

Method for producing milk like products

Field of the invention

5 The present invention concerns *in vitro* methods for producing isolated mammalian breast milk exosomes, the method comprising generating lactocytes, derived from mammalian mammary epithelial cells, for example human mammary epithelial cells, through culture and differentiation, and/or mammary-like gland organoids comprising such lactocytes, expressing a mammalian milk like product, for example
10 the human milk like product from such lactocytes and/or mammary-like gland organoids, and purifying the exosomes from the mammalian milk like product. The present invention also relates to the isolated mammalian breast milk exosomes. The invention further relates to exosomes obtainable from such method.

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Background of the invention

Mammalian and especially human milk is a complex fluid with a multitude of components, each of which may contribute substantially to infant and perhaps maternal health. It is becoming increasingly clear that human breastmilk is the most
20 appropriate source of nutrition at least up to the age of 6 months. Many components of human milk are simply not found or poorly found or less active in cow's milk upon which infant formula manufacture is based. This includes for instance protein lactoferrin, growth factors, long chain polyunsaturated fatty acids or oligosaccharides. Human milk composition has been used as a gold standard to
25 develop current infant formula, despite recent major development in infant formula composition, it is illusory to think that human milk replication or replication of components of said human milk will be achieved with current manufacturing processes.

Today the only source of human milk is human donors (breastfeeding mothers). Donation are reported for non-commercial use (human milk biobank) and commercial use. However, this is limited and has strong regulatory, safety and sometime ethical or religious constraints.

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Stem cells were found in mammalian and especially human milk called human breastmilk stem cells (hBSC). hBSCs were shown to be highly plastic and to differentiate in culture into multiple cell types and more importantly into the three lineages required to shape the lobulo-alveolar structure of the human mammary gland (Hassiotou F. et al. Stem Cells. 2012). However, the use of hBSC to produce human breast milk is neither practical nor sustainable, as it requires human donors.

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A technology based on a cell line with stem cell functionality - called induced pluripotent stem cells (iPSC) is known. A reliable two step protocol to generate human mammary like organoids from human iPSC (hiPSC) was developed (Ying Qu et al, Stem Cell Report vol 8, 205-215, February 14th 2017).

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Accordingly, it is an object of the present invention to provide alternative and improved methods for producing mammalian milk and key components of mammalian milk such as exosomes using cultured cells, without the use of early stage undifferentiated stem cells. It is also an object of the present invention to prepare customized mammalian milk like product, for example human milk like product, in cultured cells which could be adapted to specific needs of the recipient and/or to produce human milk bioactives such as exosomes to complement existing cow-based solutions for infant nutrition, again without the use of stem cells. It is also an object of the present invention to provide therapeutic uses of the isolated human breast milk exosomes, and compositions thereof.

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Summary of the invention

The present invention solves the above-mentioned technical problem.

5 Provided herein is an isolated human breast milk exosome, wherein the exosome does not comprise one or more proteins selected from Hypoxia up-regulated protein 1 (HYOU1), Apolipoprotein A-IV (APOA4) and Procollagen-lysine,2-oxoglutarate 5-dioxygenase 1 (PLOD1).

10 Also provided herein is an isolated human breast milk exosome, wherein the exosome does not comprise one or more proteins selected from C4a anaphylatoxin (CFA), Hypoxia up-regulated protein 1 (HYOU1), Apolipoprotein A-IV (APOA4), Procollagen-lysine,2-oxoglutarate 5-dioxygenase 1 (PLOD1), Threonine—tRNA ligase 1, cytoplasmic (TARS1), and Cytosolic non-specific dipeptidase (CNDP2).

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Also provided herein is an isolated human breast milk exosome, wherein Hypoxia up-regulated protein 1 (HYOU1), Apolipoprotein A-IV (APOA4) and Procollagen-lysine,2-oxoglutarate 5-dioxygenase 1 (PLOD1) are not detected by liquid chromatography – mass spectrometry in the exosome. Also provided herein is an
20 isolated human breast milk exosome, wherein Hypoxia up-regulated protein 1 (HYOU1), Apolipoprotein A-IV (APOA4) and Procollagen-lysine,2-oxoglutarate 5-dioxygenase 1 (PLOD1) are not detected by liquid chromatography – mass spectrometry in the exosome under the same conditions in which Hypoxia up-regulated protein 1 (HYOU1), Apolipoprotein A-IV (APOA4) and Procollagen-lysine,2-oxoglutarate 5-dioxygenase 1 (PLOD1) are detected by liquid
25 chromatography – mass spectrometry in an exosome isolated from human breast milk.

Also provided herein is an isolated human breast milk exosome, wherein C4a
30 anaphylatoxin (CFA), Hypoxia up-regulated protein 1 (HYOU1), Apolipoprotein A-IV

(APOA4), Procollagen-lysine,2-oxoglutarate 5-dioxygenase 1 (PLOD1), Threonine—tRNA ligase 1, cytoplasmic (TARS1), and Cytosolic non-specific dipeptidase (CNDP2) are not detected by liquid chromatography – mass spectrometry in the exosome. Also provided herein is an isolated human breast milk exosome, wherein C4a anaphylatoxin (CFA), Hypoxia up-regulated protein 1 (HYOU1), Apolipoprotein A-IV (APOA4), Procollagen-lysine,2-oxoglutarate 5-dioxygenase 1 (PLOD1), Threonine—tRNA ligase 1, cytoplasmic (TARS1), and Cytosolic non-specific dipeptidase (CNDP2) are not detected by liquid chromatography – mass spectrometry in the exosome under the same conditions in which C4a anaphylatoxin (CFA), Hypoxia up-regulated protein 1 (HYOU1), Apolipoprotein A-IV (APOA4), Procollagen-lysine,2-oxoglutarate 5-dioxygenase 1 (PLOD1), Threonine—tRNA ligase 1, cytoplasmic (TARS1), and Cytosolic non-specific dipeptidase (CNDP2) are detected in an exosome isolated from human breast milk.

Also provided herein is a population of exosomes, wherein the population comprises exosomes as described anywhere herein.

Also provided herein is a composition comprising exosomes or a population of exosomes as described anywhere herein.

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The present invention also relates to the exosome as described anywhere herein, the population of exosomes as described anywhere herein, the composition as described anywhere herein, or the exosome product which is obtainable according to the method as described anywhere herein, for use in therapy.

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Also provided herein is a method of treating a disease or disorder comprising administering to a subject in need thereof a therapeutically effective amount of the exosome as described anywhere herein, the population of exosomes as described

anywhere herein, the composition as described anywhere herein, or the exosome product which is obtainable according to the method as described anywhere herein.

The invention also relates to the exosome as described anywhere herein, the
5 population of exosomes as described anywhere herein, the composition as
described anywhere herein, or the exosome product which is obtainable according
to the method as described anywhere herein, for use in preventing, treating, or
ameliorating symptoms associated with inflammation. In some embodiments,
inflammation is intestinal inflammation. In some embodiments, inflammation is
10 bronchial inflammation. In some embodiments, inflammation is respiratory
inflammation.

Also provided herein is a method of preventing, treating, or ameliorating symptoms
associated with inflammation, the method comprising administering to a subject in
15 need thereof a therapeutically effective amount of the exosome as described
anywhere herein, the population of exosomes as described anywhere herein, the
composition as described anywhere herein, or the exosome product which is
obtainable according to the method as described anywhere herein. In some
embodiments, inflammation is intestinal inflammation. In some embodiments,
20 inflammation is bronchial inflammation. In some embodiments, inflammation is
respiratory inflammation.

Also provided herein is a mammary breast milk exosome product which is
obtainable according to the method as described anywhere herein, optionally a
25 human breast milk exosome.

Finally, provided herein is a method of producing a mammalian milk fortifier,
comprising conducting the method as described anywhere herein.

Detailed description of the invention

Definitions

5 Within the context of the present invention, the term “in vitro” means performed or taking place in a test tube, culture dish, bioreactor or elsewhere outside a living organism.

Within the context of the present invention, the term “mammalian” identify an
10 animal belonging to the mammalian species, for example human, cow, monkey, camel, sheep, goat etc.

Within the context of the present invention, the terms “lactocytes” or “mammary-like cells” identify secretory epithelial cells expressing CK18 cell marker and derived
15 from mammalian mammary epithelial cells and in particular from human mammary epithelial cells. Human mammary epithelial cells as used herein are commercially available and may be selected from any suitable cell line. A suitable human mammary epithelial cell line in the context of the current invention is e.g., a non-tumorigenic cell line such as MCF-10, or may be a tumorigenic cell line such as MCF-
20 7.

Within the context of the present invention the terms “mammary gland like organoids” or “mammary like organoids” mean a miniaturized and simplified version of a mammary gland which develops in two or three dimensions (2D/3D)
25 and which comprises lactocytes as above defined.

Within the context of the present invention, the term “human milk like product” is a cell cultured milk product. It is an edible product which is expressed by the lactocytes and/or mammary gland like organoids generated according to the
30 process of the present invention.

The "human milk like product" according to the present invention can have the same components as human breast milk of a well-nourished mother (for example in terms of bioactives, macro and micronutrients and levels thereof). This is referred to herein as a "standard human milk product". Alternatively, "human milk like product" according to the present invention can have altered ratios and concentrations of components found naturally in human breast milk of a well-nourished mother. This is referred to herein as a "non-standard milk like product". A "human milk like product" according to the present invention can be modified such that it includes components that are not found naturally in human breast milk of a well-nourished mother (a "modified milk like product"). Non-limiting examples of human milk like products are selected from the group consisting of: supplement, fortifier, human breast milk substitute (or replacer) and ingredient enriched in only one and/or a portion of bioactives, macro- and micronutrients which can be typically found in human breast milk of a well-nourished mother.

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The "human milk like product" can be used to replace consumption of naturally lactated milk (a "human milk substitute"). The milk substitute product can be used as a supplement (a "human milk supplement") or as a fortifier (a "human milk fortifier") to be consumed in combination with naturally lactated milk.

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In an embodiment, the standard human milk like product according to the present invention comprises at least macro- and micronutrients which can be typically found in human breast milk of a well-nourished mother. In one embodiment, the human milk like product according to the present invention comprises: proteins, peptides, lipids (including linoleic acid and alpha-linolenic acid), carbohydrates, Vitamins (including Vitamin A, Vitamin D3, Vitamin E, Vitamin K, Thiamin, Riboflavin, Niacin, Vitamin B6, Vitamin B12, Pantothenic acid, folic acid, Vitamin C and Biotin), minerals (including iron, calcium, phosphorus, magnesium, sodium, chloride, potassium, manganese, iodine, selenium, copper and zinc), choline, myoinositol and L-carnitine.

In one embodiment, the human milk like product according to the present invention also comprises at least one bioactive selected from the group consisting of: growth factors, cytokines, probiotics, extracellular vesicles (e.g. milk fat globules and or exosomes), bioactives from exosome (for example miRNA) and secretory IgA. The
5 standard human milk like product according to the present invention is not the product of human breast lactation as occurring in nature.

In another embodiment, the human milk like product according to the present invention can be adapted to specific needs of the infant who will receive it. It may
10 comprise only one and/or a portion of bioactives, macro and micro nutrients which can be typically found in human breast milk of a well-nourished mother. In such embodiment, the human breast milk like product may also be referred to with the term "non-standard human milk like product". In one embodiment, the non-standard human milk like product according to the present invention comprises one
15 or more of the nutrients or bioactives selected from the group consisting of proteins, peptides, lipids (including linoleic acid and alpha-linolenic acid), carbohydrates (including human milk oligosaccharides), Vitamins (including Vitamin A, Vitamin D3, Vitamin E, Vitamin K, Thiamin, Riboflavin, Niacin, Vitamin B6, Vitamin B12, Pantothenic acid, folic acid, Vitamin C and Biotin), minerals (including iron, calcium,
20 phosphorus, magnesium, sodium, chloride, potassium, manganese, iodine, selenium, copper and zinc), choline, myoinositol, L-carnitine, growth factors, cytokines, probiotics, extracellular vesicles (e.g. milk fat globules and or exosomes), bioactives from exosome (for example miRNA) and secretory IgA.

25 Within the context of the present invention, the term "non-modified human milk like product" indicates a human milk like product which is expressed by lactocytes and/or by the mammary gland like organoids generated according to steps A) and B) of the process of the present invention and which is not subject to the further treatment according to optional step C) of the process of present invention. Non-

modified human milk like product may comprise both standard and non-standard human milk like products. Non limiting examples of non-standard human milk like products are selected from the group consisting of: supplement, fortifier, and ingredient enriched in only one and/or a portion of bioactives, macro and micro nutrients which can be typically found in human breast milk of a well-nourished mother.

Within the context of the present invention, the term “modified human milk like product” indicates a human milk like product which is expressed by lactocytes and/or by the mammary gland like organoids generated according to steps A) and B) of the process of the present invention and which is subject to the further treatment according to optional step C) of the process of present invention.

Modified human milk like products may comprise both standard and non-standard human milk like products.

Within the context of the present invention the term “EBs” means “embryoid bodies”.

Within the context of the present invention the term “mEBs” means “MammoCult medium-cultured embryoid bodies”.

MammoCult medium refers to a serum-free culture medium comprising basal medium, at least one proliferation supplement, heparin and hydrocortisone.

Within the context of the present invention the terms “embryoid bodies (EBs)”, “MammoCult medium-cultured embryoid bodies (mEBs)”, “mammospheres” and/or “spheroids” refer to three-dimensional aggregates formed in suspension under step A) of the process of the present invention.

The term “infant” in the context of the present invention identifies a child under the age of 12 months, such as under the age of 9 months, particularly under the age of 6 months.

- 5 In the context of the present invention the infant may be any term infant or preterm infant. In an embodiment of the invention, the infant is selected from the group of preterm infants and term infants.

The term “term infant” refers to infants born at term or at a gestational age of 37
10 weeks or more.

The term “preterm infant” refers to infants who are born at a gestational age of less than 37 weeks.

- 15 In the context of the present invention, the term “birth weight” means the first weight of the fetus or newborn obtained after birth.

Within the context of the present invention, the term “low birth weight” means a birth weight of less than 2500 g (up to and including 2499 g).
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Within the context of the present invention, the term “very low birth weight” means a birth weight of less than 1500 g (up to and including 1499 g).

Within the context of the present invention, the term “extremely low birth weight”
25 means a birth weight of less than 1000 g (up to and including 999 g).

The term “small for gestational age infant” refers to infants having a birth weight that is more than 2 standard deviations below the mean reference to a birth weight for gestational growth chart or having a birth weight that is less than the 10th
30 percentile of population-based weight data obtained from infants at the same

gestational age. The term “small for gestational age infants” includes infants who are small at birth either from a constitutive or genetic origin or, as a consequence of intrauterine growth restriction.

- 5 Within the context of the present invention, the term “young children” or “toddler” indicates a child between the age of 1 and 3 years.

The term “child” as used herein refers to a person greater than three years of age up to twelve years of age.

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The term “adult” as used herein refers to a person greater than twelve years of age.

- The term “infant formula” as used herein refers to a nutritional composition intended for infants and as defined in *Codex Alimentarius*, (Codex STAN 72-1981) and Infant Specialities (incl. Food for Special Medical Purpose) as defined in *Codex Alimentarius*, (Codex STAN 72-1981). It also refers to a foodstuff intended for particular nutritional use by infants during the first months of life and satisfying by itself the nutritional requirements of this category of person (Article 2(c) of the European Commission Directive 91/321/EEC 2006/141/EC of 22 December 2006 on infant formulae and follow-on formulae). The infant formulas encompass the starter infant formulas and the follow-up or follow-on formulas. Generally, a starter formula is for infants from birth as breast-milk substitute, and a follow-up or follow-on formula from the 6th month onwards.
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- 25 The “growing-up milks” (or GUMs) are given from one year onwards. It is generally a milk-based beverage adapted for the specific nutritional needs of young children. They are nutritional compositions used for feeding children from 12 months to 2-3 years old in combination with other foods.

Within the context of the present invention, the term “fortifier” refers to a composition which comprises one or more nutrients having a nutritional benefit for infants or young children.

- 5 By the term “milk fortifier”, it is meant any composition used to fortify or supplement either human breast milk, infant formula, growing-up milk or human breast milk fortified with other nutrients. Accordingly, the human milk fortifier of the present invention can be administered after dissolution in human breast milk, infant formula, growing-up milk or human breast milk fortified with other nutrients
10 or otherwise it can be administered as a standalone composition.

When administered as a stand-alone composition, the human milk fortifier of the present invention can be also identified as being a “supplement”. In one embodiment, the milk fortifier of the present invention is a supplement.

- 15 By the term “human milk fortifier”, it is meant any composition used to fortify or supplement human breast milk, or human breast milk fortified with other nutrients. The “human milk fortifier” according to the present invention may be intended to be administered to infants who were born preterm, with very low birth weight
20 (VLBW) or with extremely low birth weight (ELBW).

The milk fortifier according to the present invention may be in powder or liquid form.

- 25 Milk fortifier compositions having a liquid form presents some particular benefits. For example, liquid formulations might be more convenient if coupled with a packaging that delivers calibrated drops of a certain weight or volume.

- In addition, liquid formulations are easier to mix with the compositions to be
30 fortified, whereas the powder ones can, in some cases, form lumps.

Within the context of the present invention, the term "nutritional composition" means a composition which nourishes a subject. This nutritional composition is usually to be taken orally or intravenously. It may include a lipid or fat source, a carbohydrate source and/or a protein source. In a particular embodiment the
5 nutritional composition is a ready-to-drink composition such as a ready-to-drink formula.

Reference herein to EpiCult or EpiCultB medium refers to a serum free culture medium comprising hydrocortisone, insulin, FGF10 and HGF.

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A culture medium as disclosed anywhere herein refers to a solid, semi-solid or liquid comprising essential nutrients, designed to support the growth and differentiation of microorganisms. MammoCult medium is one example of a culture medium that may be used in the present invention.

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Methods and Uses

The present invention relates to methods of producing mammary gland cells using mammalian epithelial cells that are cultured in specific conditions described below
20 and using said mammary gland cells for producing a mammalian milk like product in vitro, wherein exosomes are purified from the mammalian milk like product in order to isolate the exosomes.

It has been surprisingly demonstrated in the present invention that mammary
25 epithelial cells can be used as the starting material in a protocol for producing a mammalian milk like product. Using mammary epithelial cells as a starting material means that there is no need for a complicated differentiation protocol to first produce the mammary epithelial cells from e.g., early stage undifferentiated stem cells. This reduces the overall time of the method for producing a mammalian milk
30 like product compared to stem cell protocols, and has cost saving benefits.

Thus, the present invention relates to a method for producing a mammalian milk like product, comprising:

- A) Culturing mammary epithelial cells in a culture medium to generate lactocyte mammary-like gland organoids; and
- 5 B) Secreting the mammalian milk like product from said lactocytes.

Mammalian milk like product production

The present invention relates to methods for producing a mammalian milk like product as defined herein, including any of steps A) and B) as defined herein and
10 optional step C) as defined herein.

Step A - Generating lactocytes and/or mammary like organoids

15 According to the method of the present invention, mammary like cells and/or organoid structures are generated under step A).

This includes the proliferation and maturation the mammary epithelial cells to produce mammary like organoids, such as lactocytes.

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In the methods disclosed herein, mammary epithelial cells are used as the starting material, i.e., culturing of the epithelial cells is the first step of the method.

The proliferation and maturation of mammary epithelial cells occurs by culturing the
25 mammary epithelial cells in specific culture medium, for example complete MammoCult medium (StemCell Technologies). Complete MammoCult medium is preferably composed of the basal medium, proliferation supplements, heparin (typically 4µg/mL), and hydrocortisone (typically 0.48µg/mL). Medium is usually changed every three days. mEBs (mammospheres) obtained in said step are then
30 enriched for non-neural ectoderm cells.

In some embodiments, the proliferation and maturation stage is between day 0 and day 7, where day 0 is the time point where the mammary epithelial cells are first added to the culture medium. In some embodiments, the proliferation and maturation stage is for 7 days. In some embodiments, the proliferation and
5 maturation stage is for no longer than 7 days.

After the proliferation and maturation stage, the cells are induced to express milk proteins. In some embodiments, said induction period is between day 7 and day 14. In some embodiments, the induction period is for 7 days. In some embodiments, the
10 induction period is for no longer than 7 days.

In one embodiment of the present invention, a method for producing a human milk like product is provided comprising generating lactocytes under step A) from human mammary epithelial cells, where such step A) comprises:
15 i) culturing human mammary epithelial cells in an appropriate culture medium (for example MammoCult medium), and after 7 days generating lactocytes.

In another embodiment, a method for producing a human milk like product is provided comprising generating lactocytes under step A) from human mammary
20 epithelial cells, where such step A) comprises:
i) culturing human mammary epithelial cells in an appropriate culture medium (for example MammoCult medium), in non-adherent conditions for at least 7 days to generate lactocytes.

25 In one embodiment, the method according to the present invention provides for culture conditions according to step A), which are adapted to generate lactocytes derived from human mammary epithelial cell capable to secrete a human milk like product.

In a preferred embodiment, a method for producing a human milk substitute product is provided comprising generating lactocytes under step A) from human mammary epithelial cells, where such step A) comprises proliferating and maturing mammary epithelial cells to differentiate towards mammary gland cells (for example
5 lactocytes) in an appropriate 3D culture system as described anywhere herein (for example 3D-suspension condition). In some embodiments, for at least 7 days. In some embodiments, for 7 days or less.

In another preferred embodiment, a method for producing a human milk like
10 product is provided comprising generating lactocytes under step A) from human mammary epithelial cells, where such step A) comprises:

i) culturing mammary epithelial cells in an appropriate culture medium (for example MammoCult medium), in an appropriate 3D culture system (for example 3D-suspension condition) for at least 7 days (day 0 to day 7), , to generate lactocytes.

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In a particularly preferred embodiment of the present invention, a method of producing a human milk like product is provided comprising generating lactocytes under step A) from human mammary epithelial cells, wherein:

i) culturing mammary epithelial cells in complete MammoCult medium (StemCell
20 Technologies) comprising the basal medium, proliferation supplement and supplemented with heparin (typically 4µg/mL), hydrocortisone (typically 0.48µg/mL), for 7 days (day 0-day 7), and

ii) induction of milk protein expression by incubating the cells in EpiCultB medium supplemented with EpiCult proliferation supplement, hydrocortisone, insulin, FBS,
25 prolactin, progesterone and β-estradiol for 7 days (day 7-day 14).

Step ii) preferably leads to differentiation into milk protein expressing cells, particularly lactocytes, and/or mammary like gland organoids.

In a further particularly preferred embodiment of the present invention, a method of producing a human milk like product is provided comprising generating lactocytes under step A) from human mammary epithelial cells, wherein:

- i) culturing mammary epithelial cells in MammoCultB medium supplemented with MammoCult proliferation supplement, hydrocortisone, heparin as described anywhere herein, for 7 days (day 0-day 7), and
- ii) induction of milk protein expression by incubating the cells in EpiCultB medium supplemented with EpiCult proliferation supplement, hydrocortisone, insulin, FBS, prolactin, progesterone and β -estradiol for 7 days (day 7 to day 14).

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Step ii) preferably leads to differentiation into milk protein expressing cells, particularly lactocytes, and/or mammary like gland organoids.

In one embodiment, steps ii) as defined above for the particularly preferred embodiments, preferably lead to formation of/differentiation into at least breast cells, luminal cells, and basal cells. In this context, breast cells preferably express one or more, preferably all of markers selected from the group consisting of: β -Casein, milk protein, and hormone receptors. Moreover, luminal cells preferably express one or more, preferably all markers selected from the group consisting of: EpCAM, MUC1, CD49F, GATA3, CK8, and CK18. Moreover, basal cells preferably express one or more markers selected from the group consisting of: CK14, α -smooth muscle actin and P63.

In one further embodiment, after step ii) as defined above for the particularly preferred embodiments, mammary like gland organoids may be obtained, that express one or more markers selected from the group consisting of: β -Casein, milk protein, and hormone receptors, luminal cells that express one or more markers selected from the group consisting of: EpCAM, MUC1, CD49F, GATA3, CK8, CK18,

and basal cells that express one or more markers selected from the group consisting of: CK14, α -smooth muscle actin and P63.

In one embodiment of the invention, the methods above described are provided for
5 producing a human milk like product.

In one embodiment (of step A), delivery of nutrients and biomimetic stimuli is controlled to influence cell growth, differentiation and tissue formation. In one embodiment (of step A), such control is performed in a bioreactor.

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In some embodiments, the mammary like gland organoids or lactocytes derived from Step A have high expression of mammary gland specific markers. In some embodiments, the mammary like gland organoids or lactocytes derived from Step A express Keratin 18 (KRT-18). In some embodiments, the mammary like gland
15 organoids or lactocytes derived from Step A express estrogen receptor (ER). In some embodiments, mammary like gland organoids or lactocytes derived from Step A express more than 59% of KRT-18 and ER, as estrogen-receptor-positive luminal mammary gland population.

20 In some embodiments, the mammary like gland organoids or lactocytes derived from Step A have high expression of key milk bioactives. In some embodiments, the mammary like gland organoids or lactocytes derived from Step A have increased expression of key milk bioactives. In some embodiments, the mammary like gland organoids or lactocytes derived from Step A have increased expression of key milk
25 bioactives post-induction (for example at day 14) compared to pre-induction (for example at day 7). In some embodiments, the mammary like gland organoids or lactocytes derived from Step A have increased mRNA expression of lactoferrin (LTF) post-induction compared to pre-induction. In some embodiments, the mammary like gland organoids or lactocytes derived from Step A have increased mRNA

expression of milk fat globule-EGF factor 8 (MFGE8) post-induction compared to pre-induction.

It will be understood that any method or method step disclosed herein may be
5 conducted in 3D suspension culture rather than using a membrane matrix as a support. Thus, in some embodiments, during all the differentiation procedure, cells are maintained in suspension culture.

Step B - Expressing a human breast milk like product

10 In one embodiment of the present invention, the methods comprise expressing the human milk like product from mammary like organoids derived from human mammary epithelial cells, preferably prepared according to step A). Expressing human milk like products preferably occurs upon induction of expression of the
15 human milk like product from such lactocytes and/or mammary-like gland organoids.

In one embodiment, lactating lactocytes are induced by applying a specific medium (for example EpiCultB) supplemented with lactogenic factors (for example prolactin,
20 hydrocortisone, and insulin).

Particularly, the human milk like product obtained from mammary like organoids derived from human mammary epithelial cells, preferably prepared according to step A), contains bioactives of human milk, selected from the group comprising or
25 consisting of proteins, lipids or oligosaccharides, preferably human milk oligosaccharides, etc.. Inventors particularly managed to identify with the particularly preferred protocol according to steps A i) to iv) as carried out above inter alia oligosaccharides (including lactose and some HMOs), lipids (including 4 fatty acids), proteins (7 detected including caseins), and miRNA (75 detected, including
30 11 typically detected in HBM).

In one embodiment, the human milk like product obtained from mammary like organoids derived from human mammary epithelial cells, preferably prepared according to step A), contains bioactives of human milk, selected from the group comprising or consisting of: oligosaccharides, lipids, proteins, exosomes and miRNA.

- 5 In one embodiment, the human milk like product obtained from mammary like organoids derived from human induced pluripotent stem cells (hiPSCs), preferably prepared according to step A), contains exosomes.

- In another embodiment, human milk like product obtained from mammary like organoids derived from human mammary epithelial cells, preferably prepared according to step A), contains bioactives of human milk, selected from the group comprising or consisting of: lactose, 6'SL, C-4:0 fatty acid, C-8:0 fatty acid, C-10:0 fatty acid, C-14:0 fatty acid, C-15:0 fatty acid, C-16:0 fatty acid, C-16:1n7 fatty acid, C-17:0 fatty acid, C-18:0 fatty acid, C-18:1 n9 fatty acid, C-18:1 fatty acid, C-18:2 n6 fatty acid, C-20:0 fatty acid, C-20:1 n9 fatty acid, C-18:3 n3 fatty acid, C-22:0 fatty acid, lactoferrin, albumin, prolactin, Alpha S1-casein, Hemoglobin subunit beta, Hemoglobin subunit alpha, α -lactalbumin, Alpha-2-macroglobulin, β -casein, bile salt-activated lipase, κ -casein, lactadherin, CD14, fatty acid synthase, IgA, plgR, Serum albumin, Xanthine dehydrogenase, exosomes, miR-21-5p, miR-181a-5p, miR-30d-5p, miR-30b-5p, miR-22-3p, miR-146b-3p, miR-30c-5p, miR-30a-5p, miR-30e-5p and miR-148b-3p.
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- In one embodiment of the present invention, the human milk like product obtained from mammary like organoids derived human mammary epithelial cells is a standard human milk product. In another embodiment of the present invention, the human milk like product obtained from mammary like organoids derived from human mammary epithelial cells, is a non-standard human milk product.
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Step C - Further treatments to produce modified human breast milk like product

In one optional embodiment of the present invention, the herein-described methods comprise an additional step C) which is performed on the human milk like
5 product obtainable from step B) and which comprises performing an additional treatment on such product to provide a modified human milk like product.

In one particular embodiment, the additional treatment step C) performed on the inventive human breast milk like product may be selected from the group consisting
10 of: a purification step, an isolation process, an extraction process, a fractionation step, an enrichment process, an enzymatic treatment, the addition of further components (for example which can't be expressed by the human mammary gland organoid (such as for example Immunoglobulins, probiotic and/or minerals) or combinations thereof.

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In particular, in some embodiments, the mammalian milk like product described anywhere herein is extracted, isolated and purified as part of the methods described anywhere herein. Thus, the invention provides an isolated mammalian milk like product, for example an isolated human milk like product. In some embodiments,
20 the mammalian milk like product described anywhere herein is extracted, isolated and purified as part of the methods described anywhere herein. Thus, the invention provides an isolated mammalian milk like product, for example an isolated human milk like product.

25 In some embodiments, the isolated mammalian milk like product is formulated into a composition. In some embodiments, the isolated mammalian milk like product is formulated into a nutritional composition. In some embodiments, the isolated human milk like product is formulated into a pharmaceutical composition.

In some embodiments, the isolated mammalian milk like product is formulated into a composition. In some embodiments, the isolated mammalian milk like product is formulated into a nutritional composition. In some embodiments, the isolated mammalian milk like product is formulated into a pharmaceutical composition.

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In some embodiments, particular bioactives of the mammalian milk like product, selected from the group comprising or consisting of: oligosaccharides, lipids, proteins, exosomes and miRNA, are extracted, isolated and purified from the mammalian milk like product. In particular, exosomes are extracted, isolated and purified from the mammalian milk like product. Thus, the invention provides isolated bioactives of the mammalian milk like product, selected from the group comprising or consisting of: oligosaccharides, lipids, proteins, exosomes and miRNA. In particular, the invention provides isolated exosomes extracted, isolated and purified from the mammalian milk like product, for example isolated human exosomes extracted, isolated and purified from the human milk like product.

In some embodiments, the isolated bioactives of the mammalian milk like product are formulated into a composition. In some embodiments, the isolated bioactives of the mammalian milk like product are formulated into a nutritional composition. In some embodiments, the isolated bioactives of the human milk like product are formulated into a pharmaceutical composition.

In some embodiments, the isolated bioactives of the mammalian milk like product are formulated into a composition. In some embodiments, the isolated bioactives of the mammalian milk like product are formulated into a nutritional composition. In some embodiments, the isolated bioactives of the mammalian milk like product are formulated into a pharmaceutical composition.

In some embodiments, the isolated exosomes of the mammalian milk like product are formulated into a composition. In some embodiments, the isolated exosomes of the mammalian milk like product are formulated into a nutritional composition. In some embodiments, the isolated exosomes of the human milk like product are
5 formulated into a pharmaceutical composition.

In some embodiments, the isolated exosomes of the mammalian milk like product are formulated into a composition. In some embodiments, the isolated exosomes of the mammalian milk like product are formulated into a nutritional composition. In
10 some embodiments, the isolated exosomes of the mammalian milk like product are formulated into a pharmaceutical composition.

In some embodiments, step C) comprises purifying exosomes from the human milk like product. In some embodiments, step C) comprises purifying exosomes from the
15 human milk like product to remove impurities, for example by chromatography or filtration or ultracentrifugation, in order to isolate the exosomes. In some embodiments, step C) comprises purifying exosomes from the human milk like product by chromatography in order to isolate the exosomes. In some
20 embodiments, step C) comprises purifying exosomes from the human milk like product by filtration in order to isolate the exosomes. In some embodiments, step C) comprises purifying exosomes from the human milk like product by ultracentrifugation in order to isolate the exosomes.

In some embodiments, the chromatography is column-based chromatography, for
25 example size inclusion or size exclusion chromatography.

It will be understood that a product obtained from a purified compared to a non-purified protocol will be different in terms of its composition. In particular, the surface composition of the product will be different as components such as proteins

that are attached to the surface of a product molecule may be removed during the purification steps. For example, proteins present on the surface of the exosomes may be removed.

- 5 In some embodiments, step C) further comprises sterilising the isolated exosomes.

In some embodiments, step C) further comprises storing the isolated exosomes at below freezing, optionally at -80°C.

- 10 In some embodiments, step C) comprises formulating the isolated exosomes into a powder form, for example by freeze drying or spray drying, or a liquid form.

In some embodiments, the method further comprises formulating the isolated exosomes with supplementary nutritional ingredients.

15

In some embodiments, the method further comprises dispensing the isolated exosomes into a container for consumption.

In some embodiments, the isolated exosomes express a tetraspanin protein. In

- 20 some embodiments, the isolated exosomes express CD9, CD63 and/or CD81.

Human milk like products

'Standard' human breast milk like product

25

In one embodiment of the present invention, the human breast milk like product is a 'standard' human breast milk like product, i.e., comprises the same components as human breast milk of a well-nourished mother.

The benefits of breast feeding are well known in the scientific literature and the possibility to have access to human breast milk like product allows its use for a number of equally well-known health benefits.

- 5 In such an embodiment, the human breast milk like product can be used as a substitute of breastfeeding under circumstances where real breastfeeding is not possible.

In such embodiment, the human breast milk like product is intended to be used for
10 example to support longer breastfeeding experience for women who have less milk or who stop to produce milk after 6 months from birth.

Similarly, the human breast milk like product is intended to be used for example to allow breastfeeding even under circumstances where sicknesses compromise real
15 breastfeeding from the mother.

In another embodiment, the human breast milk like product is intended to be used under circumstances whereby breastmilk production would not naturally be initiated, for example if an infant is adopted.
20

In one embodiment, the human milk like product according to the present invention is not the product of human breast milk lactation as occurring in nature.

In one embodiment, the human breast milk like product is for use in providing
25 optimal nutrition for infant.

In one embodiment, the human breast milk like product is for use in providing healthy growth in infants.

In one embodiment, the human breast milk like product is for use in preventing infection, obesity and promoting immunity development in infants.

In one embodiment, the human breast milk like product is a non-modified human
5 breast milk like product.

In another embodiment, the human breast milk like product is a modified human breast milk like product.

10 In one embodiment, the human milk like product according to the present invention comprises: proteins, lipids, carbohydrates, vitamins and minerals.

In another embodiment, the human milk like product according to the present invention comprises: proteins, lipids, carbohydrates, vitamins, minerals and
15 bioactives.

In one embodiment, the human milk like product according to the present invention comprises: proteins, lipids (including linoleic acid and alpha-linolenic acid), carbohydrates, Vitamins (including Vitamin A, Vitamin D3, Vitamin E, Vitamin K,
20 Thiamin, Riboflavin, Niacin, Vitamin B6, Vitamin B12, Pantothenic acid, folic acid, Vitamin C and Biotin), minerals (including iron, calcium, phosphorus, magnesium, sodium, chloride, potassium, manganese, iodine, selenium, copper and zinc), choline, myoinositol and L-carnitine.

25 In a further embodiment, the human milk like product according to the present invention also comprises at least one bioactive selected in the group consisting of: growth factors, cytokines, probiotics, extracellular vesicles (e.g. milk fat globules and or exosomes), bioactives from exosomes (for example miRNA) and secretory IgA.

Such human breast milk like product may be prepared according to the method of the present invention for example by including a step C) of addition of growth factors, cytokines, probiotics, extracellular vesicles (e.g. milk fat globules and or exosomes), bioactives from exosomes (for example miRNA) and secretory IgA.

5

In one embodiment, the human breast milk like product contains probiotics.

Such human breast milk like product may be prepared according to the method of the present invention for example by including a step C) of addition of probiotics
10 (for example *B.Lactis*, *B.Infantis*, *L. Ramnhosus*) which can be obtained from several commercially available sources.

In such embodiment, the human breast milk like product may be used for optimizing gastro intestinal function and/or promoting Immunity.

15

In one embodiment, the human breast milk like product contains secretory IgA and probiotics.

Such human breast milk like product may be prepared according to the method of
20 the present invention for example by including a step C) of addition of a combination of probiotics and secretory IgA which may be prepared as described for example in patent applications WO2009/156301 and WO2009/156367 which are hereby incorporated by reference. In such embodiment, the human breast milk like product may be used for preventing Immunoglobulin deficiency and/or in the prevention of
25 recurrent infection in infants and young children.

'Non-standard' human breast milk like product

In one embodiment of the present invention, the human milk like product can have altered ratios and concentrations of components found naturally in human breast milk of a well-nourished mother. This is referred to herein as a “non-standard milk like product”.

5

In one embodiment, the human milk like product according to the present invention may be selected from the group consisting of a milk fortifier, a supplement, and/or a human breast milk replacer adapted for special purposes.

10 Human Milk fortifiers and Human milk bioactive supplements

In one embodiment, the method of the present invention provides for a human breast milk like product which may be used to fortify human breast milk naturally obtained from a nursing mother or to fortify infant formulas.

15

In another embodiment, the method of the present invention provides for a human breast milk like product which may be used as a supplement for infants or young children in need thereof.

20 In such embodiments the human breast milk like products may be used for providing healthy growth and/or to reduce the risk of developing a disease typically associated to specific conditions in an infant or young child (such as for example asthma, allergy, cognitive alterations) and /or to promote catch up growth, development of immunity, protection from infections.

25

Remarkably, the human origin of the constituents (especially bioactive constituents) in such fortifiers or supplements combined with the fact that they are according to the method of the invention, is supposed to provide to such constituents an intact or higher functionality.

The human breast milk like product is preferably intended to be used as a fortifier. Such human breast milk like product intended to be used as a fortifier and may be prepared according to the method of the present invention for example by including a step C) of isolation and/or enrichment of (certain) bioactives from the human breast milk like product obtainable from step B). Such isolation step may be performed via classical fractionation, enrichment and/or purification of the non-modified human breast milk like product obtainable from step B).

The human breast milk like product intended to be used as a supplement may comprise one or more bioactives selected from the group consisting of: human milk oligosaccharides (for example 2FL, 3FL, LNT, LnNT, DiFI, 6SL and /or 3SL), lipids, growth factors (for example epidermal growth factor (EGF), heparin binding epidermal growth factor), cytokines (for example transforming growth factor -beta 2 (TGFbeta-2), IL-1, IL-2, IL-6, IL-10, IL-18, interferon gamma (INF-gamma), TNF-alpha), extracellular vesicles (e.g. milk fat globules and or exosomes), exosome comprising microRNAs and antimicrobial/protecting bioactives (for example IgA, lactoferrin, lysozyme, lactadherine). Such human breast milk like product intended to be used as a supplement may be prepared according to the method of the present invention for example by including a step C) of isolation of the bioactives from the non-modified human breast milk like product obtainable from step B). Such isolation step may be performed via classical fractionation, enrichment and/or purification of the non -modified human breast milk like product obtainable from step B).

In one embodiment, the human breast milk like product is a supplement or milk fortifier which contains fucosylated human milk oligosaccharides, for example 2FL and/or 3FL. Such supplement or milk fortifier is for use in completing the profile of human breast milk of women who do not secrete fucosylated oligosaccharides because of the inactivity of their FUT2 gene.

Such human breast milk like product intended to be used as a fortifier or supplement may be prepared according to the method of the present invention for example by including a step C) of isolation and/or enrichment of fucosylated oligosaccharides (for example 2FL and or 3FL) from the non-modified human breast milk like product
5 obtainable from step B).

In such an embodiment, the human breast milk like product may be used for optimizing gastro intestinal function and/or promoting Immunity.

10 Human breast milk like product for infants with genetic diseases

In one embodiment, the human breast milk like product according to the present invention may be adapted to address the specific need of infants who are born with a genetic disease.

15

Galactossemia

In such embodiment, the human breast milk like product may be adapted to the needs on infants suffering from Galactossemia. Galactossemia is a rare genetic
20 disease that affects babies' ability to metabolize galactose.

In such embodiment, the human breast milk like product should be deprived of lactose and/or lactose containing saccharides. In such embodiment human breast milk like product may be used for providing healthy growth to the infants affected
25 by galactossemia.

In one embodiment, a human breast milk like product deprived of lactose and/or lactose containing saccharides may be obtained according to the method of the present invention by including a step C) of enzymatic treatment (lactase treatment),

or of membrane fractionation and ultrafiltration of the non-modified human breast milk like product obtainable from step B).

Phenyl Keturonia

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In such an embodiment, the human breast milk like product may be adapted to the needs on infants suffering from Phenyl Keturonia (PKU). PKU is due to absent or dysfunctional phenylalanine hydroxylase, which converts phenylalanine to tyrosine. Untreated, it leads to severe mental retardation due to brain toxicity.

10

In such an embodiment, the human breast milk like product should be deprived or depleted of phenylalanine.

15

In such an embodiment, the human breast milk like product may be used for providing healthy growth to the infants affected by PKU.

20

In one embodiment, the human breast milk like product is depleted of phenylalanine in such a way that phenylalanine content is kept below 20 mg/kg body weight of the subject receiving it.

25

In one embodiment, a human breast milk like product depleted or deprived of phenylalanine may be obtained according to the method of the present invention by including a step C) of enzymatic treatment (protein hydrolysis) or of filtration of the non -modified human breast milk like product obtainable from step B).

In one embodiment, a human breast milk like product depleted of phenylalanine may be obtained according to the method of the present invention by including a step C) of enzymatic treatment (protein hydrolysis) or of filtration of the non -modified human breast milk like product obtainable from step B).

In another embodiment, a human breast milk like product depleted of phenylalanine may be obtained according to the method of the present invention by providing in step B) a culture medium providing limited or zero amounts of phenylalanine, such as for example a culture medium containing
5 Glycomacropeptide (GMP) from whey.

Components of human milk like products

As previously described herein, the human milk like product according to the
10 present invention comprises at least one bioactive. One such bioactive is exosomes.

Exosomes

Human breast milk exosomes are small microvesicles involved in infant
15 development, for example in immunity, metabolism and development. They are thought to play an important role in therapy and disease prevention.

The exosomes comprise a lipid bilayer membrane that encapsulates a combination of molecules including proteins, lipids, messengerRNA (mRNA) and microRNA
20 (miRNA). The types and concentrations of these molecules determine the exosome's fingerprint and function.

The present invention provides isolated human breast milk exosomes. The exosomes of present invention possess the same lipid bilayer vesicle structure and
25 function to that of a naturally derived human breast milk exosome.

The exosomes of the invention are not produced from naturally derived human breast milk.

In some embodiments, the present invention provides exosomes that have been isolated from the human milk like product described anywhere herein, wherein the human milk like product is produced from any method described herein.

- 5 Thus, for example, the present invention relates to an *in vitro* method for producing exosomes, the method comprising:

comprising:

- A) Culturing mammary epithelial cells in a culture medium to generate lactocyte mammary-like gland organoids; and
 - 10 B) Secreting a mammalian milk like product from said lactocytes, and
 - C) Purifying the exosomes from the mammalian milk like product to remove impurities, optionally by chromatography or filtration or ultracentrifugation, in order to isolate the exosomes,
- optionally wherein step A) is conducted in 3D-suspension conditions.

15

The exosomes of the present invention differ from exosomes derived from natural human breast milk by their composition.

- In some embodiments, the exosomes of the present invention differ from exosomes
- 20 derived from natural human breast milk by their proteome profile. It will be understood that a proteome profile relates to the types of proteins present in the exosomes and/or the concentration of the proteins present in the exosomes.

- In some embodiments, the exosomes of the present invention are substantially the
- 25 same as exosomes derived from natural human breast milk by one or more characteristics selected from: size, miRNA profile and/or lipid profile. In some embodiments, the size, miRNA profile and lipid profile of the exosomes of the present invention are substantially the same as exosomes derived from natural human breast milk.

In some embodiments, differences in the proteome profile is the only difference between exosome of the present invention and exosomes derived from natural human breast milk.

- 5 The present invention also relates to a population of exosomes as described anywhere herein. In some embodiments, the exosomes in said population are identical or different in terms of their structure, function and/or composition. In some embodiments, the exosomes in said population are identical. In some embodiments, the exosomes in said population differ by their composition.

10

In some embodiments, the exosome or population of exosomes as described herein are formulated in a powder form, for example by freeze drying or spray drying, or a liquid form. In some embodiments, the exosome or population of exosomes as described herein are frozen, optionally at -80°C. In some embodiments, the
15 exosome or population of exosomes as described herein are sterilised.

In some embodiments, the exosome or population of exosomes as described herein are packaged into a container for consumption.

- 20 In some embodiments, the present invention relates to a composition comprising the exosomes or population of exosomes as described anywhere herein. In some embodiments, the present invention relates to a nutritional composition comprising the exosomes or population of exosomes as described anywhere herein.

- 25 In some embodiments, the present invention provides a composition comprising the exosome or population of exosomes as described anywhere herein. In some embodiments, the composition is a nutritional composition. In some embodiments, the composition is a pharmaceutical composition.

In some embodiments, the composition comprises supplementary nutritional ingredients.

In some embodiments, the present invention provides a pharmaceutical
5 composition comprising the exosome or population of exosomes as described
anywhere herein and a pharmaceutically acceptable excipient.

The term "pharmaceutically acceptable" as used herein means approved by a
regulatory agency of the Federal or a state government, or listed in the U.S.
10 Pharmacopeia, European Pharmacopeia or other generally recognized
pharmacopeia for use in animals, and more particularly in humans.

In some embodiments, the composition as described anywhere herein (e.g. the
nutritional or pharmaceutical composition) comprises exosomes that have been
15 isolated from the human milk like product described anywhere herein, wherein the
human milk like product is produced from any method described herein.

Thus, for example, the present invention relates to a composition (e.g. a nutritional
or pharmaceutical composition) comprising exosomes, wherein the exosomes are
20 produced according to an *in vitro* method comprising:
comprising:

- A) Culturing mammary epithelial cells in a culture medium to generate
lactocyte mammary-like gland organoids; and
- B) Secreting a mammalian milk like product from said lactocytes, and
- 25 C) Purifying the exosomes from the mammalian milk like product to
remove impurities, optionally by chromatography or filtration or
ultracentrifugation, in order to isolate the exosomes,
Optionally wherein step A) is conducted in 3D-suspension conditions.

In some embodiments, the composition as described anywhere herein is formulated into a powder form, for example by freeze drying or spray drying, or a liquid form. In some embodiments, the composition as described anywhere herein is frozen, optionally at -80°C.

5

In some embodiments, the composition as described anywhere herein is packaged into a container for consumption.

Exosome proteomics

10

Through proteomics analysis, about 2000 proteins have been identified in human breast milk exosomes in the literature.

The inventors of the present invention carried out proteomics analysis of the exosomes of the present invention and surprisingly demonstrated that the exosomes possess similar but not identical proteomes to those of exosomes derived from natural human breast milk.

In particular, exosomes of the present invention were found to be missing one or more proteins that are present in exosomes derived from natural human breast. In some embodiments, these proteins include but are not limited to Hypoxia up-regulated protein 1 (HYOU1), Apolipoprotein A-IV (APOA4) and Procollagen-lysine,2-oxoglutarate 5-dioxygenase 1 (PLOD1). In some embodiments, these proteins include but are not limited to C4a anaphylatoxin (CFA), Hypoxia up-regulated protein 1 (HYOU1), Apolipoprotein A-IV (APOA4), Procollagen-lysine,2-oxoglutarate 5-dioxygenase 1 (PLOD1), Threonine—tRNA ligase 1, cytoplasmic (TARS1), and Cytosolic non-specific dipeptidase (CNDP2).

In some embodiments, Hypoxia up-regulated protein 1 (HYOU1), Apolipoprotein A-IV (APOA4) and Procollagen-lysine,2-oxoglutarate 5-dioxygenase 1 (PLOD1) are not

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detected by liquid chromatography – mass spectrometry in an isolated human breast milk exosome of the invention. In some embodiments, Hypoxia up-regulated protein 1 (HYOU1), Apolipoprotein A-IV (APOA4) and Procollagen-lysine,2-oxoglutarate 5-dioxygenase 1 (PLOD1) are detected by liquid chromatography –
5 mass spectrometry conducted under the same conditions in an exosome isolated from human breast milk.

In some embodiments, C4a anaphylatoxin (CFA), Hypoxia up-regulated protein 1 (HYOU1), Apolipoprotein A-IV (APOA4), Procollagen-lysine,2-oxoglutarate 5-
10 dioxygenase 1 (PLOD1), Threonine—tRNA ligase 1, cytoplasmic (TARS1), and Cytosolic non-specific dipeptidase (CNDP2) are not detected by liquid chromatography – mass spectrometry in an isolated human breast milk exosome of the invention. In some embodiments, C4a anaphylatoxin (CFA), Hypoxia up-regulated protein 1 (HYOU1), Apolipoprotein A-IV (APOA4), Procollagen-lysine,2-
15 oxoglutarate 5-dioxygenase 1 (PLOD1), Threonine—tRNA ligase 1, cytoplasmic (TARS1), and Cytosolic non-specific dipeptidase (CNDP2) are detected by liquid chromatography – mass spectrometry conducted under the same conditions in an exosome isolated from human breast milk.

20 In the present invention, detection of the exosome protein profile was assayed by liquid chromatography – mass spectrometry (LC-MS). The sensitivity of LC/MS analysis is at the femtomole (fmol) level.

Exemplary, but not limiting, suitable mass spectrometers include Orbitrap Fusion™
25 Lumos™ Tribrid™. Exemplary, but not limiting, suitable HPLC systems include Vanquish™ Neo UHPLC System, 300 nL/min, and exemplary, but not limiting, suitable HPLC column include Acclaim™ PepMap™ RSLC C18 2µm 50 cm×75 µm. Overall HPLC gradient time is, for example, about 100 min to about 200 min,

preferably about 180 min. In some embodiments, the overall HPLC gradient time is 180 min.

- 5 In another embodiment, the mass spectrometer is Orbitrap Fusion™ Lumos™ Tribrid™, the HPLC system is Vanquish™ Neo UHPLC System, 300 nL/min, and the HPLC column is Acclaim™ PepMap™ RSLC C18 2µm 50 cm×75 µm. In some embodiments, the mass spectrometer is Orbitrap Fusion™ Lumos™ Tribrid™, the HPLC system is Vanquish™ Neo UHPLC System, 300 nL/min, and the HPLC column is
- 10 Acclaim™ PepMap™ RSLC C18 2µm 50 cm×75 µm using an overall HPLC gradient time of 180 min.

- The amount of proteins used to prepare the samples for proteomics LC/MS analysis is about 15 µg to about 50 µg, preferably about 20 µg to about 35 µg, more
- 15 preferably about 25 µg. The amount of peptides injected per LC MS/MS analysis (i.e., on column) is about 0.5 µg to about 5 µg, preferably about 1 µg to about 5 µg, more preferable about 1 µg to about 2.5 µg, In some embodiments, about 1 µg of proteins used to prepare the samples for proteomics LC/MS analysis. In other embodiments, about 2.5 µg of proteins used to prepare the samples for proteomics LC/MS analysis.

20

- In some embodiments, the mass spectrometer is Orbitrap Fusion™ Lumos™ Tribrid™, the HPLC system is Vanquish™ Neo UHPLC System, 300 nL/min, the HPLC column is Acclaim™ PepMap™ RSLC C18 2µm 50 cm×75 µm, and about 1 µg of
- 25 proteins used to prepare the samples for proteomics LC/MS analysis, optionally using an overall HPLC gradient time of 180 min. In another embodiment, the mass spectrometer is Orbitrap Fusion™ Lumos™ Tribrid™, the HPLC system is Vanquish™ Neo UHPLC System, 300 nL/min, the HPLC column is Acclaim™ PepMap™ RSLC C18 2µm 50 cm×75 µm, and about 2.5 µg of proteins used to prepare the samples for

proteomics LC/MS analysis, optionally using an overall HPLC gradient time of 180 min.

- Identification of the proteins is carried out using a software such as, for example, Mascot v2.8.2 and Scaffold v5.0.0. Database for protein identification include, for example, SwissProt Human (20221118, 20607 entries). The criteria of protein identifications is a peptide false discovery rate (FDR) of 1% (1 or 2 peptide(s) minimum). FDR relates to the probability to correctly identify a peptide/protein. Thus, a FDR of 1% indicates that from a list of 100 identified proteins, 1 of these proteins may be misidentified.

Exemplary LC/MS detection conditions are provided in the table below:

Mass spectrometer	Orbitrap Fusion™ Lumos™ Tribid™
HPLC system	Vanquish™ Neo UHPLC System, 300 nL/min
HPLC column	Acclaim™ PepMap™ RSLC C18 2µm 50 cm×75 µm
Overall HPLC gradient time	180 min
Amount of proteins to prepare the samples	25 µg
Amount of peptides injected per LC MS/MS analysis (<i>i.e.</i>, on column)	1 or 2.5 µg
Software for protein/peptide identification	Mascot v2.8.2 and Scaffold v5.0.0
Database for protein identification	SwissProt Human (20221118, 20607 entries)

Criteria of protein identifications	Peptide FDR: 1% Protein FDR: 1% 1 or 2 peptide(s) minimum
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In some embodiments, one or more of Hypoxia up-regulated protein 1 (HYOU1), Apolipoprotein A-IV (APOA4) and Procollagen-lysine,2-oxoglutarate 5-dioxygenase 1 (PLOD1) is not detected in the exosomes of the present invention by LC/MS analysis as described herein. In some embodiments, Hypoxia up-regulated protein 1 (HYOU1), Apolipoprotein A-IV (APOA4) and Procollagen-lysine,2-oxoglutarate 5-dioxygenase 1 (PLOD1) are not detected in the exosomes of the present invention by LC/MS analysis as described herein.

In some embodiments, one or more of C4a anaphylatoxin (CFA), Hypoxia up-regulated protein 1 (HYOU1), Apolipoprotein A-IV (APOA4), Procollagen-lysine,2-oxoglutarate 5-dioxygenase 1 (PLOD1), Threonine—tRNA ligase 1, cytoplasmic (TARS1), and Cytosolic non-specific dipeptidase (CNDP2) is not detected in the exosomes of the present invention by LC/MS analysis as described herein. In some embodiments, C4a anaphylatoxin (CFA), Hypoxia up-regulated protein 1 (HYOU1), Apolipoprotein A-IV (APOA4), Procollagen-lysine,2-oxoglutarate 5-dioxygenase 1 (PLOD1), Threonine—tRNA ligase 1, cytoplasmic (TARS1), and Cytosolic non-specific dipeptidase (CNDP2) are not detected in the exosomes of the present invention by LC/MS analysis as described herein.

In some embodiments, Hypoxia up-regulated protein 1 (HYOU1) is not detected in the exosomes of the present invention by LC/MS analysis as described herein.

In some embodiments, Apolipoprotein A-IV (APOA4) is not detected in the exosomes of the present invention by LC/MS analysis as described herein.

In some embodiments, Procollagen-lysine,2-oxoglutarate 5-dioxygenase 1 (PLOD1) is not detected in the exosomes of the present invention by LC/MS analysis as described herein.

- 5 The term “not detected” as used herein include proteins or peptides not present in a sample, or present in an amount too low to be detected by LC/MS analysis, for example less than fmol concentration. In some embodiments, reference to proteins and peptides not detected refers to proteins and peptides sequences not detected by the LC/MS analysis according to any of the embodiments for detection by LC/MS
- 10 described above.

However, importantly, the exosomes of the present invention were found to comprise proteins known in the literature to be present in exosomes derived from natural human breast milk that are key to infant health and development. These

15 proteins include Lactotransferrin (LTF), Annexin A2 (ANXA2) and Lactadherin (MFGE8). In some embodiments, these proteins include Lactotransferrin (LTF), Annexin A2 (ANXA2) and Lactadherin (MFGE8), MUC1 and tetraspanins CD9, CD81 and CD63.

20 Thus, in some embodiments, the invention provides a human breast milk exosome, wherein the exosome does not comprise one or more proteins selected from C4a anaphylatoxin (CFA), Hypoxia up-regulated protein 1 (HYOU1), Apolipoprotein A-IV (APOA4), Procollagen-lysine,2-oxoglutarate 5-dioxygenase 1 (PLOD1), Threonine—tRNA ligase 1, cytoplasmic (TARS1), and Cytosolic non-specific dipeptidase (CNDP2).

25

In some embodiments, the exosome does not comprise C4a anaphylatoxin (CFA).

In some embodiments, the exosome does not comprise Hypoxia up-regulated protein 1 (HYOU1).

In some embodiments, the exosome does not comprise Apolipoprotein A-IV (APOA4).

5 In some embodiments, the exosome does not comprise Procollagen-lysine,2-oxoglutarate 5-dioxygenase 1 (PLOD1).

In some embodiments, the exosome does not comprise Threonine—tRNA ligase 1 cytoplasmic (TARS1).

10

In some embodiments, the exosome does not comprise Cytosolic non-specific dipeptidase (CNDP2).

In some embodiments, the exosome does not comprise one or more of Hypoxia up-regulated protein 1 (HYOU1), Apolipoprotein A-IV (APOA4) and Procollagen-lysine,2-oxoglutarate 5-dioxygenase 1 (PLOD1).

15

In some embodiments, the exosome does not comprise proteins C4a anaphylatoxin (CFA), Hypoxia up-regulated protein 1 (HYOU1), Apolipoprotein A-IV (APOA4), Procollagen-lysine,2-oxoglutarate 5-dioxygenase 1 (PLOD1), Threonine—tRNA ligase 1, cytoplasmic (TARS1), and Cytosolic non-specific dipeptidase (CNDP2).

20

In some embodiments, the exosome comprises one or more of the proteins listed in Table 1. In some embodiments, the exosome comprises all of the proteins listed in Table 1.

25

Table 1

Description	Gene
Fatty acid synthase	FASN

Fibronectin	FN1
Xanthine dehydrogenase/oxidase	XDH
Ras GTPase-activating-like protein IQGAP1	IQGAP1
Cytoplasmic dynein 1 heavy chain 1	DYNC1H1
Programmed cell death 6-interacting protein	PDCD6IP
Lactotransferrin (Fragment)	LTF
Alpha-actinin-4	ACTN4
Keratin, type II cytoskeletal 1	KRT1
Keratin, type II cytoskeletal 2 epidermal	KRT2
Tenascin	TNC
Transitional endoplasmic reticulum ATPase	VCP
Unconventional myosin-Ic	MYO1C
Coatamer subunit alpha	COPA
ATP-citrate synthase	ACLY
Ezrin	EZR
Elongation factor 2	EEF2
Keratin, type I cytoskeletal 9	KRT9
Glycogen phosphorylase, brain form	PYGB
Annexin A2	ANXA2
Butyrophilin subfamily 1 member A1	BTN1A1
Galectin-3-binding protein	LGALS3BP
Keratin, type I cytoskeletal 10	KRT10
Apolipoprotein A-I	APOA1
Bile salt-activated lipase	CEL
Protein Niban 2	NIBAN2
Heat shock cognate 71 kDa protein	HSPA8
Lactadherin	MFGE8
Prostaglandin F2 receptor negative regulator	PTGFRN

Myoferlin	MYOF
Bifunctional glutamate/proline--tRNA ligase	EPRS1
Perilipin-2	PLIN2
Perilipin-3	PLIN3
Glutamine--fructose-6-phosphate aminotransferase [isomerizing] 1	GFPT1
Endoplasmin	HSP90B1
Actin-depolymerizing factor	GSN
4F2 cell-surface antigen heavy chain	SLC3A2
Endoplasmic reticulum chaperone BiP	HSPA5
Heat shock protein HSP 90-beta	HSP90AB1
Clusterin	CLU
Moesin	MSN
Heat shock 70 kDa protein 1B	HSPA1B
Phosphoglycerate kinase 1	PGK1
Aminopeptidase N	ANPEP

In some embodiments, the exosome comprises Lactotransferrin (LTF), Annexin A2 (ANXA2) and/or Lactadherin (MFGE8). In some embodiments, the exosome comprises Lactotransferrin (LTF), Annexin A2 (ANXA2) and Lactadherin (MFGE8). In

5 some embodiments, the exosome comprises Lactotransferrin (LTF), Annexin A2 (ANXA2) and Lactadherin (MFGE8), MUC1 and tetraspanins CD9, CD81 and CD63.

In some embodiments, the exosome comprises said one or more proteins described anywhere herein in substantially the same concentration as the same one or more

10 proteins in exosomes derived from natural human breast milk.

In some embodiments, the exosome comprises said one or more proteins described anywhere herein in different concentrations as the same one or more proteins in

exosomes derived from natural human breast milk. In some embodiments, said one or more proteins may be present in a higher concentration compared to the concentration of said one or more proteins in exosomes derived from natural human breast milk. In some embodiments, said one or more proteins may be present in a lower concentration compared to the concentration of said one or more proteins in exosomes derived from natural human breast milk.

As described herein, in some embodiments, the present invention relates to a composition comprising the exosomes or population of exosomes as described anywhere herein. In some further embodiments, the present invention relates to a composition comprising i) the exosomes or population of exosomes as described anywhere herein and ii) one or more proteins that are absent from the exosomes of the present invention but are present in exosomes derived from natural human breast milk. Said proteins may be selected from but not limited to one or more of proteins C4a anaphylatoxin (CFA), Hypoxia up-regulated protein 1 (HYOU1), Apolipoprotein A-IV (APOA4), Procollagen-lysine,2-oxoglutarate 5-dioxygenase 1 (PLOD1), Threonine—tRNA ligase 1, cytoplasmic (TARS1), and Cytosolic non-specific dipeptidase (CNDP2).

In some embodiments, the composition is a nutritional composition. Accordingly, in some embodiments, the present invention relates to a nutritional composition comprising i) the exosomes or population of exosomes as described anywhere herein and ii) one or more proteins that are absent from the exosomes of the present invention but are present in exosomes derived from natural human breast milk. Said proteins may be selected from but not limited to one or more of proteins Hypoxia up-regulated protein 1 (HYOU1), Apolipoprotein A-IV (APOA4) and Procollagen-lysine,2-oxoglutarate 5-dioxygenase 1 (PLOD1). In another embodiment, said proteins may be selected from but not limited to one or more of proteins C4a anaphylatoxin (CFA), Hypoxia up-regulated protein 1 (HYOU1),

Apolipoprotein A-IV (APOA4), Procollagen-lysine,2-oxoglutarate 5-dioxygenase 1 (PLOD1), Threonine—tRNA ligase 1, cytoplasmic (TARS1), and Cytosolic non-specific dipeptidase (CNDP2).

- 5 In some embodiments, the composition is a pharmaceutical composition. Accordingly, in some embodiments, the present invention relates to a pharmaceutical composition comprising i) the exosome or population of exosomes as described anywhere herein, ii) a pharmaceutically acceptable excipient and iii) one or more proteins that are absent from the exosomes of the present invention
- 10 but are present in exosomes derived from natural human breast milk. Hypoxia up-regulated protein 1 (HYOU1), Apolipoprotein A-IV (APOA4) and Procollagen-lysine,2-oxoglutarate 5-dioxygenase 1 (PLOD1). In another embodiment, said proteins may be selected from but not limited to one or more of proteins C4a anaphylatoxin (CFA), Hypoxia up-regulated protein 1 (HYOU1), Apolipoprotein A-IV
- 15 (APOA4), Procollagen-lysine,2-oxoglutarate 5-dioxygenase 1 (PLOD1), Threonine—tRNA ligase 1, cytoplasmic (TARS1), and Cytosolic non-specific dipeptidase (CNDP2).

Exosome miRNA profile

- 20 The inventors of the present invention carried out miRNA analysis of the exosomes of the present invention and surprisingly demonstrated that the exosomes possess substantially the same miRNA profile to that of exosomes derived from natural human breast milk.
- 25 In particular, exosomes of the present invention were surprisingly found to comprise miRNAs known in the literature to be present in exosomes derived from natural human breast milk that are key to infant health and development. These miRNAs include miR-148a-3p, miR-22-3p, miR-125b-5b and miR-6126.

Thus, in some embodiments, the invention provides a human breast milk exosome, wherein the exosome comprises one or more miRNAs selected from miR-148a-3p, miR-22-3p, miR-125b-5b and miR-6126.

- 5 In some embodiments, the exosome comprises miR-148a-3p, miR-22-3p, miR-125b-5b and miR-6126.

In some embodiments, the exosome comprises said one or more miRNAs described anywhere herein in substantially the same concentration as the same one or more
10 miRNAs in exosomes derived from natural human breast milk.

In some embodiments, the exosome comprises said one or more miRNAs described anywhere herein in different concentrations as the same one or more miRNAs in exosomes derived from natural human breast milk. In some embodiments, said one
15 or more miRNAs may be present in a higher concentration compared to the concentration of said one or more miRNAs in exosomes derived from natural human breast milk. In some embodiments, said one or more miRNAs may be present in a lower concentration compared to the concentration of said one or more miRNAs in exosomes derived from natural human breast milk.

20

Exosome lipidomics

The inventors of the present invention carried out lipid analysis of the exosomes of the present invention and surprisingly demonstrated that the exosomes possess
25 substantially the same lipid profile to that of exosomes derived from natural human breast milk.

In particular, exosomes of the present invention were surprisingly found to comprise lipid classes known in the literature to be present in exosomes derived from natural
30 human breast milk that are key to infant health and development. These lipids

include cholesterol esters, ceramides, triacylglycerides, diacylglycerides, lyso-phospholipids, total phospholipids, alkanyl phospholipids, and alkenyl phospholipids.

- 5 Of these key lipid classes, phospholipids in particular are considered to be key for exosome structure and function. Exosomes of the present invention were also surprisingly found to comprise phospholipid sub-classes known in the literature to be present in exosomes derived from natural human breast milk. These phospholipids include sphingomyelins, phosphatidylcholine,
10 phosphatidylethanolamine, phosphatidylinositol, and phosphatidylserine.

Thus, in some embodiments, the invention provides a human breast milk exosome, wherein the exosome comprises one or more lipids selected from cholesterol esters, ceramides, triacylglycerides, diacylglycerides, total phospholipids, alkanyl
15 phospholipids, and alkenyl phospholipids.

In some embodiments, the exosome comprises cholesterol esters, ceramides, triacylglycerides, diacylglycerides, total phospholipids, alkanyl phospholipids, and alkenyl phospholipids.

20

In some embodiments, the exosome comprises one or more phospholipids selected from sphingomyelins, phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, and phosphatidylserine.

- 25 In some embodiments, the exosome comprises sphingomyelins, phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, and phosphatidylserine.

In some embodiments, the exosome comprises said one or more lipids described anywhere herein in substantially the same concentration as the same one or more lipids in exosomes derived from natural human breast milk.

- 5 In some embodiments, the exosome comprises said one or more lipids described anywhere herein in different concentrations as the same one or more lipids in exosomes derived from natural human breast milk. In some embodiments, said one or more lipids may be present in a higher concentration compared to the concentration of said one or more lipids in exosomes derived from natural human
- 10 breast milk. In some embodiments, said one or more lipids may be present in a lower concentration compared to the concentration of said one or more lipids in exosomes derived from natural human breast milk.

Exosome size

- 15 The inventors of the present invention carried out size analysis of the exosomes of the present invention and surprisingly demonstrated that the exosomes are substantially the same size in diameter as exosomes derived from natural human breast milk.

- 20 Thus, in some embodiments, the invention provides a human breast milk exosome, wherein the exosome has a diameter size of 50 to 100nm, preferably 60-80nm, more preferably 65 to 75nm.

- 25 In some embodiments, the exosome has a diameter size of 65 to 75nm.

- It will be understood that any features of the exosomes described anywhere herein may be combined. For example, features relating to the proteome of the exosome may be combine with features relating to the miRNA profile, lipid profile and/or size
- 30 profile.

Therapeutic methods

5 The exosomes of the invention or a composition of the invention, e.g. a pharmaceutical composition, may be used in therapy, for example for treating a disease or disorder. Also provided is a method of treating a disease or disorder comprising administering to a subject in need thereof a therapeutically effective amount of the exosomes or a composition, e.g. a pharmaceutical composition) as described herein.

10

The exosomes of the invention have been shown to have a protective effect toward alteration of barrier integrity triggered by proinflammatory cytokines on epithelial Caco-2 HTB-37 cells (Example 7). The exosomes of the invention effectively promote the epithelial barrier function and prevent inflammation of the epithelium.

15 Accordingly, the present invention provides therapies which involve administering the exosomes of the invention or a composition of the invention, e.g. a pharmaceutical composition to a subject for preventing, treating, or ameliorating symptoms associated with inflammation, in particular inflammation of the epithelium. In some embodiments, inflammation is intestinal inflammation. In some

20 embodiments, inflammation is bronchial inflammation. In some embodiments, inflammation is respiratory inflammation.

Thus, provided herein are the exosomes of the invention or a composition of the invention, e.g. a pharmaceutical composition for use in preventing, treating, or

25 ameliorating symptoms associated with inflammation, in particular inflammation of the epithelium. Also provided is a method of preventing, treating, or ameliorating symptoms associated with inflammation, in particular inflammation of the epithelium, the method comprising administering to a subject in need thereof a therapeutically effective amount of the exosomes or a composition, e.g. a

pharmaceutical composition as described herein. In some embodiments, inflammation is intestinal inflammation. In some embodiments, inflammation is bronchial inflammation. In some embodiments, inflammation is respiratory inflammation.

5

Inflammation is a reaction of living tissue to injury or infection. The exosomes of the invention or a composition of the invention, e.g. a pharmaceutical composition are also useful in treating inflammation-related diseases (inflammatory diseases).

10 Inflammatory disease includes inflammatory diseases of the digestive tract, including oesophagitis (i.e., inflammation of the oesophagus, such as oesophageal ulcer), gastroenteritis (i.e., inflammation of the mucous membranes of the stomach and intestine, such as gastritis, duodenal ulcer, ileitis, or enterocolitis), colitis (i.e., inflammation of the colon, such as diverticulitis), cholangitis (i.e., inflammation of
15 the bile duct), appendicitis (i.e., inflammation of the appendix), inflammatory bowel diseases (e.g., Crohn's disease and ulcerative colitis) and irritable bowel syndrome. Inflammatory disease also includes respiratory inflammation diseases and bronchial inflammatory diseases. For example, respiratory distress syndrome and respiratory infections.

20

The exosomes of the invention or a composition of the invention, e.g. a pharmaceutical composition may be used to increase cell proliferation, decreased apoptosis and cell death in epithelial cells, for example intestinal cells, and reduced inflammation. Also provided is a method of increasing cell proliferation, decreasing
25 apoptosis in epithelial cells, for example intestinal cells, and reducing inflammation comprising administering to a subject in need thereof a therapeutically effective amount of the exosomes or a composition, e.g. a pharmaceutical composition as described herein.

The exosomes of the invention or a composition of the invention, e.g. a pharmaceutical composition may be used in restoring the intestinal barrier function of a subject. Accordingly, also provided is a method of restoring the intestinal barrier function of a subject comprising administering to the subject in need thereof a
5 therapeutically effective amount of the exosomes or a composition, e.g. a pharmaceutical composition as described herein.

The term “subject” as used herein refers to an animal, in particular a mammal, or a human. Preferably, the subject is a human. In some embodiments, the subject is
10 selected from preterm infants, infants, toddlers, children and adults. Preferably, the subject is selected from preterm infants, infants, toddlers, and children.

Additional Embodiments of the Invention

15 There is provided:

S1. An *in vitro* method for producing mammalian breast milk exosomes, the method comprising:

- 20 A) Culturing mammary epithelial cells in a culture medium to generate lactocyte mammary-like gland organoids; and
- B) Secreting a mammalian milk like product from said lactocytes, and
- C) Purifying the exosomes from the mammalian milk like product to remove impurities, optionally by chromatography or filtration or ultracentrifugation, in order to isolate the exosomes.

25

S2. The method of statement 2, wherein the time duration of step A) is no longer than 14 days, optionally is 14 days.

- S3. The method of statement 1 or 2, wherein the lactocyte mammary-like gland organoids from step A) express one or more mammary gland positive cell markers, optionally selected from KRT-18 and ER.
- 5 S4. The method of any one of statements 1 to 3, wherein step A) further comprises:
- i) culturing the mammary epithelial cells, and
 - ii) inducing milk protein expression.
- 10 S5. The method of any one of statements 1 to 4, wherein the lactocyte mammary-like gland organoids from step A) have increased mRNA expression of one or more milk bioactive markers post-induction compared to pre-induction.
- S6. The method of any one of statements 1 to 5, wherein the lactocyte
- 15 mammary-like gland organoids from step A) have increased mRNA expression of LTF post-induction compared to pre-induction.
- S7. The method of any one of statements 1 to 6, wherein the lactocyte
- 20 mammary-like gland organoids from step A) have increased mRNA expression of MFGE8 post-induction compared to pre-induction.
- S8. The method of any one of statements 1 to 7, wherein the culture medium is a MammoCult medium, in an appropriate 3D culture system, for example 3D-suspension condition.
- 25 S9. The method of statement 8, wherein step Ai) is for no more than 7 days, and optionally is for 7 days.

S10. The method of statement 8 or 9, wherein step Aii) is for no more than 7 days, and optionally is for 7 days.

5 S11. The method of any one of statements 1 to 10, wherein step A) further comprises:

i) culturing the mammary epithelial cells in complete MammoCult medium comprising the basal medium, proliferation supplement and supplemented with heparin, and hydrocortisone for 7 days, and

10 ii) inducing milk protein expression by incubating the cells in EpiCultB medium supplemented with EpiCult proliferation supplement, hydrocortisone, insulin, FBS, prolactin, progesterone and β -estradiol for 7 days.

S12. The method of any one of statements 1 to 10, wherein step A) further comprises:

15 i) culturing the mammary epithelial cells in MammoCultB medium supplemented with MammoCult proliferation supplement, hydrocortisone and heparin for 7 days, and

20 ii) inducing milk protein expression by incubating the in EpiCultB medium supplemented with EpiCult proliferation supplement, hydrocortisone, insulin, FBS, prolactin, progesterone and β -estradiol for 7 days.

S13. The method of any one of statements 1 to 12, wherein step C) further comprises treating the modified milk like product to generate a modified mammalian milk like product.

25

S14. The method of any one of statements 1 to 13, wherein the mammary epithelial cells are human mammary epithelial cells.

S15. The method of any preceding statement, wherein step C) further comprises formulating the isolated exosomes into a powder form, optionally by spray drying or freeze drying, or liquid form.

5 S16. The method of any preceding statement, wherein step C) further comprises sterilising the isolated exosomes.

S17. The method of any preceding statement, wherein step C) further comprises storing the isolated exosomes at below freezing, optionally at -80°C.

10

S18. The method of any preceding statement, further comprising formulating the isolated exosomes with supplementary nutritional ingredients.

S19. The method of any preceding statement, wherein the isolated exosomes are
15 dispensed into a container for consumption.

S20. A mammary breast milk exosome product which is obtainable according to the method of any preceding statement, optionally a human breast milk exosome.

S21. A method of producing a mammalian milk fortifier, comprising conducting
20 the method of any one of statements 1 to 19.

It should be appreciated that the various aspects and embodiments of the detailed description as disclosed herein are illustrative of the specific ways to make and use the invention and do not limit the scope of invention when taken into consideration
25 with the claims and the detailed description. It will also be appreciated that features from aspects and embodiments of the invention may be combined with further features from the same or different aspects and embodiments of the invention.

As used in this detailed description and the appended claims, the singular forms “a,” “an” and “the” include plural referents unless the context clearly dictates otherwise.

Figures

5 **Fig. 1:** shows the differentiation of human induced pluripotent stem cells (hiPSCs) according to the protocol outlined in Ying Qu and as applied in one alternative in step A) of the inventive methods.

Fig. 2: shows the differentiation of human induced pluripotent stem cells (hiPSCs)
10 according to step A).

Fig. 3: shows that three-dimensional organotypic cultures of hiPSCs as produced according to the methods of **Fig. 2** are highly permissive for mammary glands specification. mRNA expression of Nanog, TUBB3, FOXA2, TP63, KR-14, EpCAM, KRT8 and CSN2 for 3D-differentiation (42 days) protocol are shown.
15 Markers from left to right: Stages of Pluripotency (Nanog), Lineage (ectoderm & endoderm) (TUBB3, FOXA2), Basal-cell/myoepithelial markers (TP63, KR-14), Luminal epithelial markers (EpCAM, KRT8), and milk proteins (CSN2 (Casein Beta)).

20

Fig. 4: shows the two-dimensional organotypic culture of hiPSCs produced as a comparative example. mRNA expression of Nanog, TUBB3, FOXA2, TP63, KR-14, EpCAM, KRT8 and CSN2 for 2D-differentiation (31 days) protocols are shown. Markers from left to right: Stages of Pluripotency (Nanog), Lineage (ectoderm & endoderm) (TUBB3, FOXA2), Basal-cell/myoepithelial markers (TP63, KR-14), Luminal epithelial markers (EpCAM, KRT8), and milk proteins (CSN2 (Casein Beta)).
25

Fig. 5: shows lactation induction in three-dimensional (3D) cultures of mammary epithelial cells. **a**, Schematic illustration of the culture protocol of mammary epithelial cells and lactation induction (D7-14). **b**, Analysis of the mammary gland markers keratin 18 and estrogen receptor by flow cytometry, during the proliferation stage of the mammary gland epithelial cell culture. **c**, RNA expression (Δ Ct) level of human lactoferrin (LTF) and milk fat globule-EGF factor 8 (MFGE8) / lactadherin during the proliferation (Day 7) and lactation induction stages (Day 14). The Δ Ct method is used in the panel presentations to rank the LTF and MFGE8 genes by calculating the average standard deviation (SD) based on the relative expression of candidate reference gene. The indicated gene with the lowest SD was identified as the most stable or most expressed gene.

Fig. 6: shows the expression of different mammary epithelium markers in mammary epithelium cells using NanoString technology for gene expression profiling (**a-h**).

Fig. 7: shows the median particle size (nm) of EVs purified from epithelial cells and from iPSC cells compared to human milk.

Fig. 8: shows a schematic illustrations of different mammary-gland differentiation protocols. Scheme summarizing different procedures for the generation of mammary gland progenitors in 42 days (a), using bone morphogenetic protein 4 (BMP4) (b), retinoic acid (RA) (c), combinations of BMP4 and RA (d) and their combination in shortened time-course (31 days) (e). The red boxes indicate the period of addition of BMP4 and/or RA.

Fig 9: shows combinatorial effects of bone morphogenetic protein 4 (BMP4) and retinoic acid (RA) on mammary-gland differentiation of induced pluripotent

stem cells (iPSCs) using a 3D-organoid model. **a-c**, Flow cytometry quantification of the number of mammary gland positive progenitor-cells using several identified markers: EpCAM, CD49f, MUC1 and GATA3 during the differentiation time-course (day 25 (for control protocols) and day 20 (for shortened time-course protocol)) and pre-induction stages (day 35 (for control protocols including BMP4 and RA), and day 26 (for shortened time-course protocol)) and **d-f**, post-induction period (days 42 (for control protocols including BMP4 and RA) and 31 (for shortened time-course protocol)). * describes shortened-modified protocols (31 days) including BMP4 and RA combinations.

Fig 10: shows expression of different mammary epithelium markers in lactocytes derived from iPSCs in normal length and shortened protocols (**a-h**).

Fig 11: shows Liquid chromatography–mass spectrometry (LC-MS/MS) based targeted proteomic analysis of osteopontin protein secretion. Osteopontin protein secretion was detected in the media of cultures of iPS-derived mammary-gland cells using control differentiation method, or with bone morphogenetic protein 4 (BMP4), retinoic acid (RA) and in the combination of the two factors.

Fig 12: a, Venn diagram shows the number of proteins identified in EVs purified from conditioned media (Day 39-41) only (circle on the left hand side), in EVs purified from human milk only (circle on the right hand side), and in both types of EVs (overlapping circles); **b**, Table shows the list of the top 50 proteins (based on FDR) detected in both types of EVs (human milk and conditioned media).

Fig 13: **a**, Venn diagram shows the number of miRNAs significantly downregulated in EVs purified from conditioned media (Day 39-41) compared to human milk EVs (circle), significantly upregulated in conditioned media EVs compared to human milk EVs (square), and not differentially expressed in both types of EVs (overlapping circle and square shapes). **b**, Venn diagram shows result obtained from subsequent repetition of the experiment of Example 6 with 2 additional batches of cell-based EVs. Venn diagram shows the number of miRNAs significantly downregulated in EVs purified from conditioned media (Day 39-41) compared to human milk EVs (circle), significantly upregulated in conditioned media EVs compared to human milk EVs (square), and not differentially expressed in both types of EVs (overlapping circle and square shapes). **c**, Table shows the list of the top 4 miRNAs (based on FDR) expressed in both types of EVs (human milk and conditioned media).

Fig 14: shows the proportions of lipid (left panel) and phospholipid (right panel) classes found in EVs purified from human milk and conditioned media (Day 39-41). Data is expressed a percentage of total lipid and phospholipid, respectively. TG: Triacylglycerides; DG: Diacylglycerides; PL: Total phospholipids; Lyso: Lyso-phospholipid; Alkanyl: Phospholipid-O; Alkenyl: Phospholipid-P; CE: Cholesterol esters; CER: Ceramides; SM: Sphingomyelins; PC: Phosphatidylcholine; PE: Phosphatidylethanolamine; PI: Phosphatidylinositol; PS: Phosphatidylserine. The specific lipids and phospholipids in the bar charts, starting from the bottom to the top of the charts, correspond to the lipids or phospholipids in the abbreviated lists, starting left to right.

Fig 15: shows the median particle size (nm) of EVs purified from iPSCs (conditioned media (Day 41)) and human milk, analysed by Nano Flow Cytometry.

Fig 16: shows the effect of treatment of Caco-2 monolayers with dexamethasone (1 μ M), human milk-derived EVs (HM EVs) (1.107 EVs/well), and lactocyte-derived EVs (1.107 EVs/well), prior to the application of IFN γ and TNF α . Control is Caco-2 cells without any challenge.

5

Fig 17: shows the effect of treatment of Caco-2 monolayers with dexamethasone (1 μ M), human milk-derived EVs (HM EVs) (1.107 EVs/well), and lactocyte-derived EVs (1.107 EVs/well), prior to the application of IFN γ and TNF α . Values show the impact after 48 hours of inflammatory challenge. Control is Caco-2 cells without any challenge.

10

Experimental section

Example 1

Cultivation and differentiation of hiPSCs into lactocytes to obtain a human milk like product

Lactocytes are cultured starting from ihPSCs according to the procedure described in Ying Qu et al, Stem Cell Report vol 8, 205-215 February 14th 2017 and the human milk like product thereby secreted is collected and can be used in therapy and/or as a breastfeeding substitute according to the present invention.

Example 2

Cultivation and differentiation of hiPSCs into 3D-lactocytes to obtain a human milk like product

Lactocytes are cultured starting from hiPSCs according to the method of the present invention following steps A) and B) as described above) and the human milk like product thereby secreted is collected and can be used in therapy and/or as a breastfeeding substitute according to the present invention.

Example 3

Alternative methods of cultivation and differentiation of hiPSCs into lactocytes to obtain a human milk like product

Efficient lactocytes differentiation from hiPSCs can be obtained from alternative culture conditions including conditions 1 to 4 as below described:

1. 2D culture on vitronectin coated plates as monolayer of cells derived from the EBs and cultured for at least 28 days in a medium containing (RPMI 1640 with L-glutamine; Fetal bovine serum (FBS); Insulin; Epidermal growth factor (EGF); hydrocortisone; Pen-Strep (penicillin/streptomycin : antibiotic-antimycotic solution).

2. 2D culture on vitronectin coated plates of attached aggregates (EBs) of cells derived from the EBs and cultured for at least 28 days in a medium containing (RPMI 1640 with L-glutamine; Fetal bovine serum (FBS); Insulin; Epidermal growth factor (EGF); hydrocortisone; Pen-Strep (antibiotic-antimycotic solution).
3. 3D culture in suspension in MammoCult medium for at least 10 days and then culture in mixed floating gels (for example Matrigel and Collagen 1) for another 5 days in a specific medium (for example EpiCultB) in presence of Parathyroid hormone followed by 25 days in presence of insulin, HGF, hydrocortisone and FGF10;
4. 3D culture of EBs in suspension (ultra low adherent plate) in MammoCult medium for at least 10 days and then in suspension culture for another 5 days in a specific medium (for example EpiCultB) in presence of Parathyroid hormone followed by 25 days in presence of insulin, HGF, hydrocortisone and FGF10.

Example 4

2D- and 3D-lactocyte differentiation based on human-induced pluripotent stem cell (hiPSC) line 603

(a) 3D-lactocyte differentiation based on human-induced pluripotent stem cell (hiPSC) line 603:

The human-induced pluripotent stem cell (hiPSC) line 603 was used for 3D-lactocyte differentiation. The human-induced pluripotent stem cell (hiPSC) line 603 was purchased from Fujifilm Cellular Dynamics, Inc (FCDI).

- (i) For the 3D differentiation protocol (according to the invention), EBs (spheroids) were formed by incubating single cells of hiPSC in E8 medium with 10uM rock inhibitor at 37°C, 5% CO₂ in rotation at 95 rpm overnight.

Second day, medium was replaced with E8 (day -2-day 0).

Next day, medium was replaced with Mammo1 medium (MammoCult - medium with proliferation supplements, heparin (4µg/mL), and hydrocortisone (0.48µg/mL) with penicillin/streptomycin) for 10 days (day 0-day 10). Medium was changed every second day.

5 (ii) The differentiation was followed by 5 days in Mammo2 medium (EpiCultB + supplements, pThrP 100ng/ml plus penicillin/streptomycin). Culture medium was changed every 3 days (day 10-day 15).

(iii) In order to induce branching epithelial structure, alveolar differentiation and mammary cell specification, mEBs (spheroids/mammospheres)
10 were fed with Mammo3 medium (complete EpiCultB, hydrocortisone (1 µg/ml), insulin (10 µg/ml), FGF10 (50 ng/ml), HGF (50 ng/ml) and penicillin/streptomycin) for 20 days. Medium was changed every 3 days (day 15-day 35).

(iv) Finally, to induce the milk bioactive production (3D), we used the Mammo4 medium (complete EpiCultB, 10% FBS, prolactin (10 µg/ml),
15 hydrocortisone (1 µg/ml), insulin (10 µg/ml), progesterone, β-estradiol and penicillin/streptomycin for 7 days and medium was changed every 3 days (day 35-day 42). During all the differentiation procedure, spheroids were maintained in the suspension culture (rotating at 95 rpm). The differentiation procedure ended at day 42. Results are displayed in Figure 3.

20

(b) 2D-lactocyte differentiation based on human-induced pluripotent stem cell (hiPSC) line 603

The human-induced pluripotent stem cell (hiPSC) line 603 was used also for 2D-lactocyte differentiation. The human-induced pluripotent stem cell
25 (hiPSC) line 603 was purchased from Fujifilm Cellular Dynamics, Inc (FCDI).

For the 2D-differentiation protocol (used for comparison), we used the Lacto medium during all the differentiation stages (RPMI 1640, 20% FBS, 1mM glutamine, 4 µg/ml insulin, 20 ng/ml EGF, 0.5µg/ml hydrocortisone with

penicillin/streptomycin). Cells were incubated at 37°C, 5% CO₂. Medium was replaced every second day. Results are displayed in Figure 4.

(c) Results

5 The different differentiation stages during lactocyte derivation were captured using quantitative RT-PCR (Figure 3, 3D-differentiation, Figure 4, 2D-differentiation). In both 2D- and 3D-settings, NaNog expression as a marker for pluripotency is decreased while cells are passing towards the maturation and differentiation. The neuroectodermal and endodermal markers, TUBB3 (Tubulin
10 Beta 3 Class III) and Forkhead box protein A2 (FOXA2) were not expressed significantly in 3D-format and TUBB3 elevation is only captured in 2D-setting. This demonstrates that hiPSCs are patterned towards the non-neural ectodermal lineage, thus enriching mammary progenitors in 3D-format. We investigated the expression pattern of commonly used basal cell/myoepithelial markers, such as p63
15 (a p53-homologous nuclear protein) and cytokeratin 14 (KRT-14). Both markers are detectable significantly in both systems. Additionally, the epithelial cell adhesion molecule (EpCAM) and cytokeratin 8 (KRT8) were tracked only in the 3D-system and KRT8 was only partially expressed in the 2D-format. Consequently, 3D-platform in an organotypic setting expressed common breast tissue, luminal, and basal markers.
20 Such mammary like organoids express human breast specific proteins including CSN2 (casein beta), milk protein peptides, and hormone receptors. The luminal cells specifically express EpCAM, MUC1, CD49F, GATA3, CK8, and CK18 while basal cells will specifically express CK14, α -smooth muscle actin and P63. Eventually EpCAM and CD49F double positive cells can be detected at an earlier progenitor stage
25 between D10 and D35. Interestingly, CSN2 expression is only captured at the last time point (D42) of the 3D-organotypic system and not in the 2D-directed differentiation platform.

Analysis of the mammary like organoids secretome showed secretion of human milk specific bioactives including oligosaccharides (including lactose and some HMOs), lipids (including 4 fatty acids), proteins (7 detected including caseins), and miRNA (75 detected, including 11 typically detected in HBM) as below described.

Primary cell supernatant was analyzed for presence of lactose or human milk oligosaccharides following the procedure described in "Austin and Benet, Quantitative determination of non-lactose milk oligosaccharides, Analytica Chimica Acta 2018, 1010, 86-96" with minor modification. The samples were analysed with UHPLC and detected lactose or human milk oligosaccharides (HMOs) were quantified against a calibration curve of lactose and a mix of 7 HMOs (2'FL, 3FL, DFL, LNT, LNnT, 3'SL and 6'SL). The method had an estimated limit of 0.1mg/L. In the primary cell supernatants, Lactose (0.22 mg/l) and 6'SL (0.32 mg/l) were detected at day 42.

Fatty acids were analysed in media and cell supernatants by gas chromatography coupled with flame ionization detector. Briefly, the supernatants obtained at day 42 is analysed to investigate the presence of fatty acids contained in several lipid classes. A 7890A gas-chromatograph with a 7693 autosampler with preparative station module equipped with a fused-silica CP-Sil 88 capillary column (100% cyanopropylpolysiloxane; 100 m, 0.25 mm id, 0.25 mm film thickness is used with a split injector (1:25 ratio) heated at 250°C and a flame-ionization detector operated at 300°C. Preparation of FAMES (fatty acids methyl esters) is performed by direct transesterification of sample with methanolic chloridric acid. Separation of FAMES is performed using capillary gas chromatography-FID (GC). Identification of FAMES is done by retention time (RT) and comparison with an external standard. Quantification of fatty acids is done by calculation using methyl C11:0 as internal standard. Transesterification performance of the method is controlled with TAG C13:0 as second internal standard. After addition of internal standards, the solution

was mixed with 2 mL of methanol, 2 mL of Methanol/HCl (3N) and 1 mL of hexane. After heating at 100°C/60min, the sample is cooled down to room temperature (about 15 min) and the reaction is stopped by adding 2mL of water. After centrifugation the organic phase is directly injected into the GC.

- 5 Fatty acid results from protocol of Example 4a at time day 42 are reported in table 1 (differences observed between media and supernatant).

The table 1 below lists the expressed fatty acids in cell supernatant sample.

Fatty acid	Detected amount in cell supernatant (mg/100 mL)
C-4:0	2.53
C-8:0	0.49
C-10:0	0.38
C-14:0	0.44
C-15:0	0.41
C-16:0	1.85
C-16:1n7	0.08
C-17:0	0.09
C-18:0	0.97
C-18:1 n9	18.82
C-18:1	0.28
C-18:2 n6	2.16
C-20:0	0.13
C-20:1 n9	0.11
C-18:3 n3	0.08
C-22:0	0.29
Other fatty acids	1.38

Proteins in the cell supernatant were analysed using SDS-PAGE profiling and then band isolation for identity confirmation by LC-MSMS. For SDS-PAGE analysis, the total volume of the prepared sample was loaded on the gel. A human milk sample was added for comparison as control. Selected gel regions (bands) were cut to look

5 for human proteins by LC-MSMS. Eventually, bands were submitted to in-gel trypsin digestion and analyzed by LC-MSMS. LC-MSMS data were analyzed with Peaks Studio and matched against the UniProt database for human proteins.

The table 2 below lists the best candidates for all the excised bands.

Name of expressed proteins in the cell supernatant
Lactoferrin
Albumin
Prolactin
Alpha S1-casein
Hemoglobin subunit beta
Hemoglobin subunit alpha
α -lactalbumin
Alpha-2-macroglobulin
β -casein
bile salt-activated lipase
κ -casein
lactadherin
CD14
fatty acid synthase
IgA
pIgR

Serum albumin
Xanthine dehydrogenase

Exosome isolation and miRNA profiling was performed using ExoQuick polymer nets. ExoQuick polymer works to precipitate exosomes by forming a network and collects all exosomes of a certain size. Once the ExoQuick mesh is formed, a simple, low-speed centrifugation easily precipitates the exosomes as a pellet. The exosomes are intact, ready for protein or RNA analysis and are bioactive for functional studies. Precipitation buffer was added in a ration 0.25X to the sample then vortex. The mix was incubated overnight at 4°C. After incubation, samples were centrifuged 30 min at 1,500xg. The exosome pellet was re-suspended by vertexing in initial volume with Buffer XE (QIAGEN) for QC or Lysis Buffer from HTG EdgeSeq miRNA Whole Transcriptome Assay for miRNA profiling. In order to assess the extracellular vesicles (EVs) isolation, the supernatant was first centrifuged at 3000g for 15 min to remove cell pellet and debris. Then 100 microliters of media was used for an overnight precipitation at 4°C with ExoQuick buffer (ratio 0.25X). EV precipitates were recovered by centrifugation for 30 min at 1500g. Two precipitations were performed for each sample, one EV precipitation was resuspended in Buffer XE (QIAGEN) for potential further analysis, and a second one in only 50 ul HTG Lysis buffer in order to concentrate by 10-fold before miRNA profiling with HTG. For miRNA profiling, samples were used directly in the first step of lysis. Thus, Whole sample was used directly and was lysed with Plasma lysis buffer in a ratio1:1. Next, proteinase K (1/10) was added and the samples were incubated 3h at 50°C at 600rpm on Thermomixer. EVs were resuspended in Lysis buffer and lysed in the same conditions, with an incubation step at 95°C for 10min added before the lysis incubation. 26 µl of lysate was process with 70 µl of oil on the HTG processor following the HTG EdgeSeq miRNA Whole Transcriptome Assay V2 procedure. For indexing and amplification libraries, samples were tagged with Illumina adaptors and indexes by PCR with OneTaq® Hot Start 2X Master Mix GC Buffer (95°C - 4 min;

- 16 cycles: 95°C-15 sec, 56°C-45 sec, 68°C- 45 sec; 68°C10 min; Hold at 4°C) and AMPure cleaned (ratio 2.5) on a robotic liquid handler SciClone NGS WorkStation (Perkin Elmer). Pools were obtained with our custom pooling program on Hamilton robot. The samples were pooled based on GX touch Chip HS quantification. The
- 5 pools were purified manually a second time with AMPure Bead (ratio 1.8) to remove potential remaining traces of primer-dimer and quantified with Qubit to adjust the final concentration to 2 nM. And as a last step, for MiSeq sequencing, pools were loaded on MiSeq at 20pM with a 5% PhiX spike and sequenced for 50 base Single read on MiSeq with 150V3 kit.
- 10 Briefly, 974 miRNAs detected in the in the cell supernatant which more than 75 of them are highly expressed miRNAs in the milk samples.

The table 3 below lists the top ten highly expressed miRNAs.

miRNA name	log2 counts	CV
miR-21-5p	9.76	0.01
miR-181a-5p	9.07	0.03
miR-30d-5p	8.63	0.01
miR-30b-5p	8.63	0.01
miR-22-3p	8.49	0.01
miR-146b-3p	8.40	0.01
miR-30c-5p	8.12	0.04
miR-30a-5p	7.63	0.02
miR-30e-5p	7.26	0.01
miR-148b-3p	6.77	0.04

- 15 Our findings provide a novel iPSC-based 3D-organotypic model for studying the regulation and development of normal mammary cell fate and function as well as breast milk bioactives production.

Example 5

Mammary epithelial cells were cultivated in 3D-formats with and without matrixes for 7 days proliferation and 7 days induction stages using differentiation media M1/M4 (Figure 5a). Other differentiation media may also be used. Briefly, 1-5 million dissociated epithelial cells were plated in the 6 well-plates (ultra-low attachment) containing 4.5 mL media using a planar shaker platform. Cultured cells can express mammary-gland specific markers such as keratin 18 and estrogen receptor using flow cytometry quantification (Figure 5b) and are able to express mRNA-level of human lactoferrin (LTF) and milk fat globule-EGF factor 8 (MFGE8) or lactadherin during the proliferation (Day 7) and induction period (Day 14) (Figure 5c, d).

Using NanoString technology for gene expression profiling, we assessed the expression of different mammary epithelium markers in mammary epithelium cells (Figure 6). There was a consistent expression of the mammary epithelial progenitor marker CD24, with a tendency for higher expression during lactation (Figure 6a). It is interesting to observe that cells show a decrease in mammary basal-like cell's marker expression such as KRT5, KRT14 and ITGA6 (CD49f) as lactation progresses (Figure 6b-d). It appears that cells maintained a luminal-like phenotype during and after lactation using different markers, such as EpCAM, KRT8, KRT18 and MUC1 (Figure 6e-h). The post-induction period is characterized by the initiation of lactocyte (luminal-like cells) specific secretory profiles for different proteins, such as lactoferrin (LTF), clusterin and fatty acid synthase (FASN) (Table 1a). Exosomes isolated from purified epithelial cells after lactation also show abundant expression of milk-specific miRNAs, which is listed in Table 1b. Expression of mammary epithelium markers in mammary epithelium cells and milk-specific miRNA in exosomes purified epithelial cells after lactation was also observed using alternative differentiation conditions.

Table 1:

a

Protein name	Before induction	Post induction
CD14	-	++
Clusterin (ApoJ)	-	+
Fatty acid synthase (FASN)	-	+
Lactadherin (MFGE8)	+	+
Lactoferrin (LTF)	-	+
Lysozyme	-	+
pIgR	-	+

+ and ++ represent relative signal intensities for a same protein / peptide
- represents not detected or not meaningful

b.

miRNA name	Condition 1 (M1-M4)
miR-21-5p	56.73
miR-181a-5p	3.33
miR-30d-5p	9.83
miR-30b-5p	20.15
miR-22-3p	1.30
miR-146b-3p	2.43
miR-30c-5p	21.98
miR-30a-5p	7.06
miR-30e-5p	0.80
miR-148b-3p	11.15

5

Flow cytometry analysis of the EVs isolated from epithelial cells was conducted using a NanoFCM machine and compared with exosomes from natural breast milk as a

control. The results are illustrated in Figure 7 showing similar size ranges for the epithelial EVs compared to the control (see Figure 7).

Further protein expression analysis and NanoFCM antibody labelling of the isolated
5 EVs was conducted to quantitatively examine expression of tetraspanins proteins
CD9, CD63 and CD81. The average expression level for these proteins was 45%,
indicating significant expression profile with a high level of confidence.

Example 6

10

In order to generate milk bioactives that closely resemble those found in human breast milk, different methods using bone morphogenetic protein 4 (BMP4) and retinoic acid (RA) in 42 days and 31 days as a shortened time-course protocol were established in a 3D-platform using iPSCs derived mammary gland organoids as a
15 biomimetic model of the human mammary gland (Figure 8).

3D-iPSC cells in 42 days protocol and shortened time-course protocol (31 days -
describes shortened-modified protocols (31 days) including BMP4 and RA combinations) using 20 ng/mL of BMP4 and 1 μ M of RA in single or combination
20 format compared to the control condition can induce the expression of mammary gland progenitor markers such as EpCAM (CD326), CD49f, MUC1 (CD227) and GATA3 using flow cytometry quantification or maintain their expression profile during the differentiation time-course and/or pre-induction stages (Figure 9a-c) and post-induction period (Figure 9d-f).

25

We assessed the expression of different mammary epithelium markers in lactocytes derived from iPSCs in normal length and shortened protocols (Figure 10). This shortened protocol induces RELA as a specific gene for determining the lineage of the mammary epithelial cells during iPSC differentiation (Figure 10a). In addition to

the mature luminal markers such as GATA3, specific lactocyte markers such as EpCAM, KRT8/18 are also induced in the shortened protocol (Figure 10b-e). In addition, when we switch to the 31-day protocol, the ESRR level is induced (Figure 10f). As well, the shortened differentiation progress is associated with elevated
5 levels of progenitor markers for mammary glands such as CD24 and ITGA6 (CD49f) (Figure 10g-h).

Human osteopontin peptides - GDSVYGLR and -YPDAVATWLNPDPSQK were specifically detected in the cell culture supernatant by LC-MS/MS (Figure 11).

10

Example 7

A more systematic analysis of one specific human breast milk bioactive component, exosomes, as produced by Step A revealed very close profiles of composition with
15 exosomes from human breast milk positive control.

Human iPSC were aggregated in 3D-culture using ultra-low binding 6-well plates with continuous rotation shaking to form embryoid bodies, and then subjected to a multi-steps differentiation protocol (41 days) to generate functional mammary
20 gland organoids. The last 7 days of the differentiation protocol (from day 35 to day 41), the mammary gland organoids were subjected to a specific culture media supplemented with lactogenic hormones and growth factors to induce the secretion of milk-specific bioactive compounds. This media was renewed every other day (Day 35, Day 37, and Day 39).

25

Conditioned media was collected at Day 39 and Day 41 from step A, and pooled (which corresponds to the media that was in contact with the cells from Day 37 to Day 41). EVs were purified from the conditioned media and from skim human milk

(commercially available at Lee Biosolutions, 991-01-P, collected from at least 5 lactating women after week 4 of lactation) by ultracentrifugation.

Analysis of the EV-associated protein profile by liquid chromatography – mass spectrometry (LC-MS) showed an overlap of approximately 30-50% when comparing breast milk control EVs and cell-based derived EVs (Figure 12a). Figure 12b shows that most of the proteins highly expressed in breast milk exosomes are also expressed in the cell-based exosomes including lactotransferrin (LTF), annexin A2 (ANXA2) and lactadherin (MFGE8), but also Muc1, CD9, CD81, and CD63. Interestingly some proteins such as C4a anaphylatoxin (CFA), Hypoxia up-regulated protein 1 (HYOU1), Apolipoprotein A-IV (APOA4), Procollagen-lysine,2-oxoglutarate 5-dioxygenase 1 (PLOD1), Threonine—tRNA ligase 1, cytoplasmic (TARS1), and Cytosolic non-specific dipeptidase (CNDP2), are not detected in cell-based EVs but only in breast milk EV positive control.

Subsequent repetition of this experiment with 2 additional batches of cell-based EVs further confirmed that proteins Hypoxia up-regulated protein 1 (HYOU1), Apolipoprotein A-IV (APOA4) and Procollagen-lysine,2-oxoglutarate 5-dioxygenase 1 (PLOD1) are not detected in cell-based EVs but only in breast milk EV positive control. In addition, proteins C4a anaphylatoxin (CFA), Threonine—tRNA ligase 1, cytoplasmic (TARS1) and Cytosolic non-specific dipeptidase (CNDP2) were also not detected in one of the 2 additional cell-based EVs batches.

The proteomics analysis may be performed according to the following apparatus and conditions:

Mass spectrometer	Orbitrap Fusion™ Lumos™ Tribrid™
HPLC system	Vanquish™ Neo UHPLC System, 300 nL/min
HPLC column	Acclaim™ PepMap™ RSLC C18 2µm 50 cm×75 µm

Overall HPLC gradient time	180 min
Amount of proteins to prepare the samples	25 µg
Amount of peptides injected per LC MS/MS analysis (<i>i.e.</i>, on column)	1 µg or 2.5 µg
Software for protein/peptide identification	Mascot v2.8.2 and Scaffold v5.0.0
Database for protein identification	SwissProt Human (20221118, 20607 entries)
Criteria of protein identifications	Peptide FDR: 1% Protein FDR: 1% 1 or 2 peptide(s) minimum

Analysis of the miRNA profile of EVs sequenced with HTG Molecular Whole Transcriptome Assay showed strong 95-99% overlap between breast milk and cell-based exosomes (Figure 13a), including detection of already validated miRNA such as miR-148a-3p, miR-22-3p, miR-125b-5b and miR-6126 (Figure 13c). The results obtained from subsequent repetition of this experiment with 2 additional batches of cell-based EVs are shown in Figure 13b.

Analysis of the lipid fractions with the Lipometrix method (LC-MS/MS untargeted lipidomic analysis) in EVs purified from both conditioned media and human milk revealed similar overall profiles of lipid classes including cholesterol esters, ceramides, triacylglycerides, diacylglycerides, total phospholipids, alkanyl phospholipids, and alkenyl phospholipids (Figure 14). Same observation when analyzing subclasses profiles as phospholipid profile.

Flow cytometry analysis of the isolated EVs either from epithelial cells, iPSCs (conditioned media (StepA)) or from breast milk control showed a similar size (see Figure 15).

5 Example 8

Tight junction functionality of gut epithelial cells by measuring transepithelial electrical resistance (TEER)

Material and Methods

10 Caco-2 HTB-37 cells were cultured on transwell filters for 3 weeks to allow them to differentiate and form a tight barrier. Cells were seeded at a concentration 6.72×10^4 cells/well in 12 well-insert plates and culture in DMEM medium supplemented with heat-inactivated fetal bovine serum (10%), non-essential aminoacids (1%), and penicillin/streptomycin solution (1%). After 3 weeks, differentiated cells were then
15 cultured in exosome-free culture medium and put in presence of the following: PBS (vehicle), 1 μ M dexamethasone, 1.107 human-milk derived (HM EV) exosomes/well or 1.107 lactocyte-derived exosomes/well (Lactocyte EV). Then cells were exposed to an inflammatory challenge consisting of a pre-stimulation with Interferon (IFN) γ for 18 hours to trigger Tumor Necrosis Factor (TNF) α receptors expression, and a
20 stimulation with TNF α for 48 hours. Transepithelial electrical resistance (TEER) was monitored as a marker of epithelial barrier integrity.

Results

25 Figure 16 shows the effect of treatment of Caco-2 monolayers with dexamethasone (1 μ M), human milk-derived EVs (HM EVs) (1.107 EVs/well), and lactocyte-derived EVs (1.107 EVs/well), prior to the application of IFN γ and TNF α . Control is Caco-2 cells without any challenge. Figure 17 shows the effect of treatment of Caco-2 monolayers with dexamethasone (1 μ M), human milk-derived EVs (HM EVs) (1.107
30 EVs/well), and lactocyte-derived EVs (1.107 EVs/well), prior to the application of IFN γ and TNF α . Values show the impact after 48 hours of inflammatory challenge.

Control is Caco-2 cells without any challenge.

Human milk EVs exerted a protective effect toward alteration of barrier integrity triggered by proinflammatory cytokines, reaching 43.1% of protection compared to
5 control (cells with no challenge). Lactocyte-derived EVs showed similar efficacy in preventing the drop of TEER.

It should be understood that various changes and modifications to the presently preferred embodiments described herein will be apparent to those skilled in the art.
10 Such changes and modifications can be made without departing from the spirit and scope of the present invention and without diminishing its attendant advantages. It is therefore intended that such changes and modifications be covered by the appended claims.

15

Claims

1. An isolated human breast milk exosome, wherein Hypoxia up-regulated protein 1 (HYOU1), Apolipoprotein A-IV (APOA4) and Procollagen-lysine,2-oxoglutarate 5-dioxygenase 1 (PLOD1) are not detected by liquid chromatography – mass spectrometry in the exosome.
5
2. The exosome of claim 1, wherein C4a anaphylatoxin (CFA), Hypoxia up-regulated protein 1 (HYOU1), Apolipoprotein A-IV (APOA4), Procollagen-lysine,2-oxoglutarate 5-dioxygenase 1 (PLOD1), Threonine—tRNA ligase 1, cytoplasmic (TARS1), and Cytosolic non-specific dipeptidase (CNDP2) are not detected by liquid chromatography – mass spectrometry in the exosome.
10
3. The exosome of claim 1 or claim 2, wherein the exosome comprises lactotransferrin (LTF), annexin A2 (ANXA2) and lactadherin (MFGE8), MUC1 and tetraspanins CD9, CD81 and CD63.
15
4. The exosome of any one of claims 1 to 3, wherein the exosome comprises one or more miRNAs selected from miR-148a-3p, miR-22-3p, miR-125b-5b and miR-6126.
20
5. The exosome of any preceding claim, wherein the exosome comprises one or more lipids selected from cholesterol esters, ceramides, triacylglycerides, diacylglycerides, lyso-phospholipids, total phospholipids, alkanyl phospholipids, and alkenyl phospholipids.
25
6. The exosome of any preceding claim, wherein the exosome comprises one or more phospholipids selected from sphingomyelins, phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, and phosphatidylserine.

7. The exosome of any preceding claim, wherein the exosome has a diameter of 50 to 100nm.
8. The exosome of claim 7, wherein the exosome has a diameter size of 65 to 75nm.
9. The exosome of any preceding claim, wherein the exosome has the same function as exosomes derived from natural human breast milk.
10. The exosome of any preceding claim, wherein the exosome has the same structure as exosomes derived from natural human breast milk.
11. A population of exosomes, wherein the population comprises exosomes of any preceding claim.
12. A composition comprising exosomes or a population of exosomes according to any preceding claim.
13. An *in vitro* method for producing an exosome according to any one of claims 1 to 10 or population or exosomes according to claim 11, the method comprising:
- A) Culturing mammary epithelial cells in a culture medium to generate lactocyte mammary-like gland organoids; and
 - B) Secreting a mammalian milk like product from said lactocytes, and
 - C) Purifying the exosomes from the mammalian milk like product to remove impurities, optionally by chromatography or filtration or ultracentrifugation, in order to isolate the exosomes.
14. The method of claim 13, wherein the time duration of step A) is no longer than 14 days, optionally is 14 days.

15. The method of claim 13 or 14, wherein Step A) is conducted in 3D suspension culture conditions.
16. The method of claims 13 to 15, wherein step C) further comprises formulating
5 the isolated exosomes into a powder form, optionally by spray drying or freeze drying, or liquid form.
17. The method of claims 13 to 16, wherein step C) further comprises sterilising the isolated exosomes.
- 10 18. The method of claims 13 to 17, wherein step C) further comprises storing the isolated exosomes at below freezing, optionally at -80°C.
19. The method of claims 13 to 18, further comprising formulating the isolated
15 exosomes with supplementary nutritional ingredients.
20. The method of claims 13 to 19, wherein the isolated exosomes are dispensed into a container for consumption.
- 20 21. A human breast milk exosome product which is obtainable according to the method of any one of claims 13 to 20.
22. A method of producing a human milk fortifier, comprising conducting the method of any one of claims 13 to 20
- 25 23. The exosome according to any one of claims 1 to 10, the population of exosomes according to claim 11, the composition according to claim 12, or the exosome product according to claim 21, for use in therapy.

24. A method of treating a disease or disorder comprising administering to a subject in need thereof a therapeutically effective amount of the exosomes according to any one of claims 1 to 10, the population of exosomes according to claim 11, the composition according to claim 12, or the exosome product according to claim 21.

5

25. The exosome according to any one of claims 1 to 10, the population of exosomes according to claim 11, the composition according to claim 12, or the exosome product according to claim 21, for use in preventing, treating, or ameliorating symptoms associated with inflammation.

10

26. A method of preventing, treating, or ameliorating symptoms associated with inflammation, the method comprising administering to a subject in need thereof a therapeutically effective amount of the exosomes according to any one of claims 1 to 10, the population of exosomes according to claim 11, the composition according to claim 12, or the exosome product according to claim 21.

15

27. The exosome according to any one of claims 1 to 10, the population of exosomes according to claim 11, the composition according to claim 12, or the exosome product according to claim 21, for use according to claim 23, or the method according to claim 24, wherein inflammation is intestinal inflammation.

20

28. The exosome according to any one of claims 1 to 10, the population of exosomes according to claim 11, the composition according to claim 12, or the exosome product according to claim 21, for use according to claim 23, or the method according to claim 24, wherein inflammation is bronchial inflammation.

25

29. The exosome according to any one of claims 1 to 10, the population of exosomes according to claim 11, the composition according to claim 12, or the exosome product

according to claim 21, for use according to claim 23, or the method according to claim 24, wherein inflammation is respiratory inflammation.