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(54) Title: TREATMENT OF HEALING DYSREGULATION IN GI TISSUE

(57) Abstract: The present disclosure relates to the treatment of dysregulation of gastrointestinal (also referred to herein as GI) tract regeneration and/or maturation, in particular in an infant, such as a preterm (also referred to herein as a neonate), a low weight for gestational age baby and/or a baby born following pre-eclampsia by administering a therapeutically effective amount of IGF-1 in particular as a complex with an IGFBP, such as IGFBP-3. The treatment is also useful in other patient populations including the adult population for those with conditions/illness/disease and/or infection of the GI tract.

TREATMENT OF HEALING DYSREGULATION IN GI TISSUE

The present disclosure relates to the treatment of dysregulation of gastrointestinal (also referred to herein as GI) tract regeneration and/or maturation, in particular in an infant, such as a preterm (also referred to herein as a neonate), a low weight for gestational age baby and/or a baby born following pre-eclampsia. The treatment is also useful in other patient populations including the adult population for those with conditions/illness/disease and/or infection of the GI tract.

BACKGROUND

Approximately 20,000 protein coding genes are expressed in human cells and 75% of these genes are expressed in at least one of the different parts of the digestive organ system. Over 600 of these genes are more specifically expressed in one or more parts of the GI tract and the corresponding proteins have functions related to digestion of food and uptake of nutrients.

The GI tract is a super complex arrangement which can be divided into four concentric layers namely: mucosa, submucosa, muscular layer and adventitia or serosa.

The mucosa is made up of:

Epithelium – the innermost layer with primary responsibility for digestive, absorption and secretion processes, which defines the internal space of the tract (the lumen),

Lamina propria – connective tissue with a cellular composition, and

Muscularis mucosae -smooth muscle involved in GI motility and peristalsis.

Embedded in the mucosa are functional elements such as nerves, lymphatic tissue, ducts from glands, and gland mucosa.

Submucosa is a dense irregular layer of connective tissue with glands, large blood vessels, lymphatics and nerves branching into the mucosa and muscularis externa.

The **Muscular Layer** comprises an inner circular layer and a longitudinal outer layer.

Adventitia and serosa comprise several layers of connective tissue.

The structure of the GI tract is critical to the health and functioning of the digestive system. Simplistically the GI tract function is to:

- keep the contents of the tract including pathogens contained and separate from other parts of the body, and
- digest food, and
- pass nutrients to the body, and
- expel waste products.

When the GI becomes inflamed and sick this containment can become leaky. Cells in the GI tissue start to be destroyed leading to trauma, damage and “holes” in the various layers of the structure. If the matter becomes very serious then the vascular system of the GI tract can become leaky allowing pathogens to breach the GI tract and become systemic, for example causing sepsis. This process can be life threatening.

There are number of illnesses and diseases that can lead to these symptoms, for example bacterial infection/autoimmune disease/premature birth. Interestingly, some individuals are better able to handle these issues and recover than other individuals who are more susceptible to this pathology.

Studying individuals with these conditions highlights some markers, for example inflammatory markers are raised and other markers, such as IGF-1 are decreased. In this disease state there may be a negative feedback loop that perpetuates and/or exacerbates the condition or illness.

However, whilst not wishing to be bound by theory it is thought that some individuals may have dysregulation of GI tract regeneration before the disease/illness and pathology is manifest or alternatively may have dysregulation of GI tract regeneration caused by the disease.

Studies have suggested that Celiac disease patients have a predisposing condition to the damaging effects of gliadin, for example a constitutive alteration of enterocyte proliferation. It has been suggested that Paneth cell dysfunction may lead to a dysbiotic microbiota, which in turn, could predispose to the development of Crohn's disease. Abnormal Paneth cells with reduced expression or secretion of defensins HD-5 and HD-6 (in human) and antimicrobial peptides are associated with inflammatory bowel disease. Thus, in these individuals when a "challenge", such as bacterial infection occurs, the body is not able to defend itself and heal efficiently.

The above applies to premature infants who are born before their GI tract is ready for use. They have an immature mucosal barrier and may also have abnormal intestinal microbiota. Thus, the ability to heal and/or mature quickly is a life and death issue in this patient population. Necrotizing enterocolitis (NEC) is a life-threatening disease in premature newborns, commonly leading to overwhelming sepsis, intestinal necrosis and death. No specific treatment is currently available to treat or prevent NEC as its pathogenesis is not well understood. While NEC appears to be a multifactorial disease with involvement of an immature immune response, lack of breastfeeding and intestinal microbiota perturbation, the present inventors suggest a role for impaired development of the neonatal intestinal systems.

Surprisingly the present inventors have established that the underlying mechanisms involving dysregulation of GI regeneration can be addressed to support the body's function and minimise severe GI injury, even in the most fragile patient populations, such as premature infants.

SUMMARY OF THE INVENTION

The disclosure is summarised in the following paragraphs:

1. A method of treatment or prophylaxis of dysregulation of GI tract regeneration (for example in an infant such as in a preterm infant (also referred to a neonate herein) and/or low weight for gestational age baby or a baby born following pre-eclampsia) by administering a therapeutic amount of a composition comprising IGF-1 and an IGF binding protein (such as IGFBP-3), in particular as a complex. In one embodiment the patient, in particular a human, has dysregulated GI tract regeneration and/or is at risk of dysregulation of GI tract regeneration.

1A. A composition comprising IGF-1 and an IGF binding protein (such as IGFBP-3), in particular as a complex, for use in the treatment or prophylaxis of dysregulation of intestinal regeneration, in particular in a patient population disclosed herein.

1B. A composition comprising IGF-1 and an IGF binding protein (such as IGFBP-3), in particular as a complex, for use in the manufacture of a medicament for the treatment or prophylaxis of dysregulation of intestinal regeneration, in particular in a patient population disclosed herein.

1C. A method of treatment or prophylaxis by stimulating GI cell maturation (for example to increase and/or maintain tight junctions and/or) in an infant such as in a preterm infant (also referred to a neonate herein) and/or low weight for gestational age baby by administering a therapeutic amount of a composition comprising IGF-1 and an IGF binding protein (such as IGFBP-3), in particular as a complex.

2. A method or composition according to any preceding paragraph, wherein the composition is administered parenterally.

3. A method or composition according to paragraph 2, wherein parenteral administration is infusion, for example continuous infusion (for example an infant, such as a preterm infant and/or a low gestational weight infant and/or baby born following pre-eclampsia) for example continuous infusion, in particular for at least 1 week, for example 2 to 6 weeks, such as 2, 3, 4, 5 or 6 weeks).

4. A method or composition according to paragraph 2 or 3, wherein the parenteral administration is intra-peritoneal and/or subcutaneous, for example at least once per day, for example 1, 2 or 3 times a day. In one embodiment there is provided treatment of an infant, for example a preterm, low weight for gestational age and/or baby born following pre-eclampsia said treatment administered for at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 days after birth, in particular for days 1 to 7 following birth. In one embodiment there is provided treatment of an infant according to the present disclosure, for example a preterm infant, is treated by subcutaneous administration, for example bolus subcutaneous, in particular for at least a 1 week, for example 2 to 6 weeks, such as 2, 3, 4, 5 or 6 weeks. In one embodiment a bolus injection is administered once a day. Treatment may start with continuous infusion, for example initiated within the first 24 hours, for example in the first 12, 6, 5, 4, 3, 2 or 1 hours following birth for a period; with intraperitoneal and/or subcutaneous administration concomitant with this dosing and/or subsequent to this infusion, i.e. subsequent refers to when infusion has ceased the administration may be continued for a period with only intra-peritoneal and/or subcutaneous dosing.

5. A method or composition according to any previous paragraph, wherein the treatment reduces inflammation.

6. A method or composition according to paragraph 5, wherein the systemic inflammation is reduced, for example reduction in IL-6 and/or CXCL1.

7. A method or composition according to paragraph 5 or 6, wherein the inflammation is located in the GI tract, for example selected from the gut, the intestines, and/or the colon, such as the gut and the intestines, in particular the intestines. In one embodiment the inflammation is not systemic.

8. A method or composition according to any preceding paragraph, wherein one or more cytokines selected from IL-4, IL-6, IL-10 and combinations of two or three thereof are reduced by treatment, for example at least 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75% or more in comparison to an untreated patient with enterocolitis (such as necrotising enterocolitis).

9. A method or composition according to any preceding paragraph, wherein inflammation-initiated destruction of IGFBP-3 in tissue is minimised and/or destruction (including elimination) of IGF-1 is minimised.

10. A method or composition according to any preceding paragraph, wherein a positive feedback loop for IGF-1 production, is supported, stabilised, maintained, initiated and/or augmented.
11. A method or composition according to any preceding paragraph, wherein vascular permeability is reduced, for example in comparison to an untreated patient with necrotising enterocolitis, in particular wherein vascular leakage into the intestinal tissue is minimised and/or leakage from the GI tract to the vascular system is minimised. In one embodiment microvascular integrity is preserved, stabilised and/or improved by treatment.
12. A method or composition according to any preceding paragraph wherein tight junctions are facilitated, preserved, supported, stabilised, maintained, initiated and/or augmented by treatment, for example the percentage of tight junctions is preserved or augmented.
13. A method or composition according to any preceding paragraph, wherein treatment supports maintenance of epithelial barrier function and/or epithelial integrity, for example including prevention of breach of basement membrane and/or underlying stromal cells are not exposed to luminal content.
14. A method or composition according to any preceding paragraph wherein VEGF and VEGFR2 protein expression is preserved, supported, maintained, initiated and/or augmented, in particular at healthy levels, particularly in GI tract tissue such as gut.
15. A method or composition according to any preceding paragraph wherein vascularisation in the GI tract, such as the gut and/or intestines, in particular the intestines, is maintained, supported, initiated and/or augmented by treatment, such as where treatment provides increased microvascular density.
16. A method or composition according to any preceding paragraph, wherein endothelial cell proliferation is preserved, supported, maintained, initiated and/or augmented by treatment, for example where the villous endothelial cell proliferation is preserved, supported, maintained, initiated and/or augmented by treatment.
17. A method of composition according to any preceding paragraph, wherein epithelial cell migration is preserved, supported, maintained, initiated and/or augmented by treatment.
18. A method or composition according to any preceding paragraph, wherein epithelial cell apoptosis is minimised (regularised) by treatment, for example apoptosis of enterocytes, for example apoptosis induced by oxidative stress.
19. A method or composition according to any preceding claim wherein treatment promotes proliferation of enterocyte, for example in comparison to an untreated patient.
20. A method or composition according to any preceding claim, wherein the enterocyte function is preserved, supported, maintained, initiated and/or augmented, for example a function selected from ion uptake, water uptake, sugar uptake, peptide uptake, amino acid uptake, lipid uptake, vitamin B12 uptake, secretion of immunoglobulins, and a combination of two or more of the same, such as all the functions.
21. A method or composition according to any preceding paragraph, wherein Paneth cell function is preserved, supported, maintained, initiated and/or augmented, for example Wnt signalling and Notch signalling and/or secretion of defensins HD-5 and HD-6.
22. A method or composition according to any preceding paragraph, wherein Paneth cell dysfunction is minimised by treatment, for example in comparison to an untreated patient.

23. A method or composition according to any preceding paragraph wherein the Paneth cell proliferation is increased by treatment, for example in comparison to an untreated patient.
24. A method or composition according to any preceding paragraph wherein treatment reduces incidences of severe injury to the GI tract, such as severe intestinal injury, for example by promoting and/or regulating healing processes.
25. A method or composition according to paragraph 24, wherein severe injury to the villi is minimised, for example destruction of villi and/or malformation of villi.
26. A method or composition according to any preceding paragraph for use in the treatment of colitis (including necrotising enterocolitis) *C. difficile* infection, shigella infection, toxic mega colon and the like.
27. A method or composition according to paragraph 22, wherein severe incidences of severe necrotising enterocolitis, for example score equal or above 2 (such as 3, 4, 5 and 6) are reduced in comparison to untreated patients with necrotising enterocolitis.
28. A method or composition according to any preceding paragraph, wherein the incidences of sepsis are reduced in treated patients.
29. A method or composition according to any preceding paragraph, wherein treatment has a paracrine component, for example which may stimulate vascularisation (such as neovascularisation) and/or stimulation of production of IGF-1.
30. A method or composition according to any preceding claim, wherein the treatment has an endocrine component.
31. A method or composition according to any preceding claim wherein the treatment has an autocrine component.
32. A method or composition according to any preceding paragraph, wherein macrophages in the GI tract, such as the gut and/or intestines (in particular the intestines), are activated, especially to secrete IGF-1, in particular macrophages have a “healthy” morphology as opposed to the morphology adopted in NEC.
33. A method or composition according to any preceding claim wherein levels of macrophages are higher in treated patients than untreated patients.
34. A method or composition according to any preceding paragraph, wherein the patient receiving treatment has a lower risk of mortality than a corresponding patient without treatment.
35. A method or composition according to any preceding paragraph, wherein the complex is provided in the ratio 0.5 to 1.5 of IGF-1 to 1 IGFBP protein (such as IGFBP-3), such as a 1:1 ratio.
36. A method or composition according to any preceding paragraph, wherein the dose for an infant, such as a preterm, low weight for gestational age and/or a baby born following preeclampsia, is 200 to 500μg/Kg/24hours of complex, for example 350 to 500μg/Kg/24hours, such as 400 μg/Kg/24hours, in particular by continuous infusion.
37. A method or composition according to any preceding paragraph, wherein 55 to 110μg/Kg/24hours of IGF-1 are administered.
38. A method or composition according to any preceding paragraph, wherein serum levels of IGF-1 for an infant, such as a preterm are maintained with the range 28 to 109ng/mL, for example

on average 28, 29, 30, 31, 32, 33, 34, 35, 40, 45, 46, 47, 48, 49, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 101, 102, 103, 104, 105, 106, 107, 108 or 109 ng/mL.

39. A method or composition according to any preceding paragraph wherein the patient is a human, for example an infant, in particular selected from a preterm infant (also referred to herein as a neonate) a low weight for gestational age baby and baby born following preeclampsia, especially a preterm.
40. A method or composition according to any preceding paragraph, wherein the infant has birth weight of 2.2 pounds or less (1Kg or less).
41. A method or composition according to any preceding paragraph, wherein a preterm infant is in the range 23 to 37 weeks post gestation, for example 23 to 34 weeks or 23 to 30 weeks or 24 to 29 weeks post gestation, when treatment is initiated.
42. A method or composition according to any preceding paragraph, wherein treatment according to the disclosure of an infant (such as a preterm infant, a low weight for gestational age and/or a baby born following preeclampsia) is initiated within 24 hours of birth, for example within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 16, 17, 18, 19, 20, 21, 22, 23 and 24 hours of birth, in particular within 1, 2 or 3 hours of birth.
43. A method of composition according to the present disclosure for use in treatment of inflammatory bowel disease and/or Celiac disease including non-responsive Celiac disease (or a predisposition to Celiacs).
44. A method or composition according to any preceding paragraph, wherein the administration is designed to increase tissue specific levels of IGF-1 and/or IGFBP-3.
45. A method or composition according to any preceding paragraph, wherein tissue specific levels of at least IGF-1 and/or IGFBP-3 is/are augmented.
46. A method or composition according to any preceding claim wherein serum levels of IGF-1 are increased by treatment.

In one embodiment destruction of IGFBP-3 is minimised, for example proteolysis by IL-6.

In one embodiment the binding protein is IGFBP-3.

In one embodiment the binding protein is IGFBP-5.

In one embodiment the complex is a mixture of IGF-1/IGFBP-3 and 5.

In one embodiment the present disclosure relates to boosting tissue specific levels of IGF-1 and/or IGFBP-3. Whilst not wishing to be bound by theory, this halt or reverse the negative feedback loop wherein inflammation destroys IGFBP (such as IGFBP-3), may in turn facilitate the rapid clearance of IGF-1.

In one embodiment the disclosure is not about raising serum levels of free IGF-1.

In one embodiment treatment preserves expression of intestinal IGF-1 receptor, which for example helps protect against intestinal injury.

In one embodiment levels of VEGF and/or VEGFR are stabilise or augmented, in particular protein expression levels, especially locally in the GI tissue.

In one embodiment treatment provides stabilised and/or improved oxygen delivery to intestinal tissue.

In one embodiment treatment provides stabilised and/or improved nutrient delivery/absorption.

In one embodiment there is stabilised blood flow to the GI tract.

In one embodiment treatment provides stabilised and/or improved removal of waste products from local cells in the GI tract, such as enterocytes, including removal of waste products via the circulatory system.

In one embodiment treatment provides stabilised and/or improved function of local cells in the GI tract, such as enterocytes.

In one embodiment treatment provides stabilised and/or improved mobility of the GI tract and/or substructure therein, such as microvilli and/or villus. In one embodiment the arrangement of microvilli and/or villus is more "normal" in treated patients, for example not flattened and/or flaccid. In one embodiment the turgidity of local GI cells, such as enterocytes, is normalised.

In one embodiment there is provided treatment of a human patient, an infant (i.e. up to 1 including preterms etc defined herein), children (for example 1 to 12 years), adolescents (for example 12 to 17 years) and adults (18 years or older). In one embodiment the patient is female, for example in the age range 15 to 60 such as 15 to 40 or 40 to 60.

In one embodiment patients, such as infants/babies receiving the treatment according to the present disclosure have a lower mortality rate (for example all causes) than those who do not receive treatment.

In one embodiment infants, such as a premature infant, a low gestation weight infant and an infant born after preeclampsia, in particular premature infants. Treatment in this patient population may provide more elaboration/differentiation/maturation of GI cells and structure, such enterocytes, villus and/or microvilli.

In one embodiment the preterm infants are in the range 23 to 34 weeks post gestation (such as 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, or 34), when treatment is initiated.

In one embodiment patients, such as infants or children, receiving treatment have less severe disease, condition, for example Crohn's disease (exist in children but not in infants), colitis, necrotising enterocolitis, toxic megacolon, in particular severe necrotising enterocolitis is reduced in an infant, such as a premature infant, a low gestation weight infant and an infant born after preeclampsia, in particular premature infants.

In one embodiment the preterm infants are treated by infusion, for example continuous infusion, in particular for at least 1 week, for example 2 to 6 weeks, such as 2, 3, 4, 5 or 6 weeks.

In one embodiment the treatment of the present disclosure acts on endothelial cells including reduced membrane permeability, increasing and/or stabilising angiogenesis, increasing and/or stabilising vascular genesis, stabilising and/or increasing epithelial cell migration, and/or inhibiting epithelial cell apoptosis.

In one embodiment epithelial cell migration, such as intestinal epithelial cell migration, is increased by treatment, for example where same is reduced by NEC. The increase may be in comparison to a patient that did not receive treatment.

In one embodiment the treatment according to the present disclosure reduces intestinal vascular permeability, for example reduces NEC- induced increases in intestinal vascular permeability. The increase may be in comparison to a patient that did not receive treatment.

In one embodiment microvascular density is increased in the GI tract, in particular in comparison to a patient (such as a patient type disclosed herein) with NEC that did not receive treatment.

In one embodiment patients treated according to the present disclosure have better, improved or quicker healing than untreated patients.

In one embodiment the treatment according to the present disclosure is given to a patient following surgery on the GI tract.

In one embodiment treatment according to the present disclosure is given to a patient with severe gastric and/or intestinal ulcers, for example to improve their prognosis.

In one embodiments patients treated according to the present disclosure are better able to cope with the onslaught of GI tract diseases, disorders, trauma and infections.

In one embodiment patients receiving treatment are protected from severe GI tract injury.

In one embodiment the IGF-1/IGFBP (such as IGFBP-3) complex is administered topically to the gut optionally in combination with other routes of administration disclosed herein.

In one embodiment the basement membrane is denser or thicker after treatment in comparison to an untreated control.

In one embodiment VEGF inhibitors and/or VEGFR2 inhibitors are contra-indicated for patients according to the present disclosure.

In one embodiment treatment according to the present disclosure is NOT about increasing VEGF levels systemically and/or locally.

In embodiment treatment reduces intestinal vascular permeability.

In one embodiment treatment improves/increases intestinal epithelial cell migration (for example towards “normal” levels.

In one embodiment decrease in bound IGF-1 levels are minimized and/or increases in bound IGF-1 are promoted (in particular IGF-1/IGFBP-3 binding).

In one embodiment proteolysis of IGFBP-3 is minimized, eg proteolysis mediated by IL-6.

In one embodiment increased clearance of free IGF-1 is minimized and/or the half-life of free IGF-1 is optimized.

The treatment according to the present disclosure has one or more (such as all) the following effects: VEGF and VEGFR2 protein expression increased, decreased vascular permeability (such as intestinal vascular permeability), and preserved endothelial cell proliferation in the intestine, promoting enterocyte proliferation and migration while decreasing enterocyte apoptosis and attenuating systemic and small intestinal tissue inflammation.

In one embodiment apoptosis of intestinal cells, such as enterocytes in minimized (for example normalized- in particular reduced to normal levels).

In one embodiment serum levels of CXCL1, are stabilized, minimized and/or reduced by treatment.

In one embodiment serum levels of IL-6, are stabilized, minimized and/or reduced by treatment.

In one embodiment intestinal levels of IL-4, IL-6 and IL-10 and combinations thereof, are stabilized, minimized and/or reduced by treatment.

In one embodiment treatment protects the intestine against injury (including severe intestinal injury) and/or promotes healing of intestinal injury.

In one embodiment neonates to NOT express ALS.

Patients treated according to the present disclosure may have improved survival and reduced the incidence of severe intestinal injury.

DETAILED DISCLOSURE

Preterm infant (or neonate) as employed herein refers to an infant born before 40 weeks of gestation, for example with a gestational age of 37 weeks or less, such as 22 to 37 weeks (gestational age), in particular 23, 24, 25, 26, 27, 28, 29, 30, 31, 33, 34, 35 or 36 weeks. At the present time neonates born at 22 weeks gestation are at the cusp of what can be saved, in that the majority may not respond to resuscitation. Having said that neonates born at 22 weeks have survived.

Generally, the preterm infant is human.

The lowest weight (not necessarily the most premature) infants may benefit the most from the therapy of the present disclosure. Some neonates do not grow well in the womb. Even though they may be "older" they are small for their gestational age. The treatment according to the present disclosure is particularly useful for these infants, such as those in the lower quartile of birth weight.

Dysregulation of GI tract regeneration as employed herein refers a reduced ability of the GI tract, such as a gut (stomach) and/or intestines, to develop, elaborate, heal and/or imbalanced or increased destruction of cell in said location. Dysregulation of GI tract regeneration includes reduced proliferation of epithelial cells (such as enterocytes which are located in microvilli), reduced migration of epithelial cells (such as enterocytes), increased apoptosis of cells in GI tissue, increased permeability (for example increased vascular permeability, such as intestinal vascular permeability), loss or reduction in the percentage of tight junctions, reduction in the levels of VEGF/VEGFR, loss or reduction of the macrophages in the GI tissue to secrete IGF-1, increased inflammatory cytokines in the GI tissue (such as a cytokine selected from IL-4, IL-6, IL-10 and combinations of two or three of the same).

Severe intestinal injury as employed herein refers to damage to the GI tract, for example caused by inflammation (including chronic inflammation), including one or more ulcers, malabsorption, necrosis, ischemia, bleeding, pain, persistent diarrhea, weight loss, fatigue, bloating, breakage of the gut barrier, leaky membranes, pathology that renders the patients more susceptible to bacterial infection, sepsis and combinations thereof.

Gastrointestinal tract and GI tract are used interchangeably herein and includes the gut (stomach), intestines and colon, in particular the gut and intestines, especially the intestines.

It appears that the tissue levels (for example gut tissue levels) of IGF-1/IGFBP (such as IGFBP-3) profiles in tissue and serum are different, for example serum levels of IGFBP-3 may be higher than levels in organ tissue, such as the intestines. This may relate to a different function.

IGF-1 and/or IGF-1/IGFBP and/or IGFBP-3 has/have endocrine, paracrine and autocrine functions in the body.

Endocrine is wherein activity comes from a component in the circulatory system.

Paracrine is wherein activity comes from a component secreted from another cell.

Autocrine is wherein activity comes from a component secreted by the cell itself.

Augmenting levels of one or more of IGF-1 and IGFBP such as IGFBP-3 in tissue, specifically intestinal tissue, may be implemented by one or more of the following methods:

- Increasing levels of one or more of IGF-1 and IGFBP such as IGFBP-3 by intravenous infusion (for example continuous infusion) such that transport or migration of the one or more of IGF-1 and IGFBP such as IGFBP-3 into the tissue is achieved, this may require a threshold level of complex to be achieved in the blood to facilitate the same. This mechanism is an endocrine mechanism.
- Increasing levels of the complex directly in epithelial tissue by subcutaneous injection, which may in particular stimulate paracrine and/or autocrine mechanisms.
- Increasing levels of the complex directly in the peritoneal cavity, for example by IP injection may in particular stimulate paracrine and/or autocrine mechanisms.

Combinations of the above delivery strategies may be employed.

An "IGFBP" or an "IGF binding protein" refers to a protein or polypeptide from the insulin-like growth factor binding protein family and normally associated with or bound or complexed to IGF-1 whether or not it is circulatory (i.e., in serum or tissue). Such binding proteins do not include receptors. This definition includes IGFBP-1, IGFBP-2, IGFBP-3, IGFBP-4, IGFBP-5, IGFBP-6, Mac 25 (IGFBP-7), and prostacyclin-stimulating factor (PSF) or endothelial cell-specific molecule (ESM-1), as well as other proteins with high homology to IGFBPs. Mac 25 is described, for example, in Swisshelm et al., Proc. Natl. Acad. Sci. USA, 92: 4472-4476 (1995) and Oh et al., J. Biol. Chem., 271: 30322-30325 (1996). PSF is described in Yamauchi et al., Biochemical Journal, 303: 591-598 (1994). ESM-1 is described in Lassalle et al., J. Biol. Chem., 271: 20458-20464 (1996). For other identified IGFBPs, see, e.g., EP 375,438 published Jun. 27, 1990; EP 369,943 published May 23, 1990; WO 89/09268 published Oct. 5, 1989; Wood et al., Molecular Endocrinology, 2: 1176-1185 (1988); Brinkman et al., The EMBO J., 7: 2417-2423 (1988); Lee et al., Mol. Endocrinol., 2: 404-411 (1988); Brewer et al., BBRC, 152: 1289-1297 (1988); EP 294,021 published Dec. 7, 1988; Baxter et al., BBRC, 147: 408-415 (1987); Leung et al., Nature, 330: 537-543 (1987); Martin et al., J. Biol. Chem., 261: 8754-8760 (1986); Baxter et al., Comp. Biochem. Physiol., 91B: 229-235 (1988); WO 89/08667 published Sep. 21, 1989; WO 89/09792 published Oct. 19, 1989; and Binkert et al., EMBO J., 8: 2497-2502 (1989).

"IGFBP-3" refers to insulin-like growth factor binding protein 3. IGFBP-3 is a member of the insulin-like growth factor binding protein family. IGFBP-3 may be from any species, including bovine, ovine, porcine and human, in native-sequence or variant form, including but not limited to naturally-occurring allelic variants, in particular human. IGFBP-3 may be from any source, whether natural, synthetic or recombinant, provided that it will bind IGF-I at the appropriate sites. IGFBP-3 can be produced recombinantly, as described in WO 95/04076.

Therapeutic composition, as used herein, is defined as comprising IGF-1 or an analogue thereof, in combination with its binding protein, such as IGFBP-3 or an analogue thereof. In some embodiments, the IGF-1 is recombinantly produced. In some embodiments, the IGFBP-3 is recombinantly produced. In some embodiments, the IGF-1 and the IGFBP-3 are complexed prior to administration to the subject. In some embodiments, the IGF-1 and IGFBP-3 are complexed in equimolar amounts.

The therapeutic composition may also contain other substances such as water, minerals, carriers such as proteins, and other excipients known to one skilled in the art.

In the context of this specification "comprising" is to be interpreted as "including". Embodiments of the invention comprising certain features/elements are also intended to extend to alternative embodiments "consisting" or "consisting essentially" of the relevant elements/features.

Where technically appropriate, embodiments of the invention may be combined.

Technical references such as patents and applications are incorporated herein by reference.

Any embodiments specifically and explicitly recited herein may form the basis of a disclaimer either alone or in combination with one or more further embodiments.

Subject headings herein are employed to divide the document into sections and are not intended to be used to construe the meaning of the disclosure provided herein.

The background section contains technical information relating to the invention and may be employed as basis for amendment.

Specific values may be employed from the examples in combination with generic disclosure of the description.

The concepts disclosed in the Examples are NOT closely associated with the parameters of the specific examples and generic concepts that apply to the whole of the presently described invention. These concepts in the examples may be isolated and used as basis for amendments to the claims.

The present specification claims priority from US 63/484,954 filed 14 February 2023 and GB 2319443.4 filed 18 December 2023 both incorporated herein by reference. The same may be used as the basis for correction to the present specification.

The present invention is further described by way of illustration only in the following examples.

BRIEF SUMMARY OF THE FIGURES

Figure 1. ***IGF Binding protein 3 (IGFBP3) bound IGF-1 is decreased in serum of NEC pups.*** <24h-old pups were either submitted to a NEC protocol for 24 hours or left with the dams to be dam fed. 1-3ml of serum from NEC pups (n=7) or dam fed control (n=7) were subjected to Western blot analysis (probed with antibody against IGF-1) under non-reducing conditions: (A) Typical blot is shown here; (B) Relative IGF-1/BP3 and IGF-1/other BPs band densities of NEC pups were normalized to the mean band density of dam fed pups, showing that IGF-1/BP3 but not IGF-1/other BPs in NEC was decreased. Data are three experiments combined. *P* value was calculated using 2-sided Student's *t* test. ***p<0.001.

Figure 2. ***Serum concentration of "human" IGF-1 in pups that were injected with rhIGF-1/BP3 (2mg/kg or 10 mg/kg, ip) or vehicle control, approximately 8 hours after birth.*** Serum was collected 30 minutes, 1 hour, 2 hours, 4 hours, 8 hours and 24 hours after rhIGF-1/BP3 injection. * $p \leq 0.05$, ** $p \leq 0.01$. n= 4-5 pups/group for the 30 minutes, 1 hour, 2 hour, and 8 hour groups, n=3 pups/group for the 4 hour and 24 hour groups, n=2 pups/group in the vehicle control 24 hour group.

Figure 3. ***rhIGF-1/BP3 preserves intestinal VEGF/VEGFR2 expression, endothelial cell proliferation and vascular permeability.*** One-day-old neonatal mice were submitted to the experimental NEC model and injected with rhIGF-1/BP3 as

described or vehicle control. Dam fed littermates were used as baseline controls. Intestinal tissues were collected at 24 hours into experimental NEC: (A) VEGF and VEGFR2 proteins were assessed by Western blot and a typical blot is shown; (B-C) Quantified VEGF and VEGFR2, respectively, band densitometry from A is shown, n=6-8/group; (D) Tissue sections were stained with antibody against BrdU (red) and CD31 (green) and typical images are shown, scale bar=100mm, (E) Bar graph represents mean proliferating endothelial cell numbers ($BrdU^+CD31^+$cells, arrows in D) per x20 field (at least 3 fields/sample) for each sample, n=5/group. (F) One-day-old neonatal mice were submitted to the experimental NEC model and injected with rhIGF-1/BP3 (n=9) as described or vehicle control (n=9). Dam fed littermates were used as baseline controls (n=8). At 24 hours into NEC, 10 µl of Evans blue solution was injected i.v., and EBA that had leaked into the small intestinal tissue was quantified 1 hour later. *P* values were calculated using one-way ANOVA followed by Tukey-Kramer multiple-comparison test (B, C, E and F). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Figure 4. ***IGF-1/IBP3 preserved epithelial cell proliferation and migration while mitigating enterocyte apoptosis during experimental NEC.*** One-day-old newborn pups were either submitted to experimental NEC protocol or left with the dams (DF). Two hours prior to NEC initiation, half the pups in each group were injected with rhIGF-1/BP3 (6 mg/kg/d, i.p.). (A-D) At NEC initiation, all pups in DF and NEC groups were injected i.p. with 50mg/kg of BrdU. 24 or 48 hours post NEC initiation, intestines were collected, and sections stained with BrdU antibody and DAPI. Typical images are shown here, scale bar=100mm (A and B). (C) The number of proliferating enterocytes per villi was counted in 24h NEC with or without rhIGF-1/BP3 treatment and in DF controls. (D) The distance of enterocyte migration was measured using photoshop. n=4 pups/group for the dam fed groups (3 litters combined), n=4-8 pups/group for NEC group from 4 litters combined. (E-F) Intestinal tissues were collected at 48 hours into NEC and stained with anti-cleaved caspase 3 antibody. The number of apoptotic cells per 20x fields was counted. (E) Representative images are shown, scale bar=25mm. (F, Apoptotic cell count per 20x field. n=4-6/group. *P* values were calculated using one-way ANOVA followed by Tukey-Kramer multiple-comparison test. *p<0.05, **p<0.01, ***p<0.001.

Figure 5. ***IGF-1/IBP3 decreases systemic and local proinflammatory cytokines production.*** One-day-old neonatal mice were submitted to the experimental NEC model and injected with rhIGF-1/BP3 (n=8 pups/8 samples) as described or vehicle control (n=8 pups/8 samples). Untreated dam fed littermates and dam fed littermates treated with LPS were used as baseline and positive controls (n=6 pups pooled in 2 samples for both DF and LPS groups). At 24 hours into NEC, intestinal tissue lysates (A-C) and sera (D-E) were prepared and cytokines were measured by ELISA. *P* values were calculated using one-way ANOVA followed by Tukey-Kramer multiple-comparison test (A-E). p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Figure 6. ***rhIGF-1/BP3 improves survival and attenuates tissue injury in experimental NEC.*** One-day-old neonatal pups were submitted to experimental NEC and treated with rhIGF-1/BP3 or vehicle control twice daily as described (see method section section): (A) 60-hour-survival curve is shown, *P* value were calculated using Log-rank (Mantel-Cox) test, n=55 and 50 in control and treatment group, respectively; (B) Intestinal injury histological scores (left graph), percentage of severe NEC (score ≥2, right graph) are shown, *P* value were calculated using Chi-square test. n=54 and 44 in the NEC and NEC-rhIGF-1/BP3 treatment group, respectively. *p<0.05, **p<0.01.

Figure 7. **IGF-1/IBP3 preserved epithelial cell proliferation, which was attenuated by inhibiting VEGFR2 signaling**. Enterocyte proliferation was assessed on tissue sections from 36-hour NEC pups that had been treated with rhIGF-1/BP3, vehicle control, or rhIGF-1/BP3 and Ki8751, and compared to dam fed (DF) controls, n=8-11 pups per group. ***p<0.001, ****p<0.0001.

EXAMPLE

Reagents

Recombinant human IGF-1/BP3 (rhIGF-1/BP3, SHP607 DS, Lot# PR20151178-ENG) was provided by Takeda Pharmaceutical U.S.A (Deerfield, IL). Anti-CD31 (ab28364, for immunofluorescence stain) and rat anti-BrdU (cat. no. ab-6326) antibodies were purchased from Abcam (Cambridge, UK). Anti-VEGF-A (sc-7269) antibodies for Western blotting were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-IGF-1 antibodies for Western blotting (NBP2-16929) were purchased from Novus Biologicals (Littleton, CO). Goat anti-rabbit (A11034) and goat anti-rat (A11007) antibodies were obtained from Fisher Scientific (Waltham, MA). Anti-VEGFR2 (2479) antibodies were purchased from Cell Signaling Technology (Danvers, MA). Evans blue, albumin, and formamide were purchased from Sigma (St. Louis, MO).

Animal strains and breeding strategy

C57BL/6 mice were purchased from Jackson Laboratory (Bar Harbor, ME). Mice of age ranging between 8 weeks and 6 months were typically used for breeding. Breeders were fed with breeder chow and housed at the ratio of one male to maximum of three females. Overnight breeding strategy was used to generate pups for experiments. Pregnant dams were separated from breeding cages and housed individually 3 days prior to expected delivery date. Newborn pups with pigmented spot on the scrotum were identified as male. Pups were distributed among experimental groups based on gender and weight to limit bias. All performed procedures, animal breeding, maintenance and procedures were approved by the Institutional Animal Care and Use Committee and followed the regulation of the Center for Comparative Medicine of Northwestern University.

Animal experiments

Pups < 24-hour-old were left with the dam to be dam fed or exposed to a mouse NEC model which includes: (1) initial orogastric inoculation with a standardized adult mouse commensal bacteria preparation (10^8 colony-forming units) and LPS (5 mg/kg) to perturb the normal intestinal colonization process; (2) gavage with formula every 3 h (Esbilac, 200 ml·kg^{-1} day^{-1}); and (3) exposure to brief episodes of hypoxia (60 s in 100% N_2) followed immediately by cold stress (10 min at 4°C) twice daily. This well-established and widely used protocol was found to induce

intestinal injuries ranging from epithelial injury to transmural necrosis resembling human NEC which typically develop after 36 hours.

rhIGF-1/BP3, or vehicle control was injected i.p. to either pups submitted to the NEC protocol or to dam fed littermates twice daily, with the first dose administered 2 hours prior to NEC initiation and a daily dose of 3 or 6 mg/kg/d. Pups were closely monitored and euthanized when showing signs of distress. Whole intestinal tissues were collected. Hematoxylin and eosin-stained tissue sections were evaluated and scored by two investigators unaware of the group assignment as described previously. Severe NEC was defined as a histological score ≥2. Pups found dead while not under direct observation were excluded from histological analysis. Alternatively, small intestinal tissues were collected at 6 to 48 hours following NEC initiation for studies described below.

Western blot

Small intestinal tissue lysates were obtained by tissue homogenization in CelLytic™ MT Cell Lysis Reagent (sigma) containing protease inhibitors. Tissue lysate protein concentration was determined using the Bradford method. Twenty to 50 µg of non-reduced proteins were separated on 4 to 15% pre-cast gradient SDS-PAGE gel. For serum IGF-1detection, non-denatured and non-reduced proteins were used for Western blot analysis. Proteins were transferred onto nitrocellulose membranes and then blocked with 5% milk/Tween 20 for 60 minutes. Primary antibody was added and incubated at 4°C overnight, followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 hour at room temperature. Target proteins were detected using the standard Pierce enhanced chemiluminescence method. β-actin was probed on the same membranes to serve as internal controls.

Endothelial cell proliferation study

50mg/kg of BrdU was injected i.p. to pups 4 hours prior to euthanasia and tissue collection. Five-mm-thick tissue sections were prepared from formalin-fixed, paraffin-embedded intestines. Sections were boiled in pH 8.5 EDTA antigen retrieval buffer for 20 min following deparaffination and serial hydration process. After being blocked with 10% normal goat serum for 1 hour at room temperature, sections were incubated with anti-CD31 (1:50) and anti-BrdU antibodies (1:200) at 4°C overnight. Fluorescent conjugated secondary antibodies were applied to the sections and incubated at room temperature for 1 hour. Images were captured on a Keyence BZ-800 microscope after mounting media including DAPI was added. Proliferating endothelial cells staining positive for both CD31 and BrdU were counted in at least 3 fields/sample.

Intestinal enterocyte proliferation and migration study

NEC pups and DF littermates were injected i.p. with 50mg/kg of BrdU (BD Biosciences) at time of NEC initiation. 24 or 48 hours post NEC initiation, pups were euthanized by decapitation and their entire small intestine was fixed in 10% buffered formalin and processed for paraffin embedding and sectioning. Antigen retrieval on deparaffinized tissue sections (5 µm) was performed for 20 minutes as described above. Sections were then stained with rat anti-BrdU antibody (1:200) and Alexa Fluor 594 conjugated goat antibody against rat IgG (1: 1000) was used to detect anti-BrdU antibody. Nuclei were counterstained with DAPI. Slides were examined under Keyence BZ-X800 fluorescence microscope using appropriate filters. The distance of epithelial cell migration was assessed by measuring the gap between the base of the villus and the highest labeled cell within the villus axis. At least 42 villi per sample were assessed and the mean migration distance was used in the

subsequent analysis. Only intact villi were selected to examine how enterocytes migrated along the villus axis. Intestinal epithelial cell proliferation was measured from tissues collected at 24 hours into NEC. To do so, the number of $BrdU^+$ epithelial cells were counted per villus with at least 9 villi counted per sample.

Apoptosis analysis

Five-micrometer-thick sections obtained from formalin-fixed, paraffin-embedded intestinal tissue were submitted to antigen retrieval as described above. Slides were incubated with anti-cleaved caspase 3 antibody (cell signaling # 9664, 1:500) at 4°C overnight after being blocked with 10% normal goat serum for 1 hour at room temperature. Fluorescent conjugated goat anti-rabbit Ab was applied to the section and incubated at room temperature for 1 hour. Images were captured on a Keyence BZ-800 microscope after mounting media including DAPI was added. Cleaved caspase positive cells were assessed in at least 3 fields/sample (20x).

Intestinal vascular permeability assay

Pups were anesthetized by ice-induced hypothermia and injected i.v. with 10 µl of Evans blue albumin (EBA) solution (0.5% Evans blue/40 mg/ml BSA). One hour later, intestinal vascular permeability was measured as previously published. Normal saline (500 µl) was infused intracardiacally to remove the dye accumulated into the circulation and small intestinal tissues were collected immediately after perfusion. Pancreatic and surrounding connective tissues were carefully removed under a dissecting microscope and small intestinal tissue lysates were prepared as described above. EBA dye that had diffused into the intestinal tissues was extracted with formamide and quantified by spectrophotometry. Results were compared to a serial dilution of EBA with known concentrations and normalized to tissue weight.

Enzyme-linked immunosorbent assay

For determination of serum rhIGF-1/BP3 concentration, pups were i.p. injected with 2 or 10 mg/kg rhIGF-1/BP3 approximately 8 hours after birth and pups euthanized after 30 minutes, 1 hour, 2 hours, 4 hours, 8 hour or 24 hours. Blood was collected following pup decapitation and serum was obtained by centrifugation. ELISA was performed using Mediagnost (E20) IGFBP blocked Human Insulin-like Growth Factor ELISA according to the manufacturer's instructions (Mediagnost; Reutlingen, Germany).

For cytokine analysis, 2 to 3 blood samples were pooled for analysis if necessary. Intestinal tissue lysates were prepared by tissue homogenization in CelLytic™ MT Cell Lysis Reagent (Sigma) containing protease inhibitors. Inflammatory cytokines including IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, KC/GRO, IL-10, IL-12p70, and TNF-α from serum and intestinal tissue lysates were detected by V-PLEX Proinflammatory Panel 1 Mouse Kit from Meso Scale Discovery according to the manufacturer's instructions. MESO QuickPlex SQ 120 instrument was used for electrochemiluminescence signal reading. Known standards were used to calculate cytokine concentration. Data analysis was performed using DISCOVERY WORKBENCH assay analysis software, and the results from intestine were normalized to tissue weight.

Statistical analysis.

The statistical software GraphPad Prism (version 8.1.2) was used for statistical analysis. Animal survival data were analyzed by log-rank or Gehan-Breslow-Wilcoxon test. Non-parametric chi-square (χ^2) test was used to compare the incidence of severe NEC (Grade ≥2) between two groups.

For other data, two-sided Student's *t*-test was used for comparison between two groups, and one-way ANOVA was used for comparison of three or more groups. For ANOVA test, a correction for multiple comparisons was applied for pairwise comparisons after ANOVA performed. Data were normalized when failed normalization test using Shapiro-Wilk test. Results are expressed as mean ± standard error of mean (SEM). Differences were considered statistically significant when $P \leq 0.05$.

RESULTS

The IGF-1/IBP3/ALS complex is decreased in the serum prior to experimental NEC development:

Both intestinal tissue and serum IGF-1 levels are decreased prior to experimental NEC development. Given that, in the serum, IGF-1 is highly regulated by its multiple binding proteins, serum IGF-1 bound to binding proteins was assessed during NEC development by Western blot using non-denaturing conditions. When pups were submitted to the experimental NEC protocol for 24 hours, the ternary complex of IGF-1 (IGF-1/IBP3/ALS) was significantly decreased in the serum, while levels of other complexes were not significantly different (Fig.1A-B).

rhIGF-1/BP3 preserves intestinal VEGF/VEGFR2 expression and endothelial cell proliferation

To define the dose of rhIGF-1/BP3 which improves plasma levels of IGF-1 in neonatal pups, dam fed pups were injected i.p. with 2 or 10 mg/kg of rhIGF-1/BP3 (or vehicle control) approximately 8 hours after birth. Pups were euthanized and serum collected 30 minutes, 1 hour, 2 hours, 4 hours, 8 hours, or 24 hours after rhIGF-1/BP3 injection for human IGF-1 detection by ELISA. The injection of 10 mg/kg, but not 2 mg/kg, of rhIGF-1/BP3 significantly increased serum IGF-1 concentration in the serum of neonatal pups within 30 minutes, with a peak (of 165.7±49.7 ng/ml) reached at 2 hours (Fig. 2), an effect that was no longer significant after 8 hours. Based on literature data from both humans and mice, the physiological range of 30-110 ng/ml was targeted and therefore a dose of 6 mg/kg/day employed in subsequent experiments.

We showed that, in the intestine, VEGF and VEGFR2 proteins are downregulated and endothelial cell proliferation and intestinal microvascular density are decreased in NEC pups compared to dam fed controls, which are improved by exogenous IGF-1. To determine whether rhIGF-1/BP3 preserves intestinal microvascular development during NEC development, VEGF and VEGFR2 expression and endothelial cell proliferation were assessed in the intestine of neonatal mice injected with rhIGF-1/BP3 or vehicle control. In pups exposed to the NEC model for 24 hours, **treatment with rhIGF-1/BP3 (6 mg/kg/day) preserved intestinal VEGF and VEGFR2 expression** (**Fig. 3A-C**). **Furthermore, exogenous rhIGF-1BP3 administration at the same dose but not vehicle control improved NEC-induced decrease in villous endothelial cell proliferation (Fig. 3D-E).**

Exogenous rhIGF-1/BP3 improves NEC-induced increased intestinal vascular permeability

Next, we sought to determine whether NEC affects **intestinal vascular permeability** and whether permeability was improved by rhIGF-1/BP3 administration. To do so, rhIGF-1/BP3 (6mg/kg/day) or vehicle control was administered to pups submitted to the NEC model. Dam fed pups treated with vehicle served as controls. Twenty four hours later, intestinal vascular permeability was assessed by quantifying EBA leakage into intestinal tissues after i.v. injection. We found the mean EBA concentration to be significantly higher in the intestinal tissues of pups exposed to the NEC protocol

compared to dam fed controls, suggesting higher intestinal vascular permeability. Furthermore, rhIGF-1/BP3 significantly improved NEC-induced intestinal EBA leakage (Fig. 3F).

Exogenous rhIGF-1/BP3 promotes epithelial cell proliferation and migration during experimental NEC

To determine the effect of rhIGF-1/BP3 on epithelial cell proliferation and migration, <24h-old pups were submitted to the NEC protocol or dam fed for 24 or 48 hours. Starting 2 hours prior to NEC initiation, a subgroup of pups was injected i.p. with rhIGF-1/BP3 6mg/kg/day or vehicle. All pups were injected i.p. with 50 mg/kg of BrdU at time of NEC initiation to label proliferating cells and intestinal tissues were collected 24 or 48 hours to assess epithelial cell proliferation and migration. At 24 hours, we found that epithelial cell proliferation was significantly decreased in intestinal tissues of NEC pups (Fig. 4A, C) and r**hIGF-1/BP3 treatment rescued epithelial cell proliferation defect induced by NEC** (Fig. 4A, C). Furthermore, at 48 hours, intestinal epithelial cell migration was impaired in NEC pups and **rhIGF-1/BP3 treatment increased enterocyte migration in both NEC and DF pups** (Fig. 4A, B, D).

IGF-1/BP3 protect animal against NEC-induced intestinal epithelial cell apoptosis

To determine whether IGF-1/BP3 protects against enterocyte apoptosis during experimental NEC, 24-hour-old pups were submitted to NEC and treated with IGF-1/BP3 (6mg/kg/day, i.p.) or vehicle. At 48 hours into NEC, intestinal tissues were stained for *cleaved caspase 3*. We observed a significantly higher number of apoptotic cells (mainly epithelial cells) in NEC tissues compared to DF controls. Furthermore, the number of **apoptotic enterocytes was decreased when exogeneous IGF-1/BP3 was administrated (Fig. 4E-F)**.

IGF-1/IBP3 decreases systemic and local proinflammatory cytokines production

To determine whether rhIGF-1/BP3 has any effect on systemic inflammation during NEC development, pups exposed for 24 hours to the NEC protocol were either treated with rhIGF-1/BP3 or vehicle control and compared to dam fed controls treated or not with LPS (1mg/kg, i.p. 6 hours – negative and positive control, respectively). Pup sera and intestinal tissue lysates were assessed for inflammatory cytokines by ELISA. Intestinal IL-4, IL-6 and IL-10 (Fig. 5A-C) as well as serum IL6 and CXCL1 (Fig. 5D-E) were significantly increased during NEC, which were **significantly decreased by exogenous rh/IGF-1/BP3 administration**.

rhIGF-1/BP3 improves survival and attenuates tissue injury in experimental NEC

To investigate whether rhGF-1/BP3 administration protects the intestine against injury in a neonatal NEC model, rhIGF-1/BP3 or vehicle control were injected to pups i.p., twice daily (6mg/kg/day) with the first dose given 2 hours prior to NEC initiation. **When treated with rhGF-1/BP3, NEC pups had significantly improved survival compared to vehicle-treated NEC controls (median survival of 64 hours in the rhIGF-1/BP3 treatment group compared to 52 hours in the vehicle-treated group) (Fig. 6A).** Also, in pups treated with rhGF-1/BP3, the incidence of severe histological intestinal injury (grade ≥ 2) was significantly lower compared to those treated with vehicle-control (13/44 (30%) vs. 27/54 (50%) - $\chi^2=4.20$, p, 0.05 - Fig. 6B).

DISCUSSION

Exogenous rhIGF-1/BP3 is undergoing phase IIb multi-center clinical trials to determine its efficacy in diseases affecting premature infants e.g. ROP and BPD (ClinicalTrials.gov registry NCT03253263

and NCT03253263). However, the effect of exogenous rhIGF-1/BP3 on the neonatal intestine remains unclear.

In pups exposed to experimental NEC, serum IGFBP3-bound IGF-1 is decreased, and exogenous administration of rhIGF-1/BP-3 (6 mg/kg/d) **preserved VEGF and VEGFR2 protein expression, decreased vascular permeability, and preserved endothelial cell proliferation in the intestine**. Furthermore, rh**IGF1/BP3 promoted enterocyte proliferation and migration while decreasing enterocyte apoptosis and attenuating systemic and intestinal tissue inflammation**. Finally, we show that rhIGF-1/BP-3 **improved survival and reduced the incidence of severe intestinal injury in experimental NEC**. Thus, supplemental rhIGF-1/BP-3 (6 mg/kg/d) protects neonatal mice against experimental NEC via multiple mechanisms.

Since IGF-1 is bound to several IGFBPs and is decreased during experimental NEC development, we wanted to determine which form of IGF-1 is decreased during NEC development.

Similar to adult human blood, when mouse serum was assessed by Western blot analysis, most of serum IGF-1 was in a protein complex sized at 160-170 kDa, which presumably corresponded to IGF-1 sequestered into ternary complexes consisting of one molecule each of IGF-1, IGFBP-3 or IGFBP-5, and acid-labile subunit. A smaller amount of serum IGF-1 was present in a complex sized around 74kDa, presumably bound to other IGFBPs, with a small amount of IGF-1 existing as free form with a size <13 kDa.

Interestingly, we found that only IGFBP3/ALS-bound IGF-1 was decreased while the other two forms remain unchanged at 24 hours of NEC development. **The decrease in bound IGF-1 levels may relate to IL-6 mediated proteolysis of IGFBP-3 with increased clearance of free IGF-1**.

We show here that rhIGF-1/BP3 (6mg/kg/d) promotes intestinal **VEGF/VEGFR2 expression, endothelial cell proliferation**, both of which are severely decreased during NEC.

Decreased enterocyte migration and proliferation in neonatal rats and mice in a number of disease states. Here, we found that epithelial cell proliferation and migration, which are substantially decreased prior to tissue injury, are significantly improved in pups treated with rhIGF-1/BP3.

Epithelial cell proliferation, differentiation, and migration are required for the maintenance of an intact epithelial layer. Intestinal epithelial cell apoptosis precedes experimental NEC. We found here that exogenous rhIGF-1/BP3 attenuated enterocyte apoptosis.

Our present study showed that rhIGF-1/BP3 administration significantly decreased the production of several inflammatory cytokines including IL-6 both locally and systematically during experimental NEC.

Together, our study suggests that, in neonatal mice, rhIGF-1/BP3 supplementation at the dose of 6 mg/kg/day may protect against sever intestinal injury via multiple mechanisms: by protecting intestinal microvascular development and integrity, by promoting enterocyte proliferation and migration while decreasing enterocyte apoptosis and by decreasing local and systemic inflammation.

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CLAIMS:

1. A method of treatment or prophylaxis of dysregulation of GI tract regeneration, for example in an infant such as in a preterm infant (also referred to a neonate herein) and/or low weight for gestational age baby or an infant born following preeclampsia, by administering a therapeutic amount of a composition comprising IGF-1 and an IGF binding protein (such as IGFBP-3), in particular as a complex.

1A. A composition comprising IGF-1 and an IGF binding protein (such as IGFBP-3), in particular as a complex, for use in the treatment or prophylaxis of dysregulation of intestinal regeneration, for example in an infant such as in a preterm infant (also referred to a neonate herein) and/or low weight for gestational age baby or an infant born following preeclampsia,.

1B. A composition comprising IGF-1 and an IGF binding protein (such as IGFBP-3), in particular as a complex, for use in the manufacture of a medicament for the treatment or prophylaxis of dysregulation of intestinal regeneration, for example in an infant such as in a preterm infant (also referred to a neonate herein) and/or low weight for gestational age baby or an infant born following preeclampsia.

1C. A method of treatment or prophylaxis by stimulating GI cell maturation (for example to increase and/or maintain tight junctions and/or) in an infant such as in a preterm infant (also referred to a neonate herein) and/or low weight for gestational age baby by administering a therapeutic amount of a composition comprising IGF-1 and an IGF binding protein (such as IGFBP-3), in particular as a complex.

2. A method or composition according to any previous claim, wherein the treatment reduces inflammation, for example inflammation located in the GI tract, in particular wherein the inflammation is generated by a cytokine selected from IL-4, IL-6, IL-10 and combinations of two or three thereof.

3. A method or composition according to any preceding claim, wherein inflammation-initiated destruction of IGFBP-3 in tissue is minimised and/or destruction (including elimination) of IGF-1 is minimised.

4. A method or composition according to any preceding claim, wherein a positive feedback loop for IGF-1 production, is supported, stabilised, maintained, initiated and/or augmented.

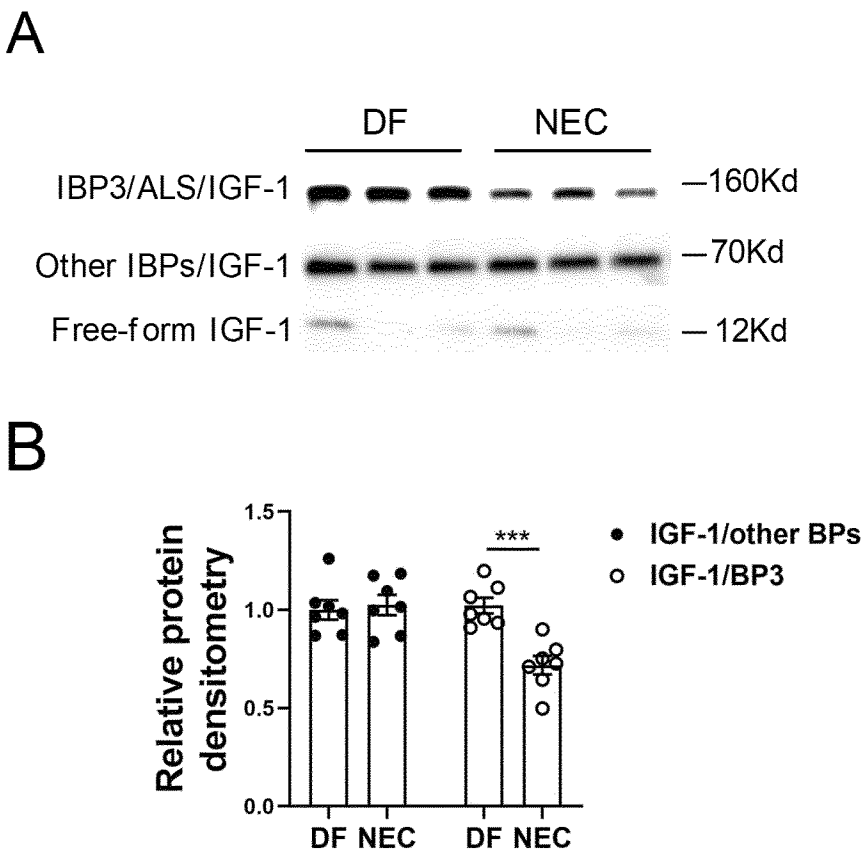
5. A method or composition according to any preceding claim, wherein vascular permeability is reduced, for example in comparison to an untreated patient with necrotising enterocolitis, in particular wherein vascular leakage into the intestinal tissue is minimised and/or leakage from the GI tract to the vascular system is minimised.

6. A method or composition according to any preceding claim, wherein tight junctions are facilitated, preserved, supported, stabilised, maintained, initiated and/or augmented by treatment, for example the percentage of tight junctions is preserved or augmented.

7. A method or composition according to any preceding claim, wherein treatment supports maintenance of epithelial barrier function and/or epithelial integrity, for example including prevention of breach of basement membrane and/or underlying stromal cells are not exposed to luminal content.

8. A method or composition according to any preceding claim wherein tight junctions are facilitated, preserved, supported, stabilised, maintained, initiated and/or augmented by treatment, for example the percentage of tight junctions is preserved or augmented.
9. A method or composition according to any preceding claim, wherein treatment supports maintenance of epithelial barrier function and/or epithelial integrity, for example including prevention of breach of basement membrane and/or underlying stromal cells are not exposed to luminal content.
10. A method of composition according to any preceding claim, wherein epithelial cell migration is preserved, supported, maintained, initiated and/or augmented by treatment.
11. A method or composition according to any preceding claim, wherein epithelial cell apoptosis is minimised (regularised) by treatment, for example apoptosis of enterocytes, for example apoptosis induced by oxidative stress.
12. A method or composition according to any preceding claim wherein treatment promotes proliferation of enterocyte, for example in comparison to an untreated patient.
13. A method or composition according to any preceding claim, wherein the enterocyte function is preserved, supported, maintained, initiated and/or augmented, for example a function selected from ion uptake, water uptake, sugar uptake, peptide uptake, amino acid uptake, lipid uptake, vitamin B12 uptake, secretion of immunoglobulins, and a combination of two or more of the same, such as all the functions.
14. A method or composition according to any preceding claim, wherein Paneth cell function is preserved, supported, maintained, initiated and/or augmented, for example Wnt signalling and Notch signalling and/or secretion of defensins HD-5 and HD-6.
15. A method or composition according to any preceding claim, wherein Paneth cell dysfunction is minimised by treatment, for example in comparison to a untreated patient.
16. A method or composition according to any preceding claim, wherein treatment reduces incidences of severe injury to the GI tract, such as severe intestinal injury, for example by promoting and/or regulating healing processes, in particular reduces severe incidences of severe necrotising enterocolitis.
17. A method or composition according to claim 16, wherein severe injury to the villi is minimised, for example destruction of villi and/or malformation of villi.
18. A method or composition according to any preceding claim, for use in the treatment of colitis (including necrotising enterocolitis) *C. difficile* infection, shigella infection, toxic mega colon.
19. A method or composition according to claim 16, wherein severe incidences of severe necrotising enterocolitis, for example score equal or above 2 (such as 3, 4, 5 and 6) are reduced in comparison to untreated patients with necrotising enterocolitis.
20. A method or composition according to any preceding claim, wherein the incidences of sepsis are reduced in treated patients.
21. A method or composition according to any preceding paragraph, wherein the dose for an infant, such as a preterm, low weight for gestational age and/or a baby born following preeclampsia, is 200 to 500μg/Kg/24hours of complex, for example 350 to 500μg/Kg/24hours, such as 400 μg/Kg/24hours, in particular by continuous infusion.

FIGURE 1:



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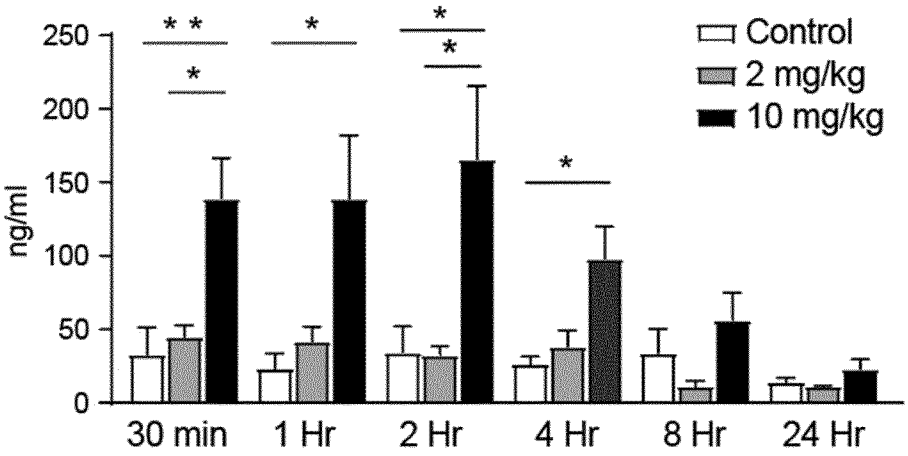

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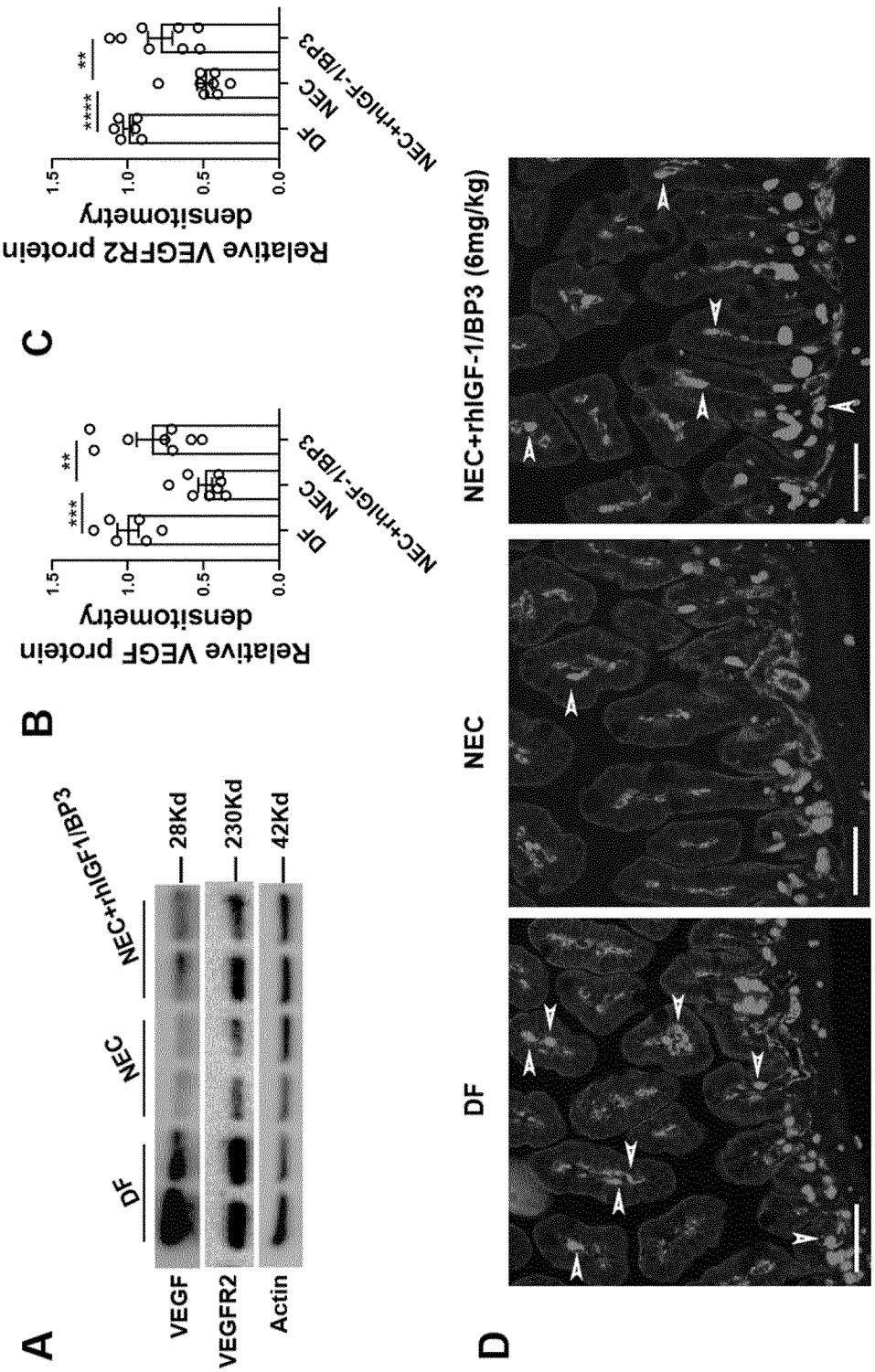
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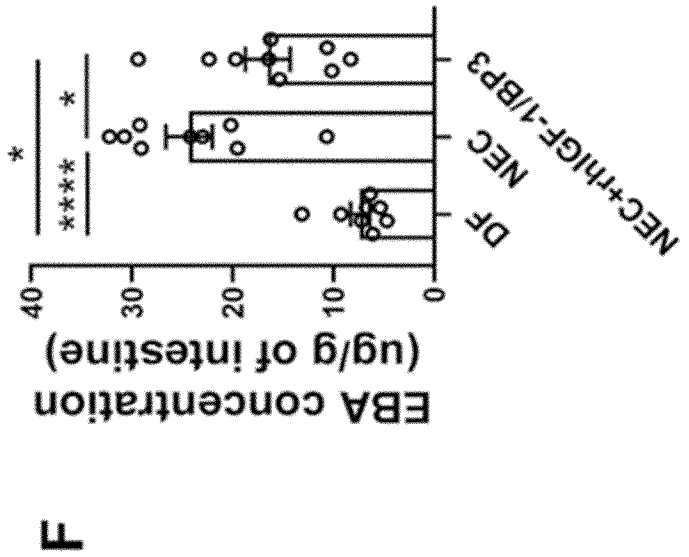
DF
NEC
NEC+rhIGF1/BP3
VEGF
VEGFR2
Actin
28Kd
230Kd
42Kd
B
Relative VEGF protein densitometry
1.5
1.0
0.5
0.0
***
**
DF
NEC
NEC+rhIGF-1/BP3
C
Relative VEGFR2 protein densitometry
****
**
D
DF
NEC
NEC+rhIGF-1/BP3 (6mg/kg)

FIGURE 3 cont:



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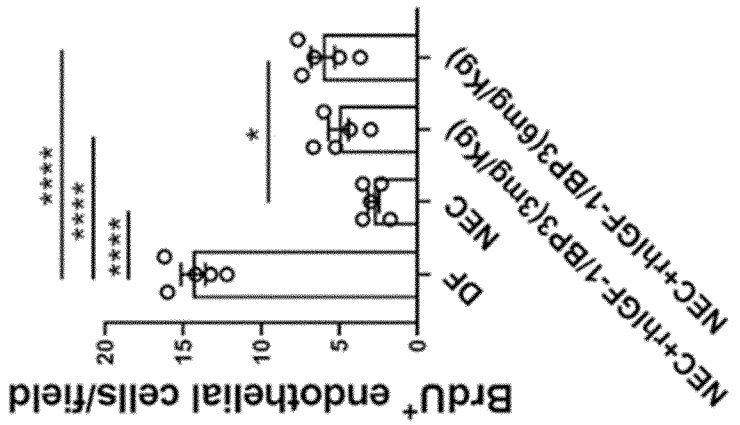

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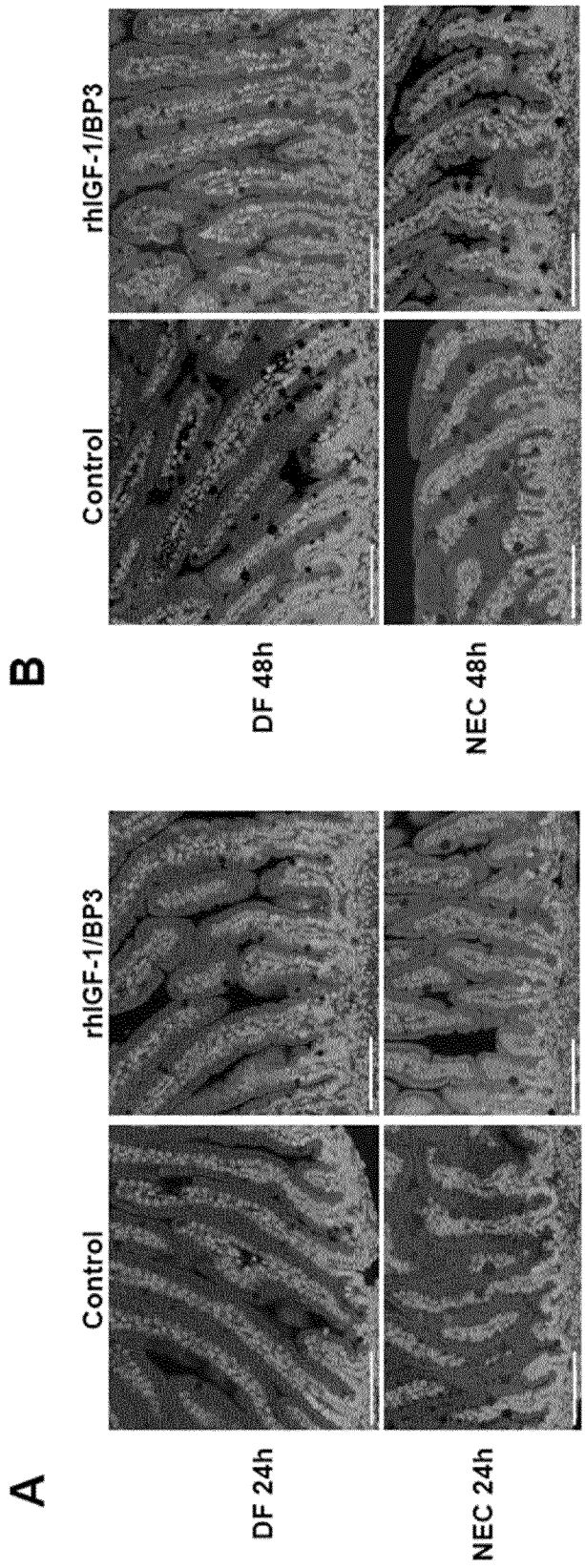
FIGURE 4:



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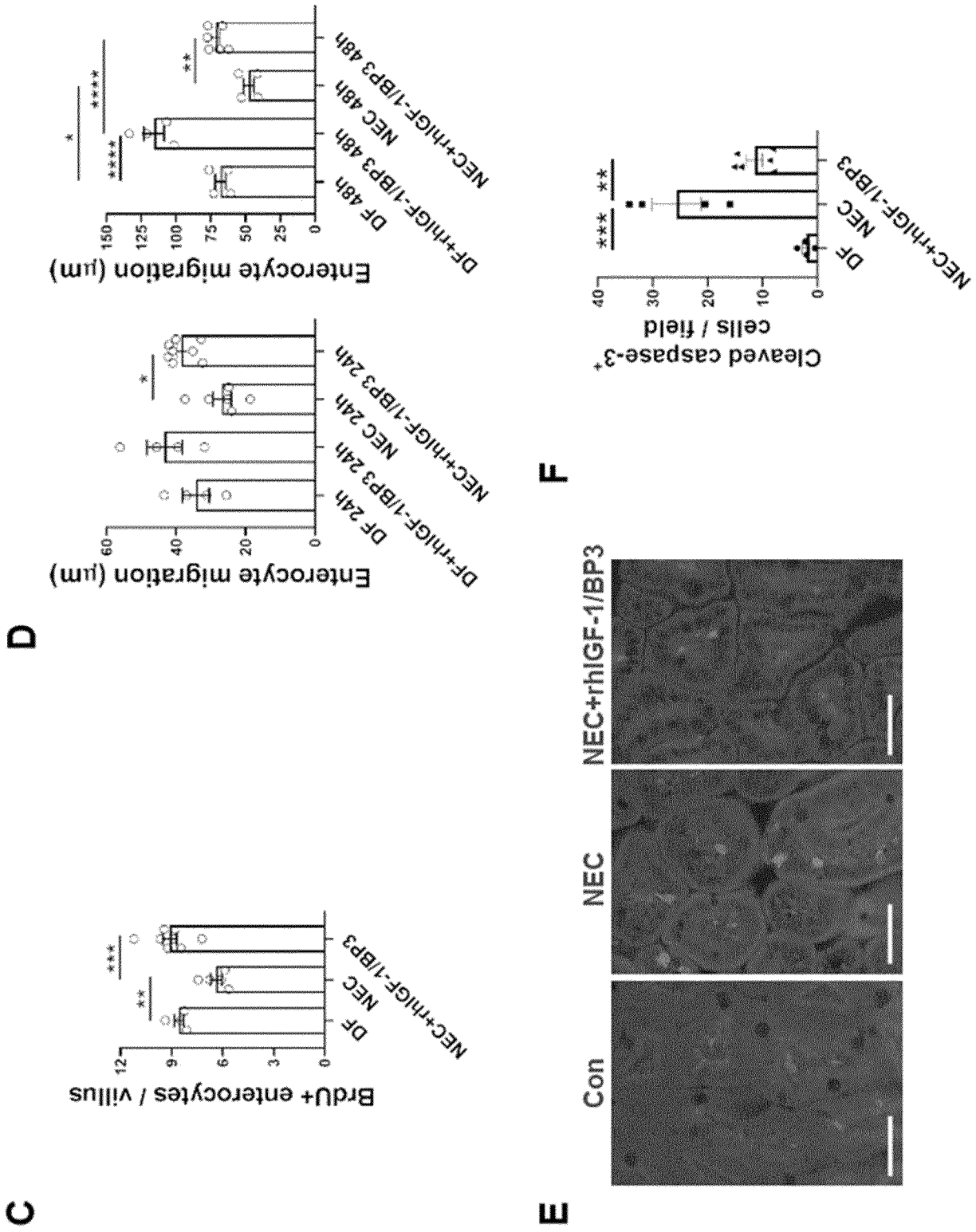

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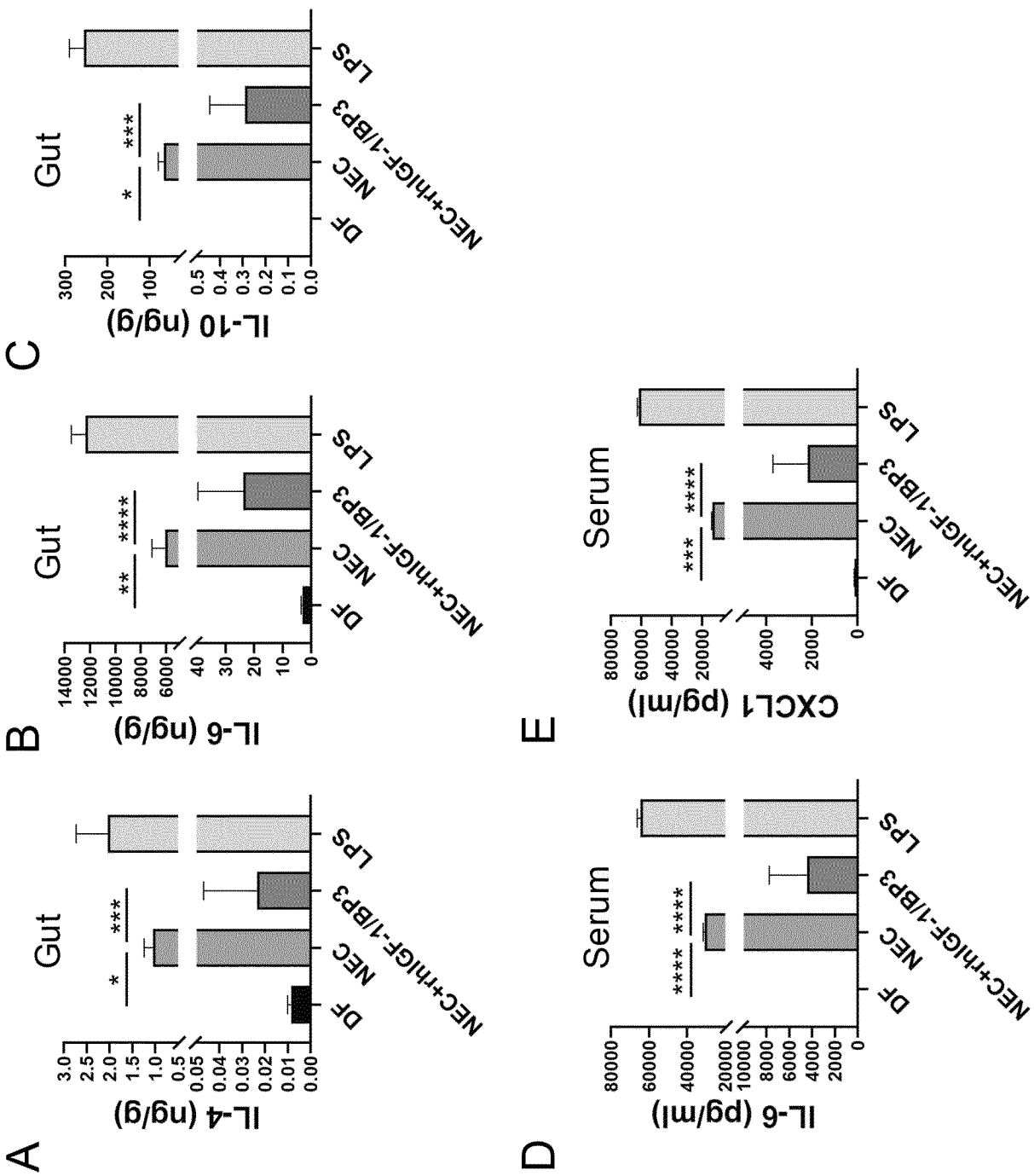
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B
Gut
IL-6 (ng/g)
C
Gut
IL-10 (ng/g)
D
Serum
IL-6 (pg/ml)
E
Serum
CXCL1 (pg/ml)
DF
NEC
NEC+rhIGF-1/BP3
LPS

FIGURE 6

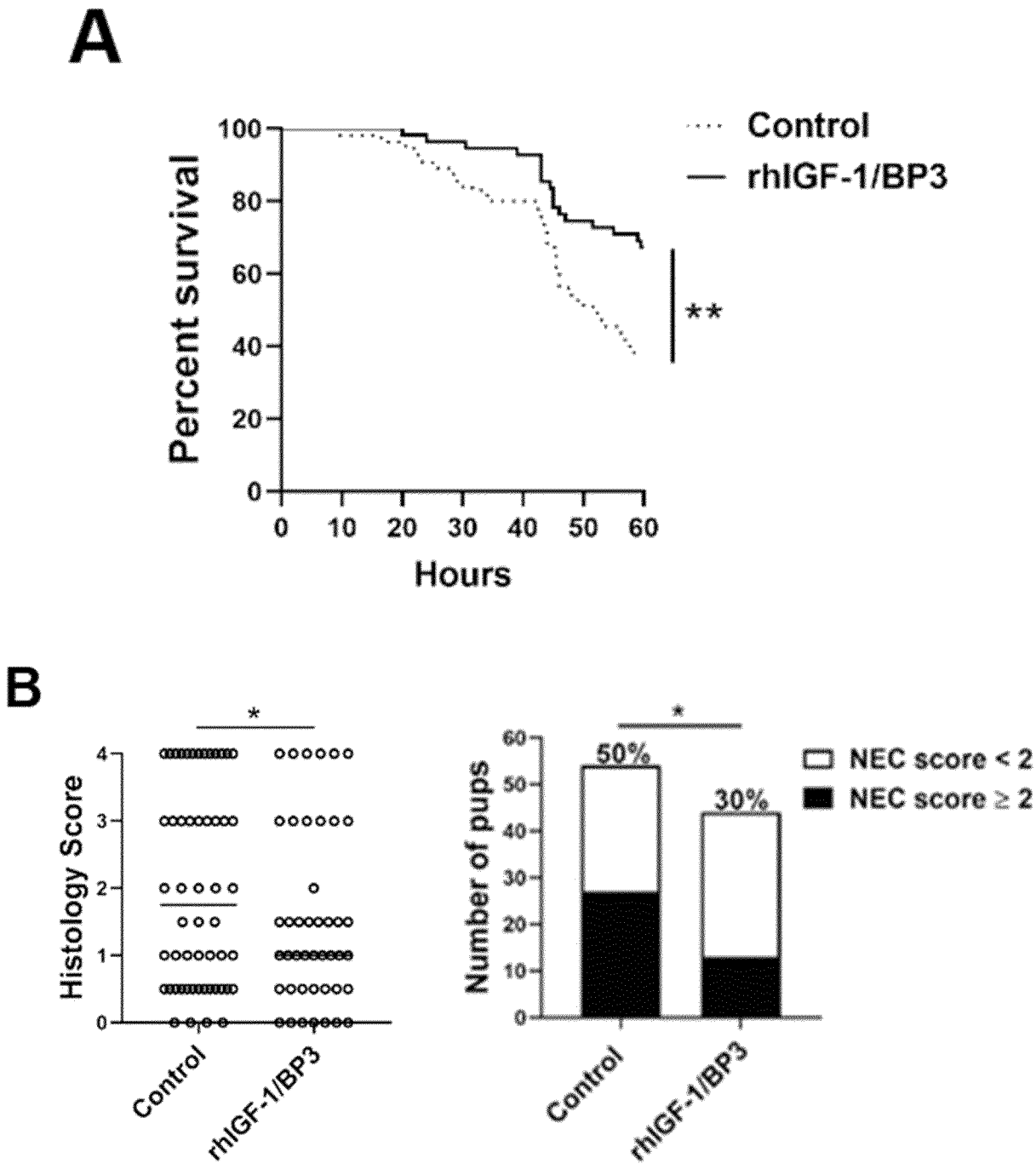
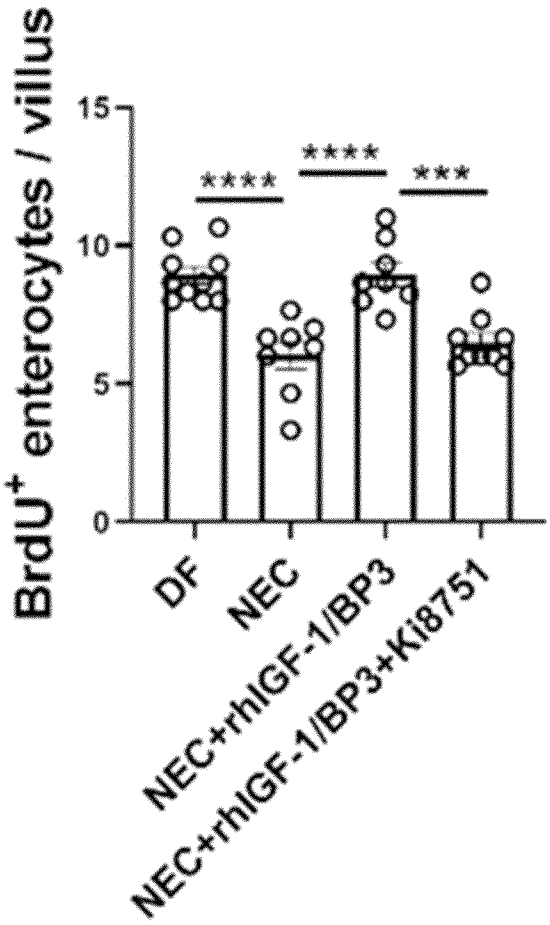

A
Percent survival
100
80
60
40
20
0
0 10 20 30 40 50 60
Hours
Control
rhIGF-1/BP3
**
B
Histology Score
4
3
2
1
0
*
Control
rhIGF-1/BP3
Number of pups
60
50
40
30
20
10
0
50%
30%
*
NEC score < 2
NEC score ≥ 2
Control
rhIGF-1/BP3

FIGURE 7


BrdU+ enterocytes / villus
15
10
5
0
****
****
***
DF
NEC
NEC+rhIGF-1/BP3
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INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2024/053705

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K38/30 A61K38/17 A61P1/12 A61P37/06 A61P1/00
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	LEY DAVID ET AL: "rhIGF-1/rhIGFBP-3 in Preterm Infants: A Phase 2 Randomized Controlled Trial", JOURNAL OF PEDIATRICS, vol. 206, 1 January 2019 (2019-01-01), page 56, XP085610894, ISSN: 0022-3476, DOI: 10.1016/J.JPEDS.2018.10.033 page 56 ----- -/--	1-21

[x] Further documents are listed in the continuation of Box C.

[x] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance;; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance;; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
8 May 2024	15/05/2024

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Zellner, Eveline

4

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	HUBERT ZATORSKI: "Is insulin-like growth factor 1 (IGF-1) system an attractive target inflammatory bowel diseases? Benefits and limitation of potential therapy", PHARMACOLOGICAL REPORTS, vol. 68, no. 4, 1 August 2016 (2016-08-01) , pages 809-815, XP093152860, PL ISSN: 1734-1140, DOI: 10.1016/j.pharep.2016.03.006 abs; page 809 page 811, column 2	1-21
Y	ALAN B R THOMSON ET AL: "REVIEW: Small Bowel Review: Normal Physiology, Part 2", DIGESTIVE DISEASES AND SCIENCES, KLUWER ACADEMIC PUBLISHERS-PLENUM PUBLISHERS, NE, vol. 48, no. 8, 1 August 2003 (2003-08-01) , pages 1565-1581, XP019236986, ISSN: 1573-2568, DOI: 10.1023/A:1024724109128 page 1556, column 2, paragraph 2 page 1570, column 1, paragraph 3	1-21
Y	BARKSDALE EDWARD M ET AL: "Insulinlike growth factor 1 and insulinlike growth factor 3: Indices of intestinal failure in children", JOURNAL OF PEDIATRIC SURGERY, vol. 34, no. 5, 30 May 1999 (1999-05-30), pages 655-662, XP029259599, ISSN: 0022-3468, DOI: 10.1016/S0022-3468(99)90350-0 abstract	1-21
Y	KUEMMERLE JOHN F: "Endogenous IGF-I regulates IGF binding protein production in human intestinal smooth muscle cells", AMERICAN JOURNAL OF PHYSIOLOGY - GASTROINTESTINAL AND LIVER PHYSIOLOGY, AMERICAN PHYSIOLOGICAL SOCIETY, US , vol. 278, no. 5 1 May 2000 (2000-05-01), pages G710-G717, XP008163880, ISSN: 0193-1857 Retrieved from the Internet: URL:http://ajpgi.physiology.org/content/278/5/G710.abstract abstract	1-21

-/--

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	EP 2 148 695 A1 (PREMACURE AB [SE]) 3 February 2010 (2010-02-03) paragraph [0018] - paragraph [0026]; claims 1-15 -----	1-21

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2024/053705

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 2148695 A1	03-02-2010	CN 101969983 A	09-02-2011
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		US 2013310316 A1	21-11-2013
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		WO 2008130315 A1	30-10-2008