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**Interstitial Cells of Cajal are Decreased in Patients with Gastroschisis
Associated Intestinal Dysmotility**

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ABSTRACT**Background:**

Gastroschisis associated intestinal dysmotility (GAID) is poorly understood. Animal experiments suggest that interstitial cells of Cajal (ICC), play an important role.

Methods:

Infants with gastroschisis (GS) and GAID (time to full feed >42 days) were selected. Age matched GS and control (NEC, ileal atresia, malrotation, and volvulus) samples from primary (T1) and secondary (T2) time points underwent standard histopathology and immunohistochemistry for identification of ICC, followed by evaluation of ICC numbers, distribution, morphology, relation to ganglion cells, and myenteric plexus architecture.

Groups were compared using parametric and non-parametric tests.

Main Results:

Twelve patients had samples available for histopathological evaluation. GAID patients had a significantly lower total number of ICC than controls (3 vs. 8, $P < 0.0029$). ICC number at T1 was 2.5 vs. 6, $P = 0.0629$ and significantly lower at T2. (3.5 vs. 11, $P = 0.0124$). GAID patients did not show a significant increase of ICC from T1 to T2. Controls showed a significant increase of ICC over time (6 vs. 11, $P = 0.0408$).

Conclusion:

Intestinal samples from infants with GAID who underwent stoma formation demonstrated fewer ICC than controls. There was no improvement or cell recovery during the study period. The ability to modulate ICC may have significant implications for the management of GAID.

Keywords : Gastroschisis, intestinal dysmotility, Cajal cells, peristalsis, outcome

INTRODUCTION:

Gastroschisis (GS) is a congenital defect of the anterior abdominal wall, typically located to the right of the umbilicus, leading to herniation and exposure of abdominal viscera to amniotic fluid [1]. Several recent studies have demonstrated that the incidence of GS is increasing in America, Asia, Australia, and Europe [2-9]. A third of patients born with GS suffer from significant intestinal dysmotility which has a recognised impact on enteral autonomy, morbidity and mortality [10-12]. Gastroschisis associated intestinal dysmotility (GAID) may be used as an umbrella term for this condition.

Theories to explain GAID include prolonged exposure of the bowel loops to the amniotic fluid and its waste products [13]. This theory is supported by experimental studies in fetal lambs, rabbits, rats and chick embryos [14-19]. In 2004, Midrio et al reported that in a fetal rat model of GS, the interstitial cells of Cajal (ICC) were reduced in number and immature [17]. ICC are c-kit positive pacemaker cells, mainly present in the myenteric plexus, responsible for the creation of bioelectrical slow wave potentials that lead to contraction of intestinal smooth muscle and peristalsis [20-22]. Since this first description, other authors have reported similar ICC derangement in both genetic and surgically induced models of GS [23-26]. Similar findings of impaired ICC differentiation have been reported in the ileum of a human infant with complicated GS [27]. It remains unknown whether human ICC demonstrate the same pattern of ICC derangement across the whole cohort of patients with GAID.

We hypothesised that ICC are reduced and immature in infants with GAID. Intestinal tissue samples were obtained from a series of GS patients at different time points and compared with similar samples from age-matched controls. We describe our findings in this report.

ACCEPTED MANUSCRIPT

MATERIALS AND METHODS:***Patients:***

Institutional approval was obtained for this study (CASS no. 3135). A prospectively maintained database was used to identify infants with GS managed at our institution between January 2004 and January 2014. Patients with GAID (defined as time to full feed > 42 days) who underwent stoma formation (ileostomy or jejunostomy) were selected. Others who underwent stoma formation due to NEC, ileal atresia, malrotation/volvulus during the same time period were used as controls. Only GAID and control patients with intestinal samples taken at two time points (stoma formation= T1; stoma closure or revision= T2) were included in the study. Data collection included patient demographic details (Table 1).

Histology:

All available intestinal tissue samples taken at T1 and T2 were analysed in blinded fashion by a Consultant Histopathologist (SDC). Samples were routinely processed (formalin-fixed, paraffin-embedded) for histopathological examination; after reviewing all available hematoxylin-eosin stained sections, representative blocks were selected for CD117 (c-kit) immunohistochemical assessment. Systematic morphological evaluation included the intensity (mild-moderate vs. severe) and distribution (mucosal-submucosal vs. transmural) of the inflammatory reaction and the level of intestinal ischemia (ulceration, transmural necrosis, and perforation with peritonitis). The immunohistochemical evaluation comprised an analysis of the ICC (as demonstrated by CD117 immunostaining) using c-kit pharmDx kit according to validated manufacturer protocol for Autostainer (Dako UK Ltd, Cambridgeshire, UK; i.e target retrieval in a Pascal pressure cooker programmed for 30 seconds at 125°C,

cooling to 90°C, followed by EnVision+ Rabbit, HRP system and a primary antibody dilution 1:600). Synaptophysin immunostaining (FLEX monoclonal mouse antihuman antibody, Dako UK Ltd, Cambridgeshire, UK) was used to highlight the myenteric ganglion cells and the relationship with the interstitial cells; samples were processed following manufacturer's protocol for Autostainer (pretreating tissues with heat-induced epitope, retrieval using diluted EnVision™ FLEX Target Retrieval Solution, High pH, and a ready-to-use monoclonal mouse primary antibody). Mast cell immunostaining was used as internal positive control for CD117 immunostaining and toluidine blue staining confirmed mast cells by the metachromatic staining of their granules. The analysis of interstitial cells involved cell density (by counting the nuclei of CD117-positive perineural cells in 25 consecutive 200x microscopic fields) and the morphology (spindle-shaped vs. dendritic) of c-kit-positive cells.

Statistics:

Groups were compared using parametric and non-parametric tests (Mann-Whitney, paired t test, χ^2) as appropriate. Data are presented as median and range or median and interquartile (IQ) range. P value of <0.05 was considered statistically significant.

RESULTS:***Patients:***

In the 11-year study period from January 2004 to January 2014, there were 112 patients with GS treated at our institution. 34 patients (30%) were identified to have GAID.

Of the 34 patients with GAID, 13 (38%) required a stoma formation. Intestinal tissue samples were available for histopathological evaluation in 12 (92%) of these patients (GS group) (Figure 1). Three patients had a closed/closing GS, defined as previously reported by Houben [28] (Table 1).

The Control group consisted of 11 patients (NEC, n= 5; ileal atresia, n= 2; malrotation/volvulus, n= 1; ileal atresia + volvulus, n=3) (Table 1). There were no differences between the two groups regarding gender (GS males n=8 vs. Control males n=7, P= 1), age in days at T1 [GS 4 (IQ range 2-41) vs Controls 4 (IQ range 3-48); P= 0.64] and age in days at T2 [GS 71 (49-246) vs controls 87 (48-154); P= 0.97] (Table1).

Histology:

A total of 46 samples from the small bowel (GS group, n= 24; Control group, n= 22) were analysed in blinded fashion.

The overall number of ICC cells was significantly lower in the GS group compared to controls (3 vs. 8, P< 0.0029; Figure 2). Similarly, samples from the GS group had a lower number of ICC than controls at T1 (2.5 vs. 6, P=0.0629) and at T2 (3.5 vs. 11, P=0.0124). The number of ICC from T1 to T2 was similar in the GS group (2.5 vs. 3.5, P=0.212), but significantly increased in the Control group (6 vs. 11, P= 0.0408, Figure 3). On morphological analysis, the

proportion of spindle shaped and dendritic ICC in the GS group (79% and 21%, respectively) was similar to that detected in control specimens (91% and 21%, respectively; $P=0.42$). There were no differences between the GS and the Control group in the degree of inflammation (severe in 10 vs. 8 samples, $P=0.78$) and its distribution (transmural in 12 vs. 10, $P=0.77$). A similar number of samples in both groups had histological evidence of necrosis (6 vs. 9, $P=0.35$), ulceration (13 vs. 16, $P=0.23$) and peritonitis (10 vs. 9, $P=1$) (Figure 4).

DISCUSSION:

This is the first report to establish the status of ICC in intestinal tissue samples from a cohort of infants with GS and bowel dysfunction. Our results demonstrate a reduction in the density of ICC in these samples when compared to those taken from age-matched controls.

These findings are important in the context of a rising worldwide incidence of GS. In 2004, the Annual Report of the Chief Medical Officer published by the Department of Health of the United Kingdom (UK) noted that over the past 30 years there has been a worldwide increase in prevalence of GS [29]. The British Isles Network of Congenital Anomaly Registers (BINOCAR) had published data showing prevalence of GS to be 4 per 10,000 total births [29]. In 2007, Loane et al, on behalf of a working group of the European Surveillance of Congenital Anomalies (EUROCAT), showed that the increase in GS prevalence over time is not restricted to young mothers only [30]. While mothers aged <20 years continue to be at greatest risk of having a GS baby, they observed increasing trends over time in mothers of all ages [30]. In 2011 the same authors reported that the trend showed a 6% increase every 2 years [7].

As the incidence of GS has risen, the mortality in the developed world has decreased to well below 10% [31,32]. Until recently it was a commonly held view that GS survivors suffered few post operative symptoms. However, recognition is increasing that a significant proportion have morbidity related to intestinal dysfunction. We have used the term GAID to describe this condition; present if the patient takes greater than 42 days to achieve enteral autonomy. Patients with GAID represent less than a third of infants with GS but account for

over half the cost of caring for the entire group. In particular, the small number of patients at the worst end of the clinical spectrum suffer considerable morbidity and constitute a significant financial burden to the National Health Service (NHS) in the UK [33]. This is, in large measure, because infants with recalcitrant GAID require long term parenteral nutritional (PN) supplementation. Historically, prolonged use of total parenteral nutrition (TPN) in infancy often resulted in liver failure. More recently, reduction of sepsis and variation of PN formulation including the use of omega oils and medium chained fatty acids has reduced its incidence [35]. However, even patients who have attained enteral autonomy over time sometimes sustain irreversible liver disease and may require liver transplantation for survival. GAID remains one of the leading causes of Intestinal Failure Associated Liver Disease (IFALD) [36].

We have previously reported the wide variation in management strategies for GAID between different European centres [37]. The survey, conducted in 2011, highlighted the lack of consensus in the management of GAID, reflecting the limited understanding of the condition and optimal interventions. A clearer understanding of the pathophysiological mechanisms involved and more effective treatment strategies are urgently needed.

Over the past decade, there has been a surge of interest in the influence of ICC and enteric nervous system cells in diseases that affect gut motility including GS [25, 38]. ICC are the pacemaker cells of the gut and generate the spontaneous slow waves of smooth muscle layers. Along with the enteric nervous system in the myenteric and submucous plexi, they play a major role in intestinal peristalsis [22].

ICC may be considered as a specialised population of smooth muscle cells that arise from mesenchyme. Unlike smooth muscle cells, they have few contractile elements but contain large numbers of mitochondria and endoplasmic reticulum. The cell itself has a spindle shaped body with thin cytoplasm, a large oval nucleus and 2-5 primary dendritic processes which then further divide into secondary and tertiary processes [39-41].

Following the initial reports by the Spanish Nobel Prize winner, Santiago Cajal at the turn of the 19th century [42, 43], the role of ICC in health and disease remained largely unknown for years. In 1986 the c-kit receptor was discovered. The c-kit receptor is a type III receptor tyrosine kinase. In the gut, c-kit is expressed only in ICCs and mast cells. The development of antibodies to c-kit has allowed for routine identification of ICCs in pathology specimens [41].

Distribution of ICC has been found to be abnormal in several diseases in which intestinal peristalsis is impaired, such as Hirschsprung disease, hypoganglionosis, neuronal intestinal dysplasia, and infantile pyloric stenosis [38,41]. It has been shown by Vargun et al. that in the absence of ICC, spontaneous depolarization and repolarization of smooth muscle cells fail to occur, and the regular pattern of peristaltic waves disappears. C-Kit is required for the development of ICC and intestinal pacemaker function. Lack of ICC causes gut hypomotility and symptoms of pseudobstruction in animal models [23].

Hitherto, the suggestion that ICC may have a role to play in GAID has come predominantly from animal studies. Vannuchi demonstrated in the foetal rat model of GS that there is delayed maturation of interstitial cells of Cajal [16]. Vargun showed in a chick embryo model

that the mean density of ICC was significantly reduced in GS when compared to controls [23]. The same group then went on to demonstrate that intrauterine treatment in the form of amniotic fluid exchange could preserve ICC density and function. They suggested that the density of ICC in the muscular layer might be a reliable numeric parameter to compare the effectiveness of experimental intrauterine treatment methods [23]. Similarly, Krebs demonstrated in a lamb model of GS that the reduction of ICC density could be reversed by intrauterine coverage of the intestines [26].

In 2008, Midrio et al reported the status of ICC in a human infant born with GS that required intestinal resection[27]. Samples taken at the time of initial operation demonstrated not only a decrease in number of ICC but also a morphological immaturity of all structures responsible for intestinal motor activities. Samples taken a month later at time of stoma closure showed that the differentiation process had continued and that the ICC were morphologically more mature.

The availability of samples for our own study was dictated by our departmental strategy for the management of infants with GAID. Those who do not respond to conservative measures such as glycerine suppositories, bowel washouts and prokinetics are put forward for stoma formation. Samples were taken from index cases and controls at two time points; first at the time of initial stoma and then at the time of the further laparotomy - which was, for stoma closure in all but one who underwent stoma revision. Consistent with Midrio's observation, our T1 samples confirmed a paucity and lack of maturity of ICC. However, in contrast to her findings, there was little evidence of ICC recovery at T2 in our series. It is not easy to reconcile the differences regarding ICC recovery with time. Our study has the advantage of

larger numbers while the Midrio case report has the advantage of greater ultrastructural detail.

A well described drawback of immunohistochemical studies of this nature is the lack of ideal control samples. In our study, we used age matched controls which included patients with NEC, atresia and malrotation/volvulus. We recognise that there is limited information about the status of ICC in these conditions and that the underlying conditions as well as the state of the specimens may affect ICC distribution and morphology. For example, it is known from animal experimental models [44, 45], that ischaemia causes immediate effects on gut ICC. The fact that there may be an element of ischaemia in all our control conditions may affect the assessment of Cajal cells in them. We sought only to perform a quantitative assessment of ICC density in representative samples taken at time of initial gut resection and then again at the time of stoma closure. However, with the aforementioned reservations, we have demonstrated there was a significant difference in ICC density between our controls and the cases of GS.

We acknowledge that the present study has limitations; these include the retrospective nature and the difficulty identifying ideal control patients and, in particular, intestinal samples. We are unable to comment on ICC distribution in GS infants who do not have GAID as they do not end up having stomas and therefore intestinal samples from them are not available. To overcome some of these limitations and confirm our current findings, we propose to prospectively evaluate the presence of ICC in GS patients with available intestinal specimens using a wide range of age matched patients undergoing intestinal surgery as controls.

In conclusion, allowing for the fact that this is a light rather than an electron microscopic evaluation, we have demonstrated that intestinal samples from infants with GAID who underwent stoma formation have fewer ICC when compared to controls. There was no significant improvement or cell recovery during the study period. Control samples were different in that they initially showed more ICC at the outset and, in addition, demonstrated enhanced ICC recovery between T1 and T2. The role of intestinal ischaemia and inflammation are unclear. Further work to elucidate the ontogeny of ICC in infants with GAID is needed. A better understanding and, potentially, the ability to modulate ICC in such a way as to enhance function may have significant implications for the management of this condition in the future.

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FIGURE LEGENDS:

Figure 1: Flow chart for gastroschisis group – data collection

Figure 2: Overall number of ICC: Significantly lower in patients with gastroschisis in comparison to Controls

Figure 3: Total number of ICC of gastroschisis compared to controls at T1 & T2

Figure 4a, b: a) Immunohistochemistry Gastroschisis at T1 and T2 (H&E and c-kit),
b) Immunohistochemistry Control at T1 and T2 (H&E and c-kit)

Fig. 1

Fig. 2

Fig. 3

Fig. 4A

Fig. 4B

Table 1: Gastroschisis and control group patients listed with details of pathology

Pathology	No	Gender		Median Age at T1 (days)	Median Age at T2 (days)	P-Value
		M	F			
Gastroschisis (n=12)						
- Simple	9	8	4	4 (2-41)	71 (49-246)	0.64
- Closing/closed	3					
Controls (n=11)						
- NEC	5	7	4	4 (3-48)	87 (48-154)	0.97
- Intestinal atresia	2					
- Malrotation/volvulus	1					
- Atresia +volvulus	3					