Digestive Diseases and Sciences (2019) 64:2709–2716
https://doi.org/10.1007/s10620-019-05778-1

PARADIGM SHIFTS IN PERSPECTIVE

Control of Intestinal Epithelial Proliferation and Differentiation: The Microbiome, Enteroendocrine L Cells, Telocytes, Enteric Nerves, and GLP, Too

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Published online: 21 August 2019

"A story has no beginning or end: arbitrarily one chooses that moment of experience from which to look back or from which to look ahead."

—— **Graham Greene**, ***The End of the Affair***

The defining characteristic of all organisms is their ability to reproduce. For most, a fully formed adult organism can arise from a single cell that contains the information, combined with environmental cues, necessary to orchestrate the near-miraculous series of controlled and timed cell divisions, migrations, differentiations, maturations, connections, and cell death needed for overall growth and maturation of the organism. The ability for cells to proliferate, divide, mature, and differentiate is retained in adults, albeit in small, defined regions termed the "stem cell niche", where many of the factors controlling embryonic growth, proliferation, and differentiation are retained [1–4]. In most organs, proliferation mostly occurs after injury, such as in the liver, which has a remarkable ability to regenerate [5]. Some stem cells continually proliferate in adults, in particular the intestinal epithelium, which has the highest proliferation rate in the adult human body, with complete turnover normally occurring every 3–5 days. The proliferating compartment is normally restricted to the crypt, where epithelial stem cells are located, with epithelial cells migrating, differentiating, and maturing as they migrate toward the villus. Clearly, regulation of this rapid division is essential, since at the extremes of dysregulation, atrophy, fibrosis, and cancer can occur.

As quoted above, the story of the regulation of intestinal differentiation and proliferation has no clearly defined beginning, although historically, several key observations have been made with regard to the mechanism controlling the rate of proliferation. One is that the intestinal proliferation rate increases following partial intestinal resection. Observations of this nature initially appeared early in the twentieth century, where surgeons noted that survival was possible following intestinal resections of up to a certain length [6–9]. This was studied in more depth in experimental animals, with experiments showing that the uptake of a short exposure or "pulse" of ^{3}H thymidine, which is incorporated into replicating DNA, was taken up in greater quantities by the intestinal crypts in rats that had undergone partial intestinal resection [10].

The first evidence supporting the existence of glucagon-like peptide (GLP)-2 was published by Sutherland and de Duve in 1948, who reported that a hormone similar to the pancreatic hyperglycemic hormone, later termed glucagon, was also present in dog stomach [11]. Further research demonstrated that intestinal glucagon stimulated adenylyl cyclase [12] and possessed immunoreactivity similar but not identical to that of glucagon, described as glucagon-like activity [13], further reinforced by the finding of immunoassayable glucagon in the gastrointestinal (GI) tract [14]. Stephen Bloom eventually reported that enteroglucagon, defined as total glucagon activity minus pancreatic activity, was necessary for post-resection adaptive intestinal proliferation to occur [15]. This conclusion was supported by a case report of a patient with a retroperitoneal tumor in whom greatly increased villous length and intestinal mucosal surface area were present, thought due to the elaboration of a glucagon-like hormone that did not affect glycemic control (in retrospect this presumably was GLP-2) [16]. With the advent of automated peptide sequencing, enteroglucagon

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was shown to be derived from the peptide proglucagon, which is processed into the peptide hormones glicentin, glucagon-like peptides (GLP)-1 and -2, and peptide YY (PYY) [17, 18]. Eventually, Dan Drucker in Pat Brubaker's laboratory convincingly demonstrated that GLP-2 is the proglucagon product controlling intestinal proliferation [19]. Proglucagon-derived hormones are primarily expressed in enteroendocrine L cells, which are a type of endocrine cells interspersed among intestinal epithelial cells, in particular in the ileum and proximal colon that release proglucagon-derived hormones in response to luminal signals [20].

Another line of evidence has been developing since the 1930s regarding the observation that the feeding of indigestible carbohydrates increased intestinal weight [21] and the proliferation rate of intestinal epithelial cells [22]. Furthermore, intestinal atrophy occurs during restriction of oral nutrients, even when nutrients are provided parenterally, consistent with the need for luminal nutrient exposure to promote villous growth [23]. These observations, repeated many times, have been mostly unexplained, but more recently data have emerged that these changes are likely due to the production of bacterial fermentative metabolites, in particular short-chain fatty acids (SCFAs), by the gut microbiota [24–26]. Since L cells express the G-protein-coupled receptors (GPCR), GPR 41 and 43 also termed free fatty acid (FFA)3 and FFA2, and since L cells in rats release GLP-2 into the portal vein in response to luminal perfusion with FFA3 ligands [27], it is likely that microbial fermentation products such as SCFAs release GLP-2 from L cells, increasing the rate of epithelial proliferation [27, 28], helping to explain the effects of high-fiber diets and starvation on the rate of intestinal proliferation.

Mesenchymal cells such as myofibroblasts are frequently present in the intestinal stem cell compartment. The importance of mesenchymal cells toward epithelial development was originally reported in the 1950s by Clifford Grobstein [29] and by Takindo Okada, and the latter who in 1960 published the first report of the effect of the mesenchyme on the embryonic differentiation of the digestive tract [30]. Mesenchymal cells express numerous bioactive molecules, such as growth factors, transcription factors, such as Wingless-related integration site (Wnts), Wnt sensitizers, such as the Roof-plate-specific (R)-spondins (RSPOs), inflammatory cytokines, prostaglandins, Hedgehog proteins, neurotransmitters, a variety of receptors, and many other bioactive substances [31–36]. It is now well accepted that mesenchymal cells are closely linked with the control of growth, proliferation, repair, and neoplastic transformation of epithelia [37, 38].

The identity of the mesenchymal cells that alter epithelial proliferation and other functions is controversial. The mesenchymal cells that directly underlie the epithelial cells in the crypt and villus have been variously termed fibroblasts, myofibroblasts, and fibroblast-like cells based on their ultrastructure and expression of smooth muscle antigens such as desmin, vimentin, and α-smooth muscle antigen [37–42]. After review of several reports, the safest way to describe these cells is that they form a network termed initially by Kaye et al as the "pericryptal fibroblast sheath" that is present from crypt base to villus tip that connect by what appears to be gap junctions with each other, the epithelial cells, enteric nerves, the microvasculature, and inflammatory cells forming what could be termed a reticular network, syncytium, or scaffold [35, 43–45]. Due to the ongoing uncertainty of the nature of these cells, I will term them generically as the "periepithelial mesenchymal syncytium" or PMS. The ultrastructure of these cells, first reported by Helen W. Deane in 1964 [46], has been extensively documented in particular by several groups in Japan working in the 1980s and 1990s, with some of their most spectacular images shown in Figs. 1 and 2, graphically depicting the three-dimensional aspect of the PMS using scanning electron micrographic images of the enterocyte basal membrane after dissolution of the underlying structures [35, 45, 47–49].

More recently, subsets of the cell comprising the PMS have been identified as specialized cells termed telocytes, mesenchymal cells implicated in the control of growth, differentiation, proliferation, migration, and repair. Telocytes, initially described due to their unusual neuron-like morphology, similar to the interstitial cells of Cajal first described by Ramòn y Cajal [50], have been primarily identified by their morphology, which is most apparent using electron microscopy (EM), with exceptionally thin (~0.10 μM) moniliform projections that can exceed 200 μm in length. Telocytes also feature what appear to be direct cell-to-cell connections with surrounding cells, including other telocytes, stem cells, inflammatory cells, the microvasculature, immune cells, and nerves [51]. The location of telocytes within the stem cell niche of multiple organs, including the intestine, combined with their expression of growth and transcription factors, have implicated telocytes as an essential component of the stem cell niche [52]. Telocytes also secrete exosomes and other membrane-bound vesicles containing potent bioactive molecules such as aforementioned transcription and growth factors [53]. On the basis of their morphology, cellular connections, and protein expression, telocytes are tempting candidates as overall coordinators of cellular growth and differentiation. Holding back progress, however, is lack of a reliable telocyte marker, with c-Kit, and the bone marrow marker cluster of differentiation (CD) 34 used frequently [54]. Platelet-derived growth factor receptor α (*PDGFRα*) has recently gained popularity as a telocyte marker, considerably strengthened by functional experiments in which proliferation of the intestinal epithelium was altered by genetic ablation of *PDGFRα*-expressing fibroblasts, confirmed by similar effects of genetic ablation

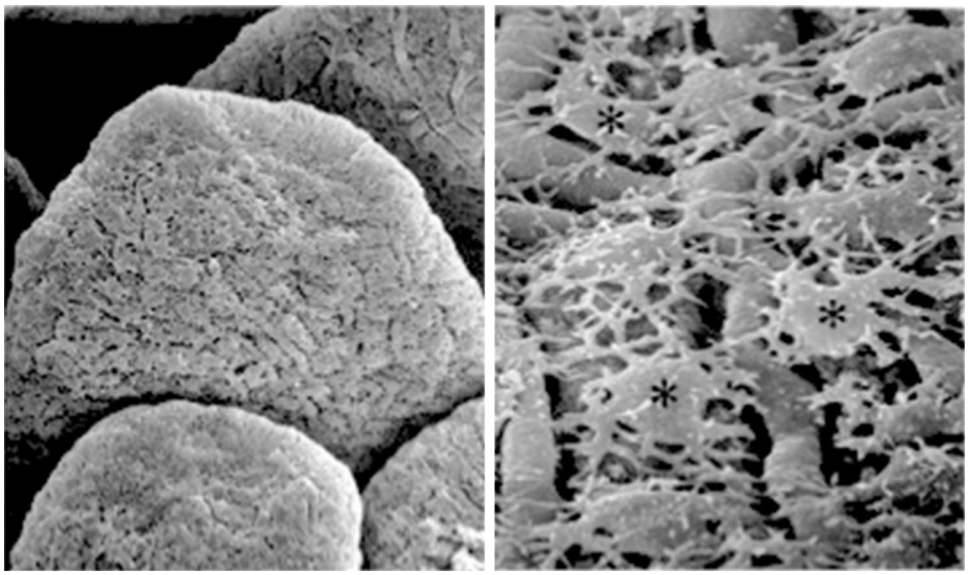

Fig. 1 Scanning electron micrograph (SEM) image of the PMS underlying the villous epithelial cells of rat intestine (*). Reproduced with permission from [49]

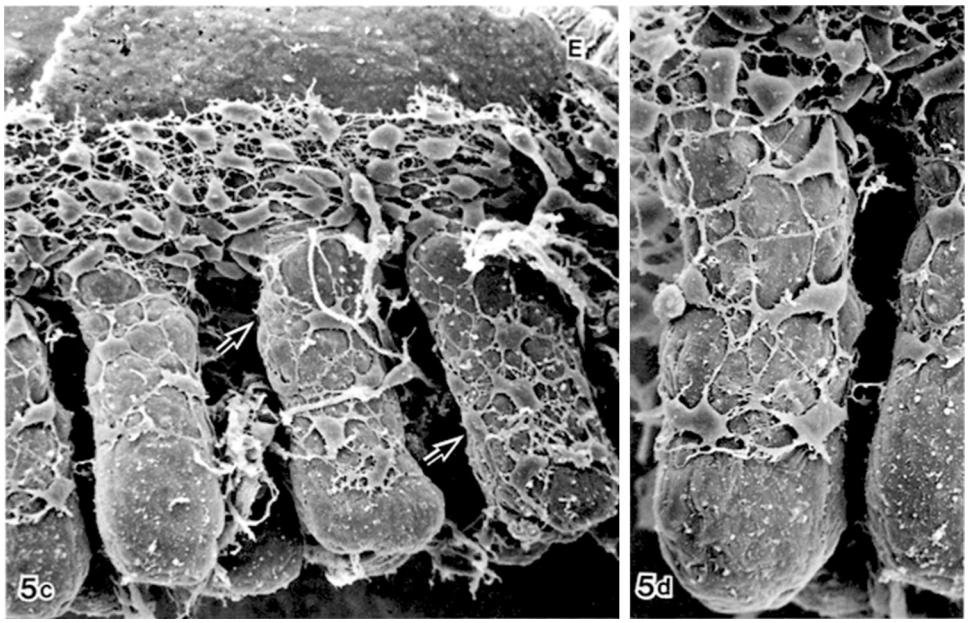

Fig. 2 SEM of the PMS underlying the crypt and villus of rat jejunum, showing the syncytium of connected fibroblast-like cells. "E": villous epithelium. Reproduced with permission from [47]

of Wnt proteins from Foxl1-expressing cells, which are also believed to be telocytes separate from *PDGFRα*-expressing fibroblasts [31, 51].

The question then arises: How does GLP-2 increase the rate of proliferation of crypt stem cells and maturation into differentiated epithelial cells so as to increase mucosal surface area, increasing intestinal absorptive capacity? This was painstakingly studied in the laboratory of Dan Drucker in 2000, who reported that GLP-2 receptors (GLP-2R) were limited to rare enteroendocrine cells in the stomach, intestine, and colon [55]. GLP-2R expression was also studied by Ørskov et al, who hypothesized that pericryptal myofibroblasts, presumably synonymous with the PMS, express GLP-2 receptors, releasing growth factors such as epidermal growth factor (EGF), keratinocyte growth factor (KGF), and insulin-like growth factor (IGF) that in turn activate non-GPCR growth factor receptors such as erythroblastic leukemia viral oncogene (ErbB) and IGF receptors expressed on replicating epithelial cells [34, 56]. Their data, however, indicate that most GLP-2R expression was limited to the myofibroblasts underlying the villous tip, an unlikely location for the promotion of cellular proliferation.

Although the hypothesis the cells of the PMS directly transduce GLP-2 signal into growth and transcription factor release is compelling, several questions remain. One concern is the identity of growth-promoting mesenchymal cells. In view of the aforementioned discussion and of recently published data, the most compelling data support that the PMS

appears to be comprised of two populations: a pericryptal population identified by Foxl1+ and a population that surrounds the mid-villus to villous tip that is *PDGFRα*+ [31, 33] Another concerns are the kinetics of the PMS. Of the few reports that describe the kinetics of the PMS, initial reports by Marsh and Trier and Kaye et al suggested a migration rate up the villus comparable to the epithelial cells [57, 58]. These reports, however, have been disputed by the more recent findings of other investigators, who found that the PMS cells are derived from the bone marrow, replicate more slowly than the epithelial cells, and also do not migrate up the villus in a linear fashion. [59, 60] Most recently, Worthley et al published compelling data supporting the origin of the PMS from stem cells located in the crypt–villus isthmus that migrated either toward to villus tip or crypt base, populating the crypt–villus unit over a year, far slower than the 3–5 days needed for epithelial cell renewal. Powell et al also provided data supporting the repopulation of the PMS from bone marrow-derived cells only after injury [61]. Combined with many reports that the cells of the PMS change from a flattened to a stellate morphology dependent on their relation to the crypt–villus axis, it is possible that at least two populations of cell comprise the PMS, including Foxl1+ cells with flattened morphology that primarily express pro-proliferative ligands for the *Lgr5*+ stem cells of the crypt and the *PDGFRα*+ stellate cells that release growth and other factors that more regulate cellular migration, differentiation, and maturation as cells migrate past the isthmus toward the villus tip.

Another question is which growth-promoting factors are most important? Data suggest the deletion of Wnts, RSPOs, and growth factors such as IGF alter intestinal structure [51, 62–67]. Furthermore, intestinal enteroids are grown in vitro using Wnt-conditioned medium supplemented by EGF, RSPOs, and a cocktail of other inhibitors and reagents designed to simulate the conditions in the stem cell niche, with Wnt deleted to promote differentiation. [66, 68] Given that for the epithelial cells, numerous processes are involved such as symmetric and asymmetric stem cell division, migration up the villus, differentiation into mature cell types, and apoptosis at the villous tip, it is not hard to imagine that numerous factors distributed over time and space are needed for maintenance of form and function of the epithelial cells, thus supporting the concept that cells of the PMS also change in form and function as they migrate up the villus, delivering the correct mix of transcription and growth factors needed for cells according to their position in the crypt–villus axis (Fig. 3).

Another question addresses the mechanism by which GLP-2 increases villous growth. Although Brubaker has

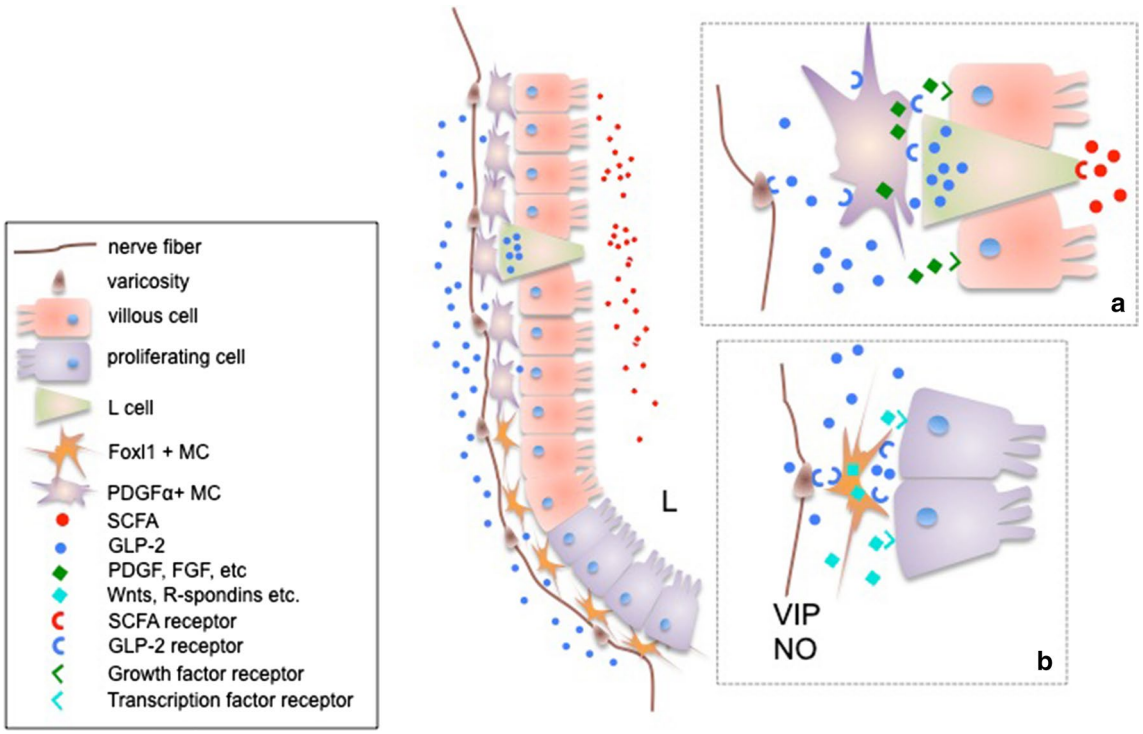

Fig. 3 Simplified proposed model of the mechanisms controlling intestinal epithelial proliferation, migration, and differentiation. Abbreviations: *SCFA* short-chain fatty acids, *GLP* glucagon-like peptide, *L* lumen, *MC* mesenchymal cell, *NO* nitric oxide, *PDGF* platelet-derived growth factor, *VIP* vasoactive intestinal peptide. Insets: **a**: detail of interactions in the villous; **b**: detail of interactions in the crypt. For further details, see text

cited data supporting the expression of GLP-2 receptors expressed on gut-derived mesenchymal cells and epithelial proliferation in vitro in response to exogenous GLP-2 [69, 70], convergent and compelling data support the expression of GLP-2 receptors on enteric nerves and activation of c-fos in villous epithelial cells after exogenous GLP-2 exposure [56, 71, 72]. Neural activation by GLP-2 is further supported by the observations that the administration of GLP-2 rapidly increases mesenteric blood flow and intestinal motility, typical of a neurally mediated mechanism. [71, 73]. Combined with anatomic evidence supporting direct contacts between the PMS and enteric nerves [45], a neural mechanism in which GLP-2 released from L cells activates enteric nerves which in turn activate the release of transcription and growth factors from PMS cells dependent on their subtype, and position on the crypt–villus axis seems to fit well with the available data, although direct GLP-2 activation of PMS cells is entirely plausible. Although vasoactive intestinal peptide and NO have been implicated in neurally mediated acute effects of GLP-2 [74–76], this mediation does not appear to extend the intestinotrophic effects of GLP-2 [77].

Putting it together, these data support the hypothesis that links the gut microbiome with intestinal proliferation via GLP-2 release that in turn activates the PMS cells to release proliferative, pro-growth, and differentiation-promoting bioactive molecules through neurally or chemically mediated activation. The beauty of this system is that bioactive factor release is limited to cells literally in contact with or proximal to the intestinal epithelium, acting in a paracrine fashion that targets only the proliferating crypt cells and maturing villous cells, ensuring precise targeting of growth-promoting factors tailored to the needs of the epithelial cells as they migrate up the crypt–villus axis, limiting potentially harmful off-target effects of powerful proteins such as Wnts and growth factors.

Is GLP-2 the only hormone that promotes growth of cells in a tiny niche? Certainly, sex steroid hormones such as testosterone, estrogen, and progesterone affect the growth of hair and the endometrium, as part of their effects on multiple organs [78, 79]. The best characterized peptide hormone that accelerates organ growth, proliferation, and differentiation is gastrin, which affects gastric epithelial cells through the Hedgehog and Wnt pathways, presumably through a mesenchymal intermediary [32], although its utility to regenerate the gastric epithelium, as an example, is severely limited by its other actions such as promoting copious gastric acid secretion. Given that many epithelia that are dependent on mesenchymal cells for growth, proliferation, differentiation, and other functions such as breast, kidney, liver, skin, heart, lung, and prostate [80], it is possible that each organ has unique sensors that "taste" its local environment, releasing appropriate molecules locally through mesenchymal cell intermediaries according to environmental cues, as presciently stated by Marsh and Trier in 1974, "…subepithelial fibroblasts and epithelial cells of mouse jejunum may interact and regulate epithelial and/or mesenchymal cell proliferation, migration, and differentiation." [58]

Table 1 Nobel Prizes in Physiology and Medicine awarded to scientists who contributed to the understanding of the regulation of intestinal proliferation

Recipient(s)	Year	Contribution
Christian de Duve	1974	Initial description of GLPs*
Rita Levi-Montalcini, Stanley Cohen	1986	Discovery of growth factors
Harold E. Varmus, J. Michael Bishop	1989	Discovery of Wnts*

*The contribution of these scientists to the understanding of the regulation of intestinal proliferation differs from the contributions for which they were awarded the Nobel Prize

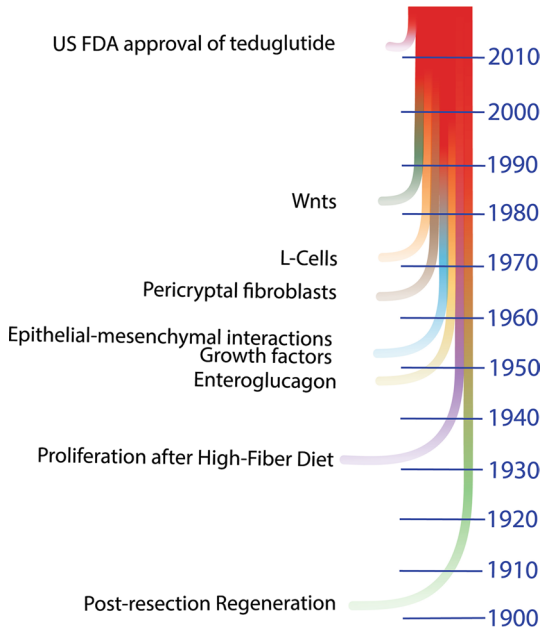

Fig. 4 Timeline of major discoveries that provided the scientific basis for the development of therapeutic GLP-2 analogs

At present, the only approved clinical treatment promoting cellular growth that has emerged from these studies is teduglutide, a stable GLP-2 analog that has proven useful in the management of severe malabsorption due to short-gut syndrome and other causes with minimal adverse effects [81, 82]. This advance is the latest innovation in over a century of discoveries on which the clinical development GLP-2 rests (Table 1; Fig. 4). In the accompanying article, Professor Palle Jeppessen, who has been deeply involved with the clinical development of GLP-2 for the treatment of intestinal failure, will detail the clinical development and impact of the GLP-2 analog teduglutide, which is due to its precise targeting and excellent safety record, and has revolutionized the

management of intestinal failure, enabling many to reduce or even discontinue parenteral nutritional support [83]. It is hoped that fundamental investigation into the control of proliferation and differentiation will yield therapies aimed at treating some of the most important medical problems, such as organ regeneration, fibrosis, and cancer [80].

Acknowledgments The authors thank Professor Don Powell for his helpful comments and suggestions.

Compliance with Ethical Standards

Conflicts of interest The authors are recipients of investigator-initiated grants from Shire Pharmaceuticals LLP.

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