFN- γ , which was not observed in healthy subjects.	3
	The observed significant relationship between serum BAFF and overall SLE disease was seen regardless of the presence of detectable IFN- γ	

Chapter	Findings	Comments
	The relationship between serum BAFF and renal SLE activity was associated with the presence of detectable serum IFN- γ	4
VI	For optimal assay performance, serum BAFF should be quantified separately from the soluble form of its receptors (sTACI and sBCMA) when using a multiplex assay	
	Serum BAFF detection by ELISA appeared to be affected by the presence of sBAFF-R	
	Effect of anti-BAFF and anti-CD20 therapies on serum soluble BAFF receptor concentrations	
	Serum sTACI was weakly correlated with C3. No significant relationship was observed with tested clinical phenotype subsets (e.g. renal, musculoskeletal) when analysed based on SLEDAI-2k scores	5
	Serum sBCMA was significantly lower in patients with active neurological SLEDAI-2k compared to those without. No significant relationship was observed with other tested clinical phenotype subsets based on SLEDAI-2k scores	6
	All data presented in section “Associations of serum sBAFF-R with SLE”	7
	Serum BAFF was not correlated with sTACI and sBAFF-R. Serum sBCMA was correlated with sBAFF-R, but serum sTACI was not correlated with either sBCMA or sBAFF-R	8
	All data presented on associations of BAFF/sTACI, BAFF/sBCMA and BAFF/sBAFF-R ratios with SLE	
	All data presented in section "Influence of ethnicity on serum soluble BAFF receptors in SLE"	

Chapter	Findings	Comments
VII	All data presented in section "Associations of serum sTACI with pSS"	
	All data presented in section "Associations of serum sBCMA with pSS"	
	All data presented in section "Relationship between serum BAFF and soluble BAFF receptors in pSS"	
	All data presented in section "Associations of BAFF/sTACI ratio and pSS"	
	All data presented in section "Associations of BAFF/sBCMA ratio and pSS"	
VIII	All data presented in this Chapter on uBAFF and uAPRIL	9

1. Only one study investigated clinical association of this ratio in SLE, and found no such association, but included only 15 patients.[176] We consider the study presented here the first significant study of sFasL/sFas in SLE.
2. No previous studies investigated sFasL/sFas ratio in SS.
3. A positive relationship between these two cytokines has previously been suggested in SLE in one study, but no direct statistical test was reported.
4. The association between BAFF and active renal disease was significant in univariable analysis, however not in multivariable analysis where only a non-significant trend toward an association was observed after adjusting for serosal domain. However, IFN- γ has not been identified as a confounder.
5. Serum sTACI has previously been reported higher in SLE patients with renal manifestations, in a study presented in abstract form only in 2001, however this was not confirmed in the present study. No other SLE phenotype has been investigated in association with serum sTACI.
6. Only four patients had active neurological disease.
7. sBAFF-R has never been measured in SLE, or in other AID, or in healthy human sera.
8. Correlation between BAFF and sBCMA was previously investigated in Laurent et al. study.[90]
9. Studies of urinary BAFF and APRIL concentrations in the setting of SLE have not previously been published at the time of the thesis submission. Urinary concentrations of BAFF have been reported in SLE, being higher in renal SLE, however in a study presented in abstract form only to date.