UNIVERSITA' POLITECNICA DELLE MARCHE

PH.D. SCHOOL OF BIOMEDICAL SCIENCES

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LIPID METABOLISM IN THE NEONATE

METABOLISMO LIPIDICO NEL NEONATO

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Abstract

English version:

Infants admitted to neonatal intensive care unit, especially those born prematurely and with a low birth weight, may require enteral and parenteral nutrition (PN) with lipids. Dietary lipids are essential for infants to achieve nutrient and energy requirements to ensure an adequate growth. Early life malnutrition and poor in-hospital growth have been associated with later impaired long-term growth and neurodevelopment.
This thesis describes a number of investigations on lipid metabolism in enterally and parenterally fed infants.
Our data suggest that human placenta tends to limit the availability of phytosterols (naturally occurring sterols found in plant-derived products, such as vegetable oils) to the foetus. Phytosterol metabolism in preterm infants on routine PN with vegetable oils is reduced in comparison with term infants and adults: lowest esterification and slow elimination from the bloodstream. In case of cholestasis, the ability of preterm infants to manage phytosterols in intravenous (IV) lipid emulsions (LE) is markedly low. A high enteral phytosterol intake as it occurs in infant milk formula-fed infants results in elevated plasma phytosterol levels both in preterm and term infants. A low phytosterol diet should be preferred for infants.
We found that IV fish oil do not negatively affect weight gain in small preterm infants. New generation IV LE containing fish oil appear to be safe for preterm infants but their effect on organ growth (such as brain and lungs) deserves further studies.
In a case-control study, we found that hypertriglyceridemia (HiTG) affects IV LE intakes in small preterm infants on routine PN. We did not find any association between HiTG and reduced growth and poorer neurodevelopment. The recommended doses of IV LE may still be in excess compared to those effectively required for adequate growth and metabolism of small preterm infants. The benefits of the extra IV LE during HiTG remain to be clarified.

Italian Version:

I neonati nel reparto di terapia intensiva neonatale, specialmente quelli nati pretermine e con basso peso alla nascita, possono aver bisogno di nutrizione enterale e parenterale (NP) con lipidi. I lipidi forniti con la dieta sono essenziali per raggiungere i requisiti nutrizionali ed energetici necessari per una corretta crescita. Uno stato di malnutrizione nella vita post-natale e una scarsa crescita intra-ospedaliera sono state associate ad un ritardo di crescita a lungo termine e del neurosviluppo.
Questa tesi descrive studi sul metabolismo lipidico di neonati alimentati per via enterale e parenterale.
I nostri dati suggeriscono che la placenta umana limita la disponibilità di fitosteroli (steroli di origine naturale presenti in prodotti derivati da piante come gli oli vegetali) al feto. Il metabolismo dei fitosteroli nel neonato pretermine in NP è ridotto rispetto a quello del neonato a termine e dell'adulto: più bassa esterificazione e lenta eliminazione dal torrente sanguigno. La difficoltà di metabolizzare i fitosteroli è marcatamente ridotta in caso di colestasi. L'assunzione orale/enterale di alimenti con elevato contenuto di fitosteroli (es. formule per l'infanzia a base di oli vegetali) è associata ad alti livelli di fitosteroli plasmatici nei neonati pretermine e a termine. Una dieta con basso contenuto di fitosteroli è preferibile per i neonati.
L'olio di pesce assunto per via intravenosa non influenza negativamente la crescita ponderale dei pretermine. Le emulsioni con olio di pesce sembrano essere sicure anche se il loro effetto sulla crescita di organi come cervello e polmoni richiede ulteriori studi.
L'ipertrigliceridemia è associata ad una riduzione degli apporti dei lipidi intravenosi ma non a ridotta crescita e neurosviluppo nei pretermine. Le dosi raccomandate di lipidi intravenosi potrebbero essere in eccesso rispetto a quelle necessarie per una corretta crescita. I benefici di lipidi extra in caso di ipertrigliceridemia restano da chiarire.

Chapter 1

Introduction

General Introduction

Lipids are an important component of the neonatal nutritional support as they provide essential fatty acids - i.e. the omega-6 polyunsaturated fatty acid (PUFA) linoleic acid (LA) and the omega-3 PUFA α-linolenic acid (ALA) -, building blocks for cell membranes and a concentrated source of calories for growth and development.

Patients in a neonatal intensive care unit (NICU), especially those born prematurely and/or with a low birth weight, may require enteral and parenteral nutrition with lipids. Dietary lipids are currently administered to meet target energy requirements as they were experimentally identified in the last century, even if small dose modifications were done according to the clinical evidence in the recent years. The optimal intake and the blend of lipids which should be used to achieve the best growth and development in infants remain debated.

Human milk (HM) is the best source of lipids for enterally fed infants. The amount of lipids per volume is strikingly different between fore- and hind HM, but the fatty acid composition is not different[1]. Infants whose mothers decided not to or were unable to provide their own HM were assigned to formulas which mainly contain a blend of vegetable oils (e.g. soybean oil, SO; olive oil, OO; medium-chain triglyceride oil, MCT oil, derived from coconut oil) as a lipid source. The different sources of lipids in commercially available formulas are combined to mimic the fatty acid composition of HM[2].

SO is a good source of the essential omega-6 PUFA LA (about 50% of fatty acids) and it also contains some (about 7% of fatty acids) of the essential omega-3 PUFA ALA. MCT oil and OO contain high amount of MCT and mono-unsaturated fatty acids (e.g. oleic acid), respectively, but low level or no omega-6 PUFA[3]. Newer infant formulas also contain algal-derived DHA to ensure an omega-3 PUFA content similar to that of HM. A non-animal alternative to fish derived DHA is used due to some evidence showing that infants who received oral fish oil had poorer growth than controls[4].

Among the additional components of vegetable oil-based formulas there is varying amount of *phytosterols*[5] (see paragraph 1.1 for more details), a naturally occurring constituents of plants that are structurally similar to cholesterol yet differ in side chain configuration, as illustrated in **Fig. 1**. These compounds are not synthesized in mammals, and therefore are derived solely from the diet. Phytosterol concentration in HM is negligible. It is well established that dietary phytosterols reduced plasma cholesterol concentrations by inhibiting intestinal cholesterol absorption[6]. The cholesterol-lowering proprieties of phytosterols are used to prevent hypercholesterolemia in human adults but they may represent disadvantages for growing infants. Accumulating evidence also suggests that high circulating levels of phytosterols may be linked to an increased risk of diseases at short- and medium-term[7].

According to the current European parenteral nutrition guidelines in paediatric, intravenous lipids should be started immediately after birth (<2nd day of life) and a continuous lipid infusion over 24 h should be provide[8]. Lipid infusion should not exceed the lipoprotein lipase (LPL) clearance capacity to avoid *hypertriglyceridemia* and its associated complications, such as fever, jaundice, respiratory distress, etc[9]. Lipid metabolism in infants on parenteral nutrition is usually monitored by repeated triglyceride measurements on micro-blood samples. Plasma

triglycerides should be monitored within 1-2 days after initiation or adjustment of intravenous lipids and thereafter on weekly to monthly basis. However, the threshold and timing of monitoring triglycerides in this group of infants is still a matter of debate.

Several intravenous lipid emulsions are now available on the market for use in paediatrics and all of them are based on vegetables (soybean, SO; olive, OO; medium-chain triglycerides, MCT). At present, Omegaven® is the only lipid intravenous lipid emulsion which does not contain vegetable oils, but exclusively *fish oil* (FO; **Table 2**). FO is a good source of omega-3 PUFA (e.i. eicosapentaenoic, EPA; and docosahexaenoic acid, DHA) which play an important role in central nervous system development, and thus it is introduced in the last generation of intravenous lipid emulsions. Recent clinical evidence support the use of FO containing lipid emulsions due to their anti-inflammatory and immune modulatory effects and their ability in improving hepatic metabolism and liver function, including reversal of cholestasis[10]. Pro-inflammatory omega-6 PUFA in vegetable oil-rich lipid emulsions were considered one of the responsible factors for parenteral nutrition-associated cholestasis in infants. Phytosterols were also associated to cholestasis in infants on parenteral nutrition[11-12]. Although, FO containing lipid emulsions have emerged as promising fat formulations for parenteral nutrition, information on medium and long-term effects of intravenous FO in infants remains scanty.

REFERENCES

1: Demmelmair H, Koletzko B. Lipids in human milk. Best Pract Res Clin Endocrinol Metab. 2018 Jan;32(1):57-68. doi: 10.1016/j.beem.2017.11.002. Epub 2017 Nov 14. Review. PubMed PMID: 29549961.

2: Hamdan IJA, Sanchez-Siles LM, Garcia-Llatas G, Lagarda MJ. Sterols in Infant Formulas: A Bioaccessibility Study. J Agric Food Chem. 2018 Feb 14;66(6):1377-1385. doi: 10.1021/acs.jafc.7b04635. Epub 2018 Feb 2. PubMed PMID: 29369630.

3: Calder PC, Adolph M, Deutz NE, Grau T, Innes JK, Klek S, Lev S, Mayer K, Michael-Titus AT, Pradelli L, Puder M, Vlaardingerbroek H, Singer P. Lipids in the intensive care unit: Recommendations from the ESPEN Expert Group. Clin Nutr. 2018 Feb;37(1):1-18. doi: 10.1016/j.clnu.2017.08.032. Epub 2017 Sep 7. Review. PubMed PMID: 28935438.

4: Carlson SE, Cooke RJ, Werkman SH, Tolley EA. First year growth of preterm infants fed standard compared to marine oil n-3 supplemented formula. Lipids. 1992 Nov;27(11):901-7. PubMed PMID: 1491608.

5: Claumarchirant L, Matencio E, Sanchez-Siles LM, Alegría A, Lagarda MJ. Sterol Composition in Infant Formulas and Estimated Intake. J Agric Food Chem. 2015 Aug 19;63(32):7245-51. doi: 10.1021/acs.jafc.5b02647. Epub 2015 Aug 11. PubMed PMID: 26242905.

6: Jesch ED, Carr TP. Food Ingredients That Inhibit Cholesterol Absorption. Prev Nutr Food Sci. 2017 Jun;22(2):67-80. doi: 10.3746/pnf.2017.22.2.67. Epub 2017 Jun 30. Review. PubMed PMID: 28702423; PubMed Central PMCID: PMC5503415

7: Zaloga GP. Phytosterols, Lipid Administration, and Liver Disease During Parenteral Nutrition. JPEN J Parenter Enteral Nutr. 2015 Sep;39(1 Suppl):39S-60S. doi: 10.1177/0148607115595978. Epub 2015 Jul 15. Review. PubMed PMID: 26177665.

8: Lapillonne A, Fidler Mis N, Goulet O, van den Akker CHP, Wu J, Koletzko B; ESPGHAN/ESPEN/ESPR/CSPEN working group on pediatric parenteral nutrition. ESPGHAN/ESPEN/ESPR/CSPEN guidelines on pediatric parenteral nutrition: Lipids. Clin Nutr. 2018 Jun 18. pii: S0261-5614(18)31165-8. doi: 10.1016/j.clnu.2018.06.946. [Epub ahead of print] PubMed PMID: 30143306.

9: Wanten GJ. Parenteral lipids: safety aspects and toxicity. World Rev Nutr Diet. 2015; 112:63-70. doi: 10.1159/000365434. Epub 2014 Nov 24. Review. PubMed PMID: 25471803.

10: Edward RR, Innes JK, Marino LV, Calder PC. Influence of different intravenous lipid emulsions on growth, development and laboratory and clinical outcomes in hospitalised paediatric patients: A systematic review. Clin Nutr. 2018 Jun;37(3):765-783. doi: 10.1016/j.clnu.2017.07.003. Epub 2017 Jul 8. Review. PubMed PMID: 28712532.

11: Clayton PT, Bowron A, Mills KA, Massoud A, Casteels M, Milla PJ. Phytosterolemia in children with parenteral nutrition-associated cholestatic liver disease. Gastroenterology. 1993 Dec;105(6):1806-13. PubMed PMID: 8253356.

12: Kurvinen A, Nissinen MJ, Andersson S, Korhonen P, Ruuska T, Taimisto M, Kalliomäki M, Lehtonen L, Sankilampi U, Arikoski P, Saarela T, Miettinen TA, Gylling H, Pakarinen MP. Parenteral plant sterols and intestinal failure-associated liver disease in neonates. J Pediatr Gastroenterol Nutr. 2012 Jun;54(6):803-11. doi: 10.1097/MPG.0b013e3182474118. PubMed PMID: 22197940.

1.1 PHYTOSTEROLS

The following paragraphs provide information on phytosterols related to their molecular structure (1.1.1), the fat of enteral and parenteral phytosterols (1.1.2), their content in human milk, infant formulas (1.1.3) and in intravenous lipid emulsions (1.1.4), and their role on the onset of parenteral nutrition-associated cholestasis (1.1.5).

1.1.1 Molecular Structure

Phytosterols are natural compound structurally similar to mammalian cell-derived cholesterol but include a methyl or ethyl group at C-24, as illustrated in **Fig. 1**. They exist in the free form and as esters of fatty acid/cinnamic acid or as glycosides. They found in all plant derived food, with the highest concentrations occurring in vegetable oils. The most abundant phytosterols in vegetable oils are β-sitosterol, campesterol and stigmasterol[1].

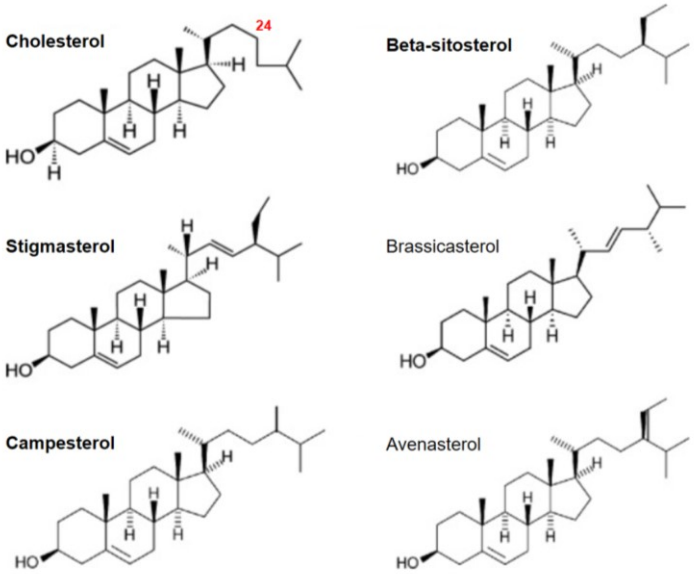

Fig 1. Structure of cholesterol and phytosterols which are abundant in vegetable oils [modified from Tong Wang. AOCS Press. 2008, ISBN 9781893997646].

1.1.2 Intestinal Absorption and Intravenous Administration

In human adults, dietary phytosterol intake has been estimated to vary from 150 mg/day (Western diet) to 450 mg/day (Asian diet)[2, 3, 4]. Vegetarians and vegans generally have the highest intake of dietary phytosterols (up to 800 mg/day)[5]. The intestinal absorption of phytosterols is very low compared with that of cholesterol, which is about 50%. Less than 5% of the phytosterols consumed are intestinally absorbed[6]. As cholesterol, dietary phytosterols must be incorporated into mixed micelles in order to be absorbed by enterocytes. Mixed micelles are mixtures of bile salts, lipids, and sterols formed in the small intestine after consumption of a fat-containing meal[7]. Cholesterol and phytosterols are taken up into enterocytes after hydrolysis by lipases in mixed micelles. Transport both of cholesterol and phytosterols across the apical membrane of enterocytes is mediated by intestinal transporter,

Niemann Pick C1-Like 1 (NPC1L1)[8]. Once inside the enterocyte, cholesterol is esterified in a reaction catalyzed by intestinal acyl-coenzyme A (CoA) cholesterol acyltransferases ($ACAT_2$; also present in the liver) and incorporated together with phytosterols into chylomicrons, which are secreted into the intestinal lymphatics[9]. Phytosterols are not as readily esterified as cholesterol, so they are incorporated into chylomicrons at much lower concentrations. A small amount of absorbed cholesterol and phytosterols are extruded through the basolateral ATP binding cassette transporter A1 (ABCA1) to enter the high-density lipoprotein (HDL) fraction, in part formed locally in the intestine from locally generated apoAI[10]. However, the absorption of phytosterols is inhibited by the activity of an efflux transporter, consisting of a pair of ATP-binding cassette (ABC) proteins known as ABCG5 and ABCG8. ABCG5/G8 transporters are the most important transmembrane proteins involved in secreting phytosterols into the intestinal lumen. Un-esterified cholesterol was also secreted by ABCG5/G8 from the enterocyte into the intestinal lumen. Phytosterols are secreted back into the intestine by ABCG5/G8 transporters at a much greater rate than cholesterol, resulting in much lower intestinal absorption of dietary phytosterols than cholesterol[11]. Campesterol has been reported to have the highest absorption rate among different phytosterols[12].

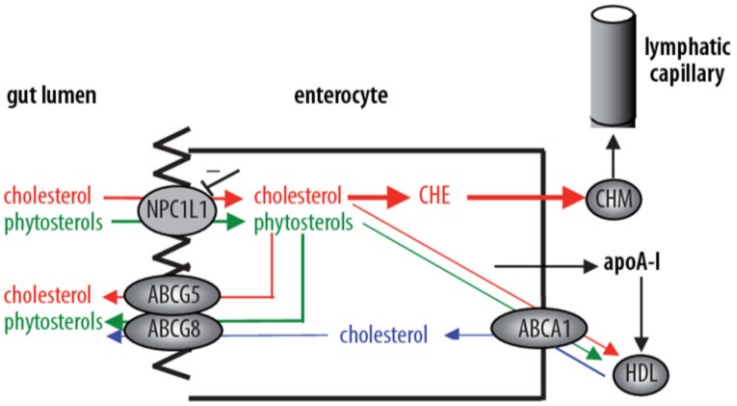

Fig. 2. Intestinal phytosterol handling (green arrows), intestinal cholesterol absorption (red arrows) and the role of enterocytes in excretion of cholesterol delivered from peripheral tissues by HDL (i.e. alternative reverse cholesterol transport, blue arrows) [modified from Wójcicka G et al. Postepy Hig Med Dosw. 2007, PMID: 18063918].

As circulating chylomicrons become depleted of triglycerides, they become chylomicron remnants, which are taken up by the liver. In the liver, cholesterol and phytosterols from chylomicron remnants or HDL may be repackaged into other lipoproteins for transport throughout the circulation or, alternatively, secreted into bile, which is released into the small intestine. Both cholesterol and phytosterols are secreted into bile by hepatic ABCG5/G8 transporters. However, the rate of phytosterol secretion into bile is much greater than cholesterol secretion. Among phytosterols, beta-sitosterol is secreted at highest rate by the liver[13]. The low serum concentrations of phytosterols relative to cholesterol can be explained by decreased intestinal absorption and increased excretion of phytosterols into bile.

In the circulation, phytosterols are carried mainly in low density lipoprotein (LDL) (70%–80%) and in high density lipoprotein (HDL) particles (20%–30%)[14]. Lecithin cholesterol acyltransferase (LCAT) in plasma and $ACAT_1$ in macrophages and many tissue are responsible for lipoprotein metabolism by cholesterol and phytosterol esterification[15].
On the contrary, intravenous administration of phytosterols, as it occurs in individuals on parenteral nutrition with vegetable oils, bypasses normal intestinal mechanisms of excretion that prevent against phytosterol accumulation in the body and thus large amount of phytosterols are arrived and handled by the liver.

1.1.3 Human Milk and Infant Formulas

HM is the best food for infants because it contains the complete essential nutrients required for growth and development. Infant formula is the food provided as replacement for HM. Infant formulas currently available on the marker are based on vegetable oils and contain large amount of phytosterols[16]. HM contains only traces of phytosterols[17]. Phytosterol content in HM and in selected infant formulas are reported in **Table 1**.

	Human milk**	Prenidina®	Aptamil pre®	Mellin 0®
Phytosterol content (mean), mg/dl	trace	3.8	5.6	6.2

Table 1. Phytosterol concentrations (sum of beta-sitosterol, campesterol and stigmasterol) in HM and infant formulas available at our institution. **Phytosterols in premature human milk were obtained from 10 samples from different mothers at about one-month post-partum. Trace<0.5.

The large amount of phytosterols ingested by infants fed with vegetable oil-based formulas may interfere with cholesterol absorption[18]. It has been reported that dietary phytosterols compete with cholesterol for incorporation into mixed micelles and with cholesterol uptake by enterocytes[19]. In addition, the cholesterol content of infant formulas (about 2-4 mg/dl) is very low in comparison with that of HM (about 10-38 mg/dl)[20]. As result, patients taking vegetable oil-based infant formulas may be exposed to a higher serum phytosterol concentrations and lower serum cholesterol concentration compared to those taking HM. In 1976, Mellies et al. first reported that infants (ages 1 to 12 months) and children fed with formulas, containing both vegetable oils and low cholesterol, quadrupled their serum phytosterol concentrations (from 2.2 mg/dl in HM fed patients to 9.2 mg/dl)[21].
To the best of our knowledge, no further data is available on phytosterols both in enterally fed preterm and term infants. Absorption of phytosterols may be potentially greater during infancy during childhood and adulthood, exposing them to potentially adverse effects. It is reported that infants receiving vegetable oil-based diets accumulate phytosterols in their tissues[22]. Medium- and long-term effects of phytosterol accumulation in the human body remain unknown. A low serum cholesterol concentration in patients fed with vegetable oils containing infant formulas has been documented by some authors[23, 24]. This is importance particularly for pre-mature infants who need to grow faster in the early stage after birth. Of note, an exclusive HM feeding has been associated with low blood cholesterol later in life and therefore

proper caution should be taken in the infant formulas production with low cholesterol and/or high phytosterol content[25].

1.1.4 Intravenous Lipid Emulsions

Intravenous lipid emulsions are an essential component of neonatal parenteral nutrition, especially for infants born prematurely[26]. Both older and newer parenteral nutrition guidelines strongly suggest starting intravenous lipids as soon as possible after birth to prevent essential fatty acid deficiency[27, 28]. An adequate intake of fatty acid precursors of long-chain PUFA, such as AA and DHA, are required for a proper growth and development[29] (**Fig. 3**). Serum triglyceride monitoring is currently recommended to check the adequate lipid intakes of infants on parenteral nutrition. Elevated triglyceride values usually determine intravenous lipid titration or halting but consequences of this clinical practice are scanty unexplored. We approached the parenteral nutrition management of preterm infants in case of hypertriglyceridemia in *Chapter 7*.

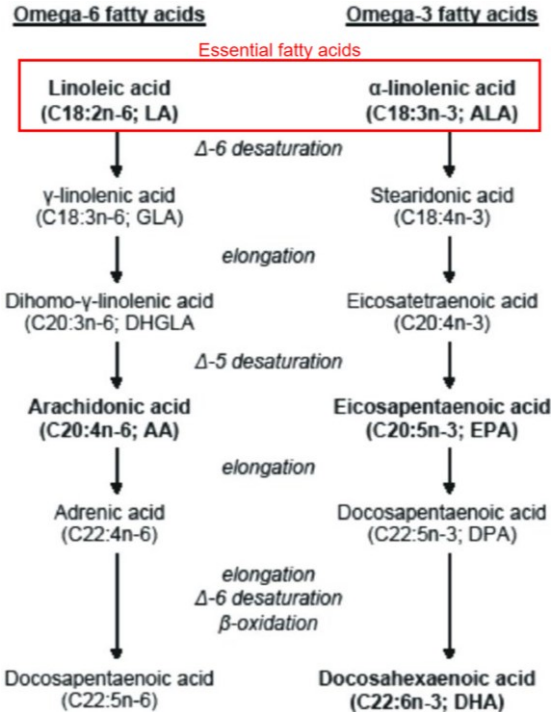

Fig 3. Metabolism of omega-3 and omega-6 fatty acids [modified from Katalin Fekete and Tamás Decsi. Nutrients. 2010, PMID: 22254065].

Vegetable oils are the main source of fat in commercially available lipid emulsions. The first generation of lipid emulsions consisted purely of SO, with the second generation lipid emulsions including MCT and the third generation beginning with the inclusion of OO. Most recently, third generation lipid emulsions containing FO have been introduced. The blend of lipids used for parenteral nutrition is rapidly evolved over time due an intensive research in

developing formulations which mimic neonatal fatty acid requirements as they were estimated for normal foetal growth in utero. SO, MCT oil and OO are used as good sources of omega-6, medium chain and unsaturated fatty acids, respectively. Omega-3 fatty acids are mainly supplied by FO. Different vegetable oil-mixtures are explored over the years to obtain a balance of omega-6/omega-3 fatty acids as recommended. Inadequate nutrition during the early postnatal period has been associated with abnormal neurodevelopment in preterm infants[30, 31]. Suboptimal nutrition is also associated with adverse outcomes which include increased susceptibility to infections, greater need for mechanical ventilation and development of chronic lung disease[32]. For this reason, Omegaven®, the only intravenous lipid emulsions containing 100% FO and thus richest in omega-3 fatty acids, should be exclusively used in addition to another one (usually in a 1:1 ratio). Additional components of intravenous lipid emulsions include phytosterols, α-tocopherol and phospholipids, usually phosphatidylcholine (sometimes called lecithin), as an emulsifier. Oil and typical fatty acid compositions (weight%) of available lipid emulsions for use in parenteral nutrition are shown in **Table 2**.

	Intralipid®	Lipofundin® MCT/LCT	Omegaven®	ClinOleic®	Lipidem®	SMOFlipid®
Oil source	100% SO	50% MCT + 50% SO	100% FO	80% OO + 20% SO	50% MCT +40% SO +10% FO	30% MCT + 30% SO + 25% OO + 15% FO
SFA, wt%	17	57	24	15	55	41
MUFA, wt%	23	12	21	63	12	32
Omega-3 PUFA, wt%	9	5	50	4	9	7
Omega-6 PUFA, wt%	51	26	5	18	24	20
Phytosterols**, mg/dl	31.6	26.6	trace	27.6	24.5	19.5

Table 2. Oil, fatty acid and phytosterol compositions of commercially available lipid emulsions for use in parenteral nutrition (FO: fish oil; MCT: medium chain triglycerides (from coconut oil); OO: olive oil; SO: soybean oil). SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; Trace<0.5. **campesterol, stigmasterol and beta-sitosterol. Data are measured in our laboratory by GC-FID and GC-MS.

Intravenous administration of large amount of phytosterols into the circulation may lead to phytosterol accumulation in the body and an increased hepatic exposure to these potential toxins. Support for phytosterol toxicity comes from patients with congenital phytosterolemia

where the toxic accumulation of phytosterols can lead to premature cardiovascular disease, haematological disorders, endocrine disruption, and possible liver cirrhosis[33]. Recently, it is reported that phytosterols are also involved in challenging normal mechanisms of hepatic sterol excretion: e.g. El Kasmi et al. in an animal model found that stigmasterol suppress canalicular bile transport expression through antagonism of FXR nuclear receptors and thus may be responsible for parenteral nutrition associated-cholestasis[34].

1.1.5 Parenteral Nutrition Associated-Cholestasis

Cholestasis represents impairment in the formation and/or secretion of bile and is one of the most common complications associated with parenteral nutrition in neonates. Infants who developed cholestasis have a higher risk to death and other complications in comparison to other infants during the hospital stay[35]. The aetiology of parenteral nutrition-associated cholestasis is so far unclear, but it is thought to result from multifactorial causes related to genetic, to dietary factors, and to liver injury. Intravenous intake of vegetable oil-based lipid emulsions containing phytosterols is felt to be an important contributing factor[36]. Phytosterols may cause cholestasis by interfering with the expression and function of transporters for sterols and bile acids within the hepatobiliary system[34, 37]. Clayton et al (1993) first hypothesized an association between phytosterols and the development of cholestasis in children receiving SO containing intravenous lipid emulsions[38]. Serum phytosterol concentrations of patients on parenteral nutrition were similar to that of sitosterolemic individuals and thus the authors speculated that phytosterols might be a responsible factor. The main mechanisms proposed were as follows:

(a) Phytosterol inhibit cholesterol 7alpha-hydroxylase, the rate-limiting step in bile acid synthesis (in vitro model). This lead to reduced bile acid-dependent bile flow[39];

(b) Phytosterols may be metabolised to polar bile acids, and these could potentially be cholestatic because they may lead to formation of biliary sludge[40, 41];

(c) Phytosterols may downregulate genes of transporters for sterols and bile acids promoting cholestasis[34, 37];

(d) Phytosterols may bind to sterol carrier proteins impairing movement of cholesterol, bile acid precursors, and other lipids across the cells[41];

(e) Similarity in structures between cholesterol and the major plant sterols could lead to substitution of cholesterol in the liver cell membranes by phytosterols interfering with membrane function[42].

As result, till date, FO containing intravenous lipid emulsions which have low phytosterol levels are more used than those with a high amount of SO in the neonatal clinical practice. A comprehensive understanding of hepatic advantages in using mixed lipid emulsions with low phytosterol levels is still understudied, but accumulating evidence demonstrate marked improvements in cholestasis after parenteral nutrition with FO[43, 44]. Parenteral FO contains mostly omega-3 fatty acids, which have anti-inflammatory properties and lack phytosterols. Whether the presence of this type of fatty acid that may play a role in improving bile flow or the absence of phytosterols or both are the reasons of its ability in reverse cholestasis in some patients is still unknown. Studies able to clarify the role of phytosterols in the development of

cholestasis are still needed. We provided additional information about phytosterol metabolism in small preterm infants receiving routine parenteral nutrition in *Chapters 2-6*.
In our unit at Azienda Ospedaliero-Universitaria Ospedali Riuniti Umberto I - G. M. Lancisi - G. Salesi, FO containing intravenous lipid emulsions are preferred to emulsions at high content of SO. Pure intravenous FO is used only for infants with severe cholestasis (conjugated bilirubin >2.0 mg/dL). However, our previous findings showed that intravenous FO interfere with lipogenesis leading to lower plasma lipid concentrations in infants[45, 46, 47]. This could be a reason of concern for neonatologists which should provide fat as soon as possible to achieve growth target. This controversial is still opened and we partially gave a response in *Chapter 8*.

REFERENCES

1. Ostlund RE, Jr. Phytosterols in human nutrition. Annual review of nutrition 2002, 22: 533-549.

2. Klingberg S, Andersson H, Mulligan A, Bhaniani A, Welch A, Bingham S, et al. Food sources of plant sterols in the EPIC Norfolk population. European journal of clinical nutrition 2008, 62(6): 695-703.

3. Valsta LM, Lemstrom A, Ovaskainen ML, Lampi AM, Toivo J, Korhonen T, et al. Estimation of plant sterol and cholesterol intake in Finland: quality of new values and their effect on intake. The British journal of nutrition 2004, 92(4): 671-678.

4. Phillips KM, Tarragó-Trani MT, Stewart KK. Phytosterol content of experimental diets differing in fatty acid composition. Food Chemistry 1999, 64(3): 415-422.

5. Vuoristo M, Miettinen TA. Absorption, metabolism, and serum concentrations of cholesterol in vegetarians: effects of cholesterol feeding. The American journal of clinical nutrition 1994, 59(6): 1325-1331.

6. Ostlund RE, Jr., McGill JB, Zeng CM, Covey DF, Stearns J, Stenson WF, et al. Gastrointestinal absorption and plasma kinetics of soy Delta(5)-phytosterols and phytostanols in humans. American journal of physiology Endocrinology and metabolism 2002, 282(4): E911-916.

7. de Jong A, Plat J, Mensink RP. Metabolic effects of plant sterols and stanols (Review). The Journal of nutritional biochemistry 2003, 14(7): 362-369.

8. Davis HR, Jr., Zhu LJ, Hoos LM, Tetzloff G, Maguire M, Liu J, et al. Niemann-Pick C1 Like 1 (NPC1L1) is the intestinal phytosterol and cholesterol transporter and a key modulator of whole-body cholesterol homeostasis. The Journal of biological chemistry 2004, 279(32): 33586-33592.

9. Howles PN. Cholesterol Absorption and Metabolism. Methods in molecular biology (Clifton, NJ) 2016, 1438: 177-197.

10. Wojcicka G, Jamroz-Wisniewska A, Horoszewicz K, Beltowski J. Liver X receptors (LXRs). Part I: structure, function, regulation of activity, and role in lipid metabolism. Postepy higieny i medycyny doswiadczalnej (Online) 2007, 61: 736-759.

11. Jones PJ, Winthrop AL, Schoeller DA, Swyer PR, Smith J, Filler RM, et al. Validation of doubly labeled water for assessing energy expenditure in infants. Pediatric research 1987, 21(3): 242-246.

12. Heinemann T, Axtmann G, von Bergmann K. Comparison of intestinal absorption of cholesterol with different plant sterols in man. European journal of clinical investigation 1993, 23(12): 827-831.

13. Sudhop T, Sahin Y, Lindenthal B, Hahn C, Luers C, Berthold HK, et al. Comparison of the hepatic clearances of campesterol, sitosterol, and cholesterol in healthy subjects suggests that efflux transporters controlling intestinal sterol absorption also regulate biliary secretion. Gut 2002, 51(6): 860-863.

14. Björkhem I. BKM, Leitersdorf E. Inborn errors in bile acid biosynthesis and storage of sterols other than cholesterol. In: Scriver CR, Beaudet AL, editors The Metabolic and Molecular Bases of Inherited Disease 8th Ed McGraw-Hill; New York, NY, USA: 2001: pp. 2961–2988.

15. Lin DS, Steiner RD, Merkens LS, Pappu AS, Connor WE. The effects of sterol structure upon sterol esterification. Atherosclerosis 2010, 208(1): 155-160.

16. Claumarchirant L, Matencio E, Sanchez-Siles LM, Alegria A, Lagarda MJ. Sterol Composition in Infant Formulas and Estimated Intake. Journal of agricultural and food chemistry 2015, 63(32): 7245-7251.

17. Jensen RG, Clark RM, Ferris AM. Composition of the lipids in human milk: a review. Lipids 1980, 15(5): 345-355.

18. Jones PJ, Hrboticky N, Hahn P, Innis SM. Comparison of breast-feeding and formula feeding on intestinal and hepatic cholesterol metabolism in neonatal pigs. The American journal of clinical nutrition 1990, 51(6): 979-984.

19. De Smet E, Mensink RP, Plat J. Effects of plant sterols and stanols on intestinal cholesterol metabolism: suggested mechanisms from past to present. Molecular nutrition & food research 2012, 56(7): 1058-1072.

20. Hodgson PA, Ellefson RD, Elveback LR, Harris LE, Nelson RA, Weidman WH. Comparison of serum cholesterol in children fed high, moderate, or low cholesterol milk diets during neonatal period. Metabolism: clinical and experimental 1976, 25(7): 739-746.

21. Mellies M, Glueck CJ, Sweeney C, Fallat RW, Tsang RC, Ishikawa TT. Plasma and dietary phytosterols in children. Pediatrics 1976, 57(1): 60-67.

22. Mellies MJ, Ishikawa TT, Glueck CJ, Bove K, Morrison J. Phytosterols in aortic tissue in adults and infants. The Journal of laboratory and clinical medicine 1976, 88(6): 914-921.

23. Normén L, Dutta P, Lia Å, Andersson H. Soy sterol esters and β-sitostanol ester as inhibitors of cholesterol absorption in human small bowel. The American journal of clinical nutrition 2000, 71(4): 908-913.

24. Tammi A, Ronnemaa T, Valsta L, Seppanen R, Rask-Nissila L, Miettinen TA, et al. Dietary plant sterols alter the serum plant sterol concentration but not the cholesterol precursor sterol concentrations in young children (the STRIP Study). Special Turku Coronary Risk Factor Intervention Project. The Journal of nutrition 2001, 131(7): 1942-1945.

25. Owen CG, Whincup PH, Kaye SJ, Martin RM, Davey Smith G, Cook DG, et al. Does initial breastfeeding lead to lower blood cholesterol in adult life? A quantitative review of the evidence. The American journal of clinical nutrition 2008, 88(2): 305-314.

26. Salama GS, Kaabneh MA, Almasaeed MN, Alquran MI. Intravenous lipids for preterm infants: a review. Clinical medicine insights Pediatrics 2015, 9: 25-36.

27. Lapillonne A, Fidler Mis N, Goulet O, van den Akker CHP, Wu J, Koletzko B, et al. ESPGHAN/ESPEN/ESPR/CSPEN guidelines on pediatric parenteral nutrition: Lipids. Clinical Nutrition 2018, 37(6): 2324-2336.

28. Koletzko B, Goulet O, Hunt J, Krohn K, Shamir R. 1. Guidelines on Paediatric Parenteral Nutrition of the European Society of Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) and the European Society for Clinical Nutrition and Metabolism (ESPEN), Supported by the European Society of Paediatric Research (ESPR). Journal of pediatric gastroenterology and nutrition 2005, 41 Suppl 2: S1-87.

29. Lapillonne A, Moltu SJ. Long-Chain Polyunsaturated Fatty Acids and Clinical Outcomes of Preterm Infants. Annals of nutrition & metabolism 2016, 69 Suppl 1: 35-44.

30. Lucas A, Morley R, Cole TJ. Randomised trial of early diet in preterm babies and later intelligence quotient. BMJ (Clinical research ed) 1998, 317(7171): 1481-1487.

31. Latal-Hajnal B, von Siebenthal K, Kovari H, Bucher HU, Largo RH. Postnatal growth in VLBW infants: significant association with neurodevelopmental outcome. The Journal of pediatrics 2003, 143(2): 163-170.

32. Ho MY, Yen Y, Hsieh MC, Chen HY, Chien SC, Hus-Lee SM. Early versus late nutrition support in premature neonates with respiratory distress syndrome. Nutrition (Burbank, Los Angeles County, Calif) 2003, 19(3): 257-260.

33. Ajagbe BO, Othman RA, Myrie SB. Plant Sterols, Stanols, and Sitosterolemia. Journal of AOAC International 2015, 98(3): 716-723.

34. El Kasmi KC, Anderson AL, Devereaux MW, Vue PM, Zhang W, Setchell KD, et al. Phytosterols promote liver injury and Kupffer cell activation in parenteral nutrition-associated liver disease. Science translational medicine 2013, 5(206): 206ra137.

35. Fischler B, Lamireau T. Cholestasis in the newborn and infant. Clinics and research in hepatology and gastroenterology 2014, 38(3): 263-267.

36. Zaloga GP. Phytosterols, Lipid Administration, and Liver Disease During Parenteral Nutrition. JPEN Journal of parenteral and enteral nutrition 2015, 39(1 Suppl): 39S-60S.

37. Carter BA, Taylor OA, Prendergast DR, Zimmerman TL, Von Furstenberg R, Moore DD, et al. Stigmasterol, a soy lipid-derived phytosterol, is an antagonist of the bile acid nuclear receptor FXR. Pediatric research 2007, 62(3): 301-306.

38. Clayton PT, Bowron A, Mills KA, Massoud A, Casteels M, Milla PJ. Phytosterolemia in children with parenteral nutrition-associated cholestatic liver disease. Gastroenterology 1993, 105(6): 1806-1813.

39. Boberg KM, Akerlund JE, Bjorkhem I. Effect of sitosterol on the rate-limiting enzymes in cholesterol synthesis and degradation. Lipids 1989, 24(1): 9-12.

40. Boberg KM, Lund E, Olund J, Bjorkhem I. Formation of C21 bile acids from plant sterols in the rat. The Journal of biological chemistry 1990, 265(14): 7967-7975.

41. Clayton PT, Whitfield P, Iyer K. The role of phytosterols in the pathogenesis of liver complications of pediatric parenteral nutrition. Nutrition (Burbank, Los Angeles County, Calif) 1998, 14(1): 158-164.

42. Pianese P, Salvia G, Campanozzi A, D'Apolito O, Dello Russo A, Pettoello-Mantovani M, et al. Sterol profiling in red blood cell membranes and plasma of newborns receiving total parenteral nutrition. Journal of pediatric gastroenterology and nutrition 2008, 47(5): 645-651.

43. Reniers D, Rajakumar I, Ratko S, Atkison P. Use of parenteral fish oil to reverse cholestasis induced by parenteral nutrition in infants with intestinal failure: single-centre case series. The Canadian journal of hospital pharmacy 2012, 65(1): 27-30.

44. Puder M, Valim C, Meisel JA, Le HD, de Meijer VE, Robinson EM, et al. Parenteral fish oil improves outcomes in patients with parenteral nutrition-associated liver injury. Annals of surgery 2009, 250(3): 395-402.

45. D'Ascenzo R, Savini S, Biagetti C, Bellagamba MP, Marchionni P, Pompilio A, et al. Higher docosahexaenoic acid, lower arachidonic acid and reduced lipid tolerance with high doses of a lipid emulsion containing 15% fish oil: a randomized clinical trial. Clinical nutrition (Edinburgh, Scotland) 2014, 33(6): 1002-1009.

46. D'Ascenzo R, D'Egidio S, Angelini L, Bellagamba MP, Manna M, Pompilio A, et al. Parenteral nutrition of preterm infants with a lipid emulsion containing 10% fish oil: effect on plasma lipids and long-chain polyunsaturated fatty acids. The Journal of pediatrics 2011, 159(1): 33-38 e31.

47. Biagetti C, Vedovelli L, Savini S, Simonato M, D'Ascenzo R, Pompilio A, et al. Double blind exploratory study on de novo lipogenesis in preterm infants on parenteral nutrition with a lipid emulsion containing 10% fish oil. Clinical nutrition (Edinburgh, Scotland) 2016, 35(2): 337-343.

AIM OF THE STUDIES

1. To study the phytosterol metabolism in preterm infants receiving vegetable oil-based intravenous lipid emulsions and infant formulas (*Chapters 2 - 6*).
2. To evaluate the impact of hypertriglyceridemia on the daily macronutrient (lipid, amino acids and carbohydrates) intakes, growth and neurodevelopment in preterm infants on routine parenteral nutrition (*Chapter 7*).
3. To study the growth of preterm infants on parenteral nutrition with FO containing lipid emulsions (*Chapter 8*).

Chapter 2

The Maternal-Fetal Gradient of Free and Esterified Phytosterols at the Time of Delivery in Humans

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Summary

Background: High dietary intakes of phytosterols (Phyto), such as those consumed by vegans and vegetarians, are not recommended for cholesterol-lowering in pregnant women (PW) because the safety of their use during pregnancy has not been fully established. Information on Phyto in pregnancy is very limited.

Objective: To characterize the maternal-fetal gradient of free and esterified Phyto at the time of delivery in humans.

Design: PW who had a term delivery at the Obstetrics and Gynecology Unit of the University Hospital of Padua (Padua, Italy), between November 2016 and March 2017, participated in the study. Fatty acids (FA), cholesterol (Chol), Chol metabolites (7-dehydrocholesterol, 7-DHChol; lathosterol, Latho; 7a-hydroxycholesterol, 7α-OHChol), and Phyto (campesterol, Camp; stigmasterol, Stigma; sitosterol, Sito) were measured in both maternal (MB) and cord blood (CB) at the time of delivery. Non-pregnant adult volunteers (Ref-NA) served as a reference.

Results: Thirty-four term PW and 12 Ref-NA signed informed consent and were studied. Plasma total Phyto concentrations in CB were up to 20-fold lower than in MB ($p < 0.05$). Positive and significant correlations were found between total Phyto of MB-CB pairs ($p < 0.01$), and between total FA and Camp of MB ($p < 0.05$). Interestingly, free Chol to Chol ester ratio of CB did not differ from that of MB, and free Phyto to Phyto ester ratios were higher in CB than in MB ($p < 0.001$). No differences were found between Phyto concentrations of MB and Ref-NA. However, free Chol to Chol ester ratio, and free Phyto to Phyto ester ratios were higher in MB than in Ref-NA ($p < 0.05$). Chol synthesis, as indicated by 7-DHChol to 7α-OHChol, Latho to 7α-OHChol, and Latho to Sito ratios, was greatest in CB and lowest in Ref-NA.

Conclusion: Our data suggest that free Phyto cross the human placenta more easily than Phyto ester. An elevated Stigma to Chol ratio in CB than in MB was also described for the first time. The impact of these findings on the neonatal outcomes remains to be elucidated.

Abbreviations: Camp, campesterol; CB, cord blood; Chol, cholesterol; FA, fatty acids; Latho, lathosterol; MB, Maternal blood; Phyto, phytosterols; PW, pregnant women; Ref-NA, reference non-pregnant adults; Sito, sitosterol; Stigma, stigmasterol; 7-DHChol, 7-dehydrocholesterol; 7α-OHChol, 7α-hydroxycholesterol.

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INTRODUCTION

Total plasma cholesterol (Chol) rises by 60% in the human pregnancy [2-4]. This appears to be mainly associated with physiological hormonal changes as concentrations of estrogen and insulin increase [5]. However, there is evidence suggesting that an excessive rise in plasma Chol may be related to complications during pregnancy and to the development of cardiovascular lesions in offspring neonates [6e8]. Because lipid-lowering drugs are contraindicated during pregnancy, natural compounds may be a safe alternative to prevent the rise of plasma Chol [9]. Phytosterol (Phyto)-based preparations are now available as natural drugs effective in reducing intestinal Chol absorption [10].

To date, information on Phyto in pregnancy is still very limited. Despite there is no evidence that high dietary intakes of Phyto, such as those consumed by vegan and vegetarian women, adversely affects pregnancy and neonates, Phyto are not recommended for pregnant women (PW) [1,11]. Nikkila et al. reported that serum Phyto concentrations in PW with and without cholestasis are not different from those of healthy non-PW [12]. Miettinen et al. did not find statistical differences between Phyto concentrations of PW with and without gestational diabetes mellitus (GDM) [13]. Gao et al. demonstrated that the daily consumption of a Phyto-enriched diet is able to improve both maternal and neonatal outcomes in patients with GDM [14]. Rideout et al. observed that Phyto supplementation in hypercholesterolemic pregnant mice produces pups with favourable liver and serum lipid responses in comparison with controls [9]. Vuorio et al. showed that Phyto are present in cord blood (CB) [15].

Overall, the available data indicate that Phyto can cross the placenta and the safety of their use during pregnancy.

On the other hand, accumulating evidence suggests that neonates receiving Phyto-rich lipid emulsions may have a high risk to develop life threatening illness, such as cholestasis [16-18]. Thus, in our view, understanding of fetal Phyto exposure in utero may be important to improve the nutritional management of PW and neonates.

For this purpose, in term pregnancies, we measured for the first-time plasma free and esterified Phyto (campesterol, Camp; stigmasterol, Stigma; sitosterol, Sito) of maternal blood (MB)-CB pairs at the time of delivery. Plasma Chol, and Chol metabolites (lathosterol, Latho; 7-dehydrocholesterol, 7-DHChol; 7α-hydroxycholesterol, 7α-OHChol) were also studied. Non-pregnant adults (Ref-NA) served as a reference.

SUBJECTS AND METHODS

Trial design and participants

PW who delivered at term (>37 gestational weeks) were recruited at the Obstetrics and Gynecology Unit of the University Hospital of Padua (Padua, Italy), between November 2016 and March 2017. Written informed consent was obtained from each PW before enrolment and the study was approved by the ethics committee and institutional review board (No. 878/DG). Inclusion criteria were a single pregnancy, a gestational age determined from known last maternal menstrual period higher than 37 weeks, and a normal diet without Phyto supplementation during pregnancy. Exclusion criteria were: prematurity, major congenital anomalies, intrauterine growth restriction, and endocrine disorders such as diabetes, hypercholesterolemia, pre-eclampsia, thyroid or adrenal problems. PW who delivered a stillborn neonate were excluded from the study at the time of data analysis. All data concerning PW, their pregnancies and deliveries were recorded according to the routine practice of the participating center. Ref-NA (age 25 to 40) consuming a normal diet served as a reference.

Sample collection and handling

We collected EDTA-treated blood samples remaining after completion of biochemical routine (scavenged blood) from PW, and umbilical cord at the time of delivery for measurements. MB were drawn from the cubital vein, whereas CB from the umbilical vein. EDTA-tubes containing 0.5 ml of venous blood were obtained from Ref-NA after an overnight fast (about 12 h). All blood samples were centrifuged at 2800 rpm (1358 g) and plasma was stored in pyrogallol-added tubes at -20°C until analysis.

Outcomes

The primary outcome was maternal-fetal concentration gradient of free and esterified sterols (Phyto and Chol) at the time of delivery. Free Chol to Chol ester ratio, and free Phyto to Phyto ester ratios of MB and CB were used to assess which sterol forms (free or esterified) had highest gradient across the placenta. The main secondary outcome was the effect of pregnancy on sterol esterification rate, which was evaluated by comparing free and esterified sterol ratios of MB and Ref-NA. Total Latho, 7-DHChol (Chol synthesis precursors), 7α-OHChol (a Chol breakdown product), and total FA concentrations were also measured to assess the Chol and FA metabolism in the study subjects

Analytical methods

Reagents were obtained from both Steraloids Inc. (Newport, USA) and Sigma-Aldrich (Milan, Italy). 5α-cholestane (internal standard for esterified sterols), epicoprostanol (internal standard for free sterols), 7α-OHChol, Camp, Stigma, and Sito were purchased from Steraloids and had a purity level of 97, 98, 98, 65, 95 and 71%, respectively. Chol, Latho, and 7-DHChol were purchased from Sigma-Aldrich and had a purity level of 99, 98 and 95%, respectively. Free and esterified sterols were measured by gas chromatography-mass spectrometry (GC-MS) as previously described [19]. In brief, 5α-cholestane and epicoprostanol were added as internal standards to plasma (200 µl), lipids were extracted twice with chloroform, and then thin-layer chromatography was used to separate free and esterified sterols. Sterol esters were saponified in methanolic potassium hydroxide (5M) for 1 h and extracted with two sequential portions of hexane and diethyl ether, before analysis. Both dried sterol samples were derivatized by bis (trimethylsilyl) trifluoro-acetamide in pyridine. A 50 µl aliquot of 200 µl hexane solution containing trimethylsilyl derivatives of free sterols was added to 100 µl of pure hexane, while those of sterol esters were added to 200 µl of pure hexane. One microliter of both diluted solutions was injected in splitless mode into an Agilent Technologies apparatus (model GC7890A/MD5975), using a capillary column with a non-polar stationary phase (HP5MS, 30 m x 0.25 mm x 0.25 mm film thickness). Sterols were identified by selected ion monitoring. The ions at 357, 355, 456, 458, 366, 443, 382, 484 and 486 m/z were used to detect 5α-cholestane, epicoprostanol, 7α-OHChol, Chol, 7-DHChol, Latho, Camp, Stigma and Sito, respectively. The appropriate calibration curve was used for the quantitation of each studied sterols. Coefficients of variation of all free sterols to sterol ester with our method were always less than 10% (data not shown).
Total Chol, Chol metabolites, and Phyto were measured in all study groups as sum of plasma free and esterified sterols. Plasma total FA were separated and quantified by GC with flame ionization detection using a standard fatty acid methyl ester mixture (NuChek-Prep GLC 461C), as reported by Pupillo et al. [20].

Statistical analysis

This study was exploratory in nature as the plasma free and esterified Phyto concentrations in MB-CB pairs at the time of delivery were unknown at the time this study began. Data on sterol and FA concentrations, and sterol ratios were presented as mean ± SD. Independent t-test was used to compare both CB and MB to Ref-NA. Paired t-test was considered appropriate for comparing CB and MB. Repeated measures ANOVA with post-hoc Bonferroni test was used to compare free sterols to sterol ester within each study group. Pearson correlation and linear regression were used to assess associations between variables. A p-value <0.05 was considered significant. All statistical analyses were performed by using SPSS (v 19.0; SPSS Inc, Chicago, Illinois).

RESULTS

Thirty-four PW who delivered a singleton term neonate signed informed consent and were studied. All neonates of enrolled PW were born alive and blood samples were collected from all PW-umbilical cord pairs at the time of delivery. Overnight fasting blood samples were also taken from 12 healthy Ref-NA: 6 women and 6 men. Selected demographic and clinical characteristics of the study subjects are reported in **Table 1**.

Table 1. Demographic and clinical characteristics of PW, term neonates, and Ref-NA at the time of enrollment[a].

Study subjects	Characteristics	
PW (=MB), n = 34	Age, years	33 ± 6
	Nullipara, no. (%)	18 (53)
	Vaginal delivery, no. (%)	20 (59)
	Elective delivery, no. (%)	5 (15)
Term neonates (=CB), n = 34	Gestational age, days	278 ± 9
	Gender (Male), no. (%)	15 (44)
	Weight, g	3315 ± 368
	Length, cm	49 ± 1
	1-min Apgar score, no.	10 ± 1
	5-min Apgar score, no.	10
Ref-NA, n = 12	Age, years	35 ± 6
	Gender (Male), no. (%)	6 (50)
	Weight, Kg	76 ± 15
	Height, cm	175 ± 11

[a] Values are presented as mean ± SD or no. (%)

MB-CB pairs

Total Chol concentration was about 5-fold higher in MB than in CB ($p < 0.001$). Chol metabolites had concentrations from 2-fold to 4-fold lower in CB than in MB ($p < 0.001$). Camp and Sito concentrations were from 7-fold to 20-fold lower in CB than in MB, whereas Stigma only about 3-fold lower ($p < 0.001$). Chol metabolites to Chol ratios were higher in CB than in MB ($p < 0.05$). Among Phyto, only Camp, and Sito were lower in CB than in MB, when they were compared to Chol ($p < 0.001$; Table 2). 7-DHChol and Latho to 7α-OHChol ratios, indicators of Chol synthesis, were highest in CB (7-DHChol to 7α-OHChol ratio e MB:77.0 ± 29.0, and CB: 93.4 ± 18.7, $p < 0.01$; Latho to 7α-OHChol ratio - MB: 85.8 ± 45.3, and CB: 184.6 ± 48.6, $p < 0.001$). Also, Latho to Sito ratio, a widely-used index for Chol synthesis [13], was highest in CB (MB: 1.3 ± 0.9 and CB: 5.2 ± 1.6, $p < 0.001$). Total plasma FA concentration was lower in CB than in MB (MB: 482.2 ± 107.5 and CB: 112.4 ± 25.3 mg/dl, $p < 0.001$). Positive correlations were found between total Chol and FA concentrations in CB and MB, and between both total Phyto and total Chol of these two groups. Only total Camp was also positively and significantly correlated to total FA in MB. No correlation was found between total FA concentration of MB and CB (**Table 3**).

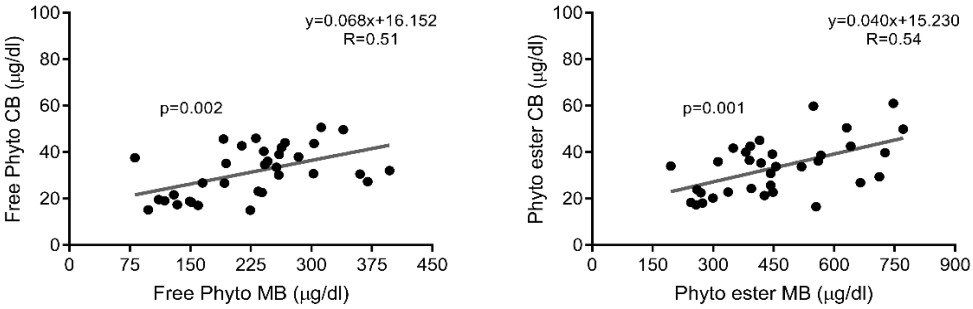
Table 3. Linear correlation and regression analysis between total plasma Chol, Phyto and total FA concentrations of MB-CB pairs[1].

Dependent variable	Independent variable	R	Linear Regression	
			Equation	p-value
Total CB Chol[a]	Total MB Chol[a]	**(+)0.40**	**y=0.076x+35.685**	**0.020**
	Total CB FA[a]	**(+)0.56**	**y=0.378x+13.593**	**0.001**
Total PW Chol[a]	Total MB FA[a]	**(+)0.35**	**y=0.290x+130.811**	**0.045**
Total CB FA[a]	Total MB FA[a]	0.10	y=0.024x+100.881	0.568
Total MB Camp[b]	Total MB FA[a]	**(+)0.34**	**y=0.354x+154.118**	**0.049**
Stigma[b]		0.17	y=0.008x+12.336	0.327
Sito [b]		0.18	y=0.206x+243.756	0.317
Total CB Camp[b]	Total MB Camp[b]	**(+)0.44**	**y=0.018x+8.021**	**0.009**
Stigma[b]	Stigma[b]	**(+)0.52**	**y=0.189x+3.331**	**0.002**
Sito[b]	Sito[b]	**(+)0.65**	**y=0.087x+15.051**	**0.000**

[1] Different superscripts (a, b) identify the units of measurement of variables which were considered for statistical analysis: a, mg/dl; b, μg/dl. p-value < 0.05 was considered significant.

Free Chol to Chol ester ratio was not different between MB and CB ($p = 0.90$). Free Phyto to Phyto ester ratios were higher in CB than in MB ($p < 0.001$). Free Chol to Chol ester ratio was not different from free Camp to Camp ester ratio in MB ($p = 0.58$). Free Stigma to Stigma ester ratio was higher than the other ratios in both groups ($p < 0.001$; Fig. 1). A significant positive correlation was found on both free Phyto and Phyto ester between MB and CB (**Fig. 2**).

Figure 2. Linear regression of both free Phyto and Phyto ester between MB and CB [1]



[1] p-value < 0.05 was considered significant.

MB versus Ref-NA

Total Chol concentration in MB was about twice as high as in Ref-NA ($p < 0.001$). 7-DHChol, and Latho (Chol precursors) had concentrations about 2-fold higher in MB than in Ref-NA ($p < 0.001$), whereas 7α-OHChol was not different between MB and Ref-NA ($p = 0.17$). No differences were also found between total Phyto concentrations of both groups. Latho to Chol ratio was higher in MB than in Ref-NA ($p = 0.002$), whereas 7α-OHChol to Chol ratio was lower ($p = 0.018$). Phyto to Chol ratios were lower in MB than in Ref-NA ($p < 0.05$; **Table 2**).

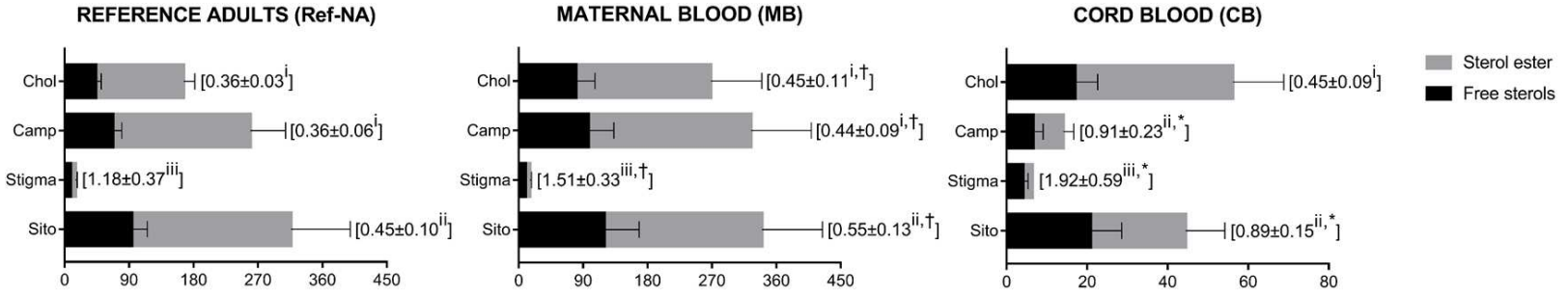
Table 2. Total plasma concentrations of Chol, Chol metabolites, and Phyto in Ref-NA and in MB-CB pairs at the time of delivery[a].

Variable	Day of delivery		
	Ref-NA	MB	CB
	(n = 12)	(n = 34)	(n = 34)
Chol	4.3±0.6	7.0±2.3 [c]	1.5±0.4 [b]
	(166.3±22.4)	(270.6±90.1) [c]	(56.1±17.2) [b]
7-DHChol	117.2±27.7	138.7±55.3	204.6±50.7 [b]
	(193.6±51.6)	(345.8±119.5) [c]	(111.1±34.3) [b]
Latho	99.5±29.6	148.8±79.1 [c]	400.9±111.7 [b]
	(169.0±63.4)	(365.7±156.5)	(214.2±58.6) [b]
7α-OHChol	2.4±0.8	1.8±0.7 [c]	2.1±0.5 [b]
	(4.2±1.1)	(4.8±1.6)	(1.2±0.4) [b]
Camp	154.8±48.8	118.4±49.2 [c]	25.1±8.2 [b]
	(259.8±57.6)	(316.1±109.0)	(14.0±4.7) [b]
Stigma	8.6±1.7	6.0±2.3 [c]	11.4±4.5 [b]
	(15.2±2.7)	(16.0±4.7)	(6.4±1.7) [b]
Sito	180.5±62.9	128.2±58.0 [c]	76.8±26.4 [b]
	(316.3±101.6)	(342.1±125.3)	(44.8±16.8) [b]

[a] Values are presented as mean±SD. Values are 10^2 millimoles per mole of Chol, except Chol, which is millimoles per liter; the values in the second line (in parentheses) are in mg/dl for Chol, and in µg/dl for Chol metabolites and Phyto. [b] $p < 0.05$ compared with MB; paired t-test between MB and CB. [c] $p < 0.05$ compared with Ref-NA; independent t-test between MB and Ref-NA.

7-DHChol to 7α-OHChol ratio, Latho to 7α-OHChol ratio, Latho to Sito ratio, and total plasma FA were highest in MB (7-DHChol to 7α-OHChol ratio e MB: 77.0 ± 29.0, and Ref-NA: 49.2 ± 17.0, $p = 0.003$; Latho to 7α-OHChol ratio - MB: 85.8 ± 45.3, and Ref-NA: 44.5 ± 22.4, $p = 0.004$; Latho to Sito ratio - MB: 1.3 ± 0.9, Ref-NA: 0.6 ± 0.3, $p = 0.012$; Total FA e MB: 482.2 ± 107.5, and Ref-NA: 254.8 ± 32.0 mg/dl, $p < 0.001$). Free Chol to Chol ester ratio, and free Phyto to Phyto ester ratios were higher in MB than in Ref-NA ($p < 0.05$). Similar to MB, free Cho to Cho ester ratio was no different from free Camp to Camp ester ratio in Ref-NA ($p < 0.001$). Free Stigma to Stigma ester ratio was higher than the other ratios in each group ($p < 0.001$; **Fig. 1**).

Figure 1. Free and esterified sterol concentrations, and free sterol to sterol ester ratios of MB, CB and Ref-NA [1]



[1] Values are presented as mean±SD. Values are in mg/dl for Chol, and in µg/dl for Camp, Stigma, and Sito. Free sterol to sterol ester ratios are in parentheses.

* $p < 0.05$ compared with MB; paired t-test between MB and CB.

† $p < 0.05$ compared with Ref-NA; independent t-test between MB and Ref-NA.

i $p < 0.05$ compared with other sterol ratios; repeated measures-ANOVA and Bonferroni post-test within each group.

Discussion

To the best of our knowledge, this is the first report on the maternal-fetal gradient of free and esterified Phyto in human pregnancy. Under normal conditions (no phytosterol supplementation), we found that: (1) free Phyto to Phyto ester ratios were higher in CB than in MB at the time of delivery, (2) Stigma to Chol ratio was higher in CB than in MB, and (3) pregnancy affected the sterol esterification rate versus non-pregnant adults. Below, we will discuss these points further. In our MB-CB pairs, Phyto ester exhibited a higher concentration gradient than free Phyto. It might mean a lower potential toxicity of free Phyto than Phyto ester for the fetus, that would make free Phyto the best candidate as Chol-lowering compounds during pregnancy. Our study is clearly unable to address this issue. However, the high gradient of Phyto between MB and CB, indicates that human placenta is designed to limit the Phyto availability to the fetus [12,13,15]. Moreover, our data suggested that high Phyto concentrations during pregnancy may lead to high Phyto concentrations in CB, which might expose offspring neonates to a higher risk of atherosclerosis, hemolysis and liver dysfunction [17,21]. To date, the proposed cause-effect relationship between maternal Phyto intakes and neonatal diseases remains elusive.

Interestingly, in CB, Stigma, a potent antagonist of a nuclear receptor critically involved in hepatoprotection from cholestasis (Farnesoid X Receptor, NR1H4) [22], was lower than the other Phyto, when they were compared to Chol. This result was in line with data previously published by Pianese et al., and Mellis et al. on Phyto content in CB of healthy term neonates, and in aortic tissue of miscarried fetuses (gestational week 30), respectively [23,24]. However, surprisingly, Stigma to Chol ratio was the only Phyto to Chol ratio higher in CB than in MB. At present, we do not have a clear explanation of why this most potentially dangerous Phyto may cross placenta more easily than both Camp, and Sito. Free Chol to Chol ester ratio in MB was lower than that of RefNA, as also reported by Neary et al. [25]. Moreover, free Phyto to Phyto ester ratios of both MB and Ref-NA had the same esterification pattern previously described by Connor et al. [26]: Camp was the most esterified Phyto, whereas Stigma was the least. Of note, we also found no significant differences in free Chol to Chol ester ratio between MB and CB as reported in some studies [27,28], even though this was at variance with other data previously published [25,29]. Anesthesia and analgesics given during labor may cause a lower Chol esterification rate, and thus an increase in free Chol to Chol ester ratio of MB [30-32]. According to this, free Chol to Chol ester ratio of the 14 PW who received anesthesia and/or analgesics during labor (cesarean delivery: n = 13, vaginal delivery: n = 1) was significant higher than that of PW who did not receive these medications (0.51 ± 0.10 vs 0.41 ± 0.10, respectively; P = 0.012). However, free Chol to Chol ester ratio of MB continued to be not significantly different from that of CB, when only MB-CB pairs who did not receive anesthesia and/or analgesics were considered (n = 20; p = 0.52). This suggests that our finding on free Chol to Chol ester ratio in MB-CB pairs was not affected by anesthesia and/or analgesics given to PW during labor.

This study has limitations. We have limited clinical data of term neonates, except for birth weight, birth length, gender, Apgar score, and gestational age which did not correlate with free Phyto to Phyto ester ratios of CB (data not shown). Ref-NA consisted of both males and females, even though it has been reported that men have a higher plasma Latho concentration than women [33]. However, the use of a mixed-gender reference group did not affect our findings on Chol and Phyto. Furthermore, study PW received anesthetics and analgesics that could have influenced the sterol esterification rate as described above.

Lastly, it is unknown whether PW consumed other drugs during pregnancy that might have been affected the acetyl-CoA acetyltransferase and lecithin-cholesterol acyltransferase activity.
In conclusion, we first looked at the maternal-fetal gradient of free and esterified Phyto at the time of delivery in humans. Our data suggest that: (a) the human placenta tends to limit the Phyto availability to the fetus, (b) free Phyto cross the placenta more easily than Phyto ester, and (c) Stigma crosses the placenta more easily than the other Phyto, when they were compared to Chol. Further studies will be needed to clarify the impact of these findings on the neonatal outcomes.

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CONFLICT OF INTEREST

No conflicts of interests to declare. This study did not receive an external funding.

AUTHORS' CONTRIBUTIONS

The authors' responsibilities were as follows:

- VPC designed the research project;
- AC, and SV analyzed data;
- EC, EP, MS, LV, and SD contributed to subject recruitment and samples collection;
- PC performed statistical analysis.

All authors read and approved the final manuscript.

REFERENCES

[1] Berger A, Jones PJH, Abumweis SS. Plant sterols: factors affecting their efficacy and safety as functional food ingredients. Lipids in Health and Disease. 2004;3:5.

[2] Montelongo A, Lasuncion MA, Pallardo LF, Herrera E. Longitudinal study of plasma lipoproteins and hormones during pregnancy in normal and diabetic women. Diabetes. 1992;41:1651-9.

[3] Brizzi P, Tonolo G, Esposito F, Puddu L, Dessole S, Maioli M, et al. Lipoprotein metabolism during normal pregnancy. American journal of obstetrics and gynecology. 1999;181:430-4.

[4] Martin U, Davies C, Hayavi S, Hartland A, Dunne F. Is normal pregnancy atherogenic? Clinical science (London, England : 1979). 1999;96:421-5.

[5] Catalano PM, Tyzbir ED, Roman NM, Amini SB, Sims EA. Longitudinal changes in insulin release and insulin resistance in nonobese pregnant women. American journal of obstetrics and gynecology. 1991;165:1667-72.

[6] Napoli C, Glass CK, Witztum JL, Deutsch R, D'Armiento FP, Palinski W. Influence of maternal hypercholesterolaemia during pregnancy on progression of early atherosclerotic lesions in childhood: Fate of Early Lesions in Children (FELIC) study. Lancet (London, England). 1999;354:1234-41.

[7] Solomon CG, Carroll JS, Okamura K, Graves SW, Seely EW. Higher cholesterol and insulin levels in pregnancy are associated with increased risk for pregnancy-induced hypertension. American journal of hypertension. 1999;12:276-82.

[8] Marseille-Tremblay C, Ethier-Chiasson M, Forest JC, Giguere Y, Masse A, Mounier C, et al. Impact of maternal circulating cholesterol and gestational diabetes mellitus on lipid metabolism in human term placenta. Molecular reproduction and development. 2008;75:1054-62.

[9] Rideout TC, Movsesian C, Tsai YT, Iqbal A, Raslawsky A, Patel MS. Maternal Phytosterol Supplementation during Pregnancy and Lactation Modulates Lipid and Lipoprotein Response in Offspring of apoE-Deficient Mice. The Journal of nutrition. 2015;145:1728-34.

[10] Ikeda I. Factors affecting intestinal absorption of cholesterol and plant sterols and stanols. Journal of oleo science. 2015;64:9-18.

[11] Piccoli GB, Clari R, Vigotti FN, Leone F, Attini R, Cabiddu G, et al. Vegan-vegetarian diets in pregnancy: danger or panacea? A systematic narrative review. BJOG : an international journal of obstetrics and gynaecology. 2015;122:623-33.

[12] Nikkila K, Riikonen S, Lindfors M, Miettinen TA. Serum squalene and noncholesterol sterols before and after delivery in normal and cholestatic pregnancy. J Lipid Res. 1996;37:2687-95.

[13] Miettinen HE, Rönö K, Koivusalo S, Stach-Lempinen B, Pöyhönen-Alho M, Eriksson JG, et al. Elevated serum squalene and cholesterol synthesis markers in pregnant obese women with gestational diabetes mellitus. Journal of Lipid Research. 2014;55:2644-54.

[14] Gao F, Wang G, Wang L, Guo N. Phytosterol nutritional supplement improves pregnancy and neonatal complications of gestational diabetes mellitus in a double-blind and placebo-controlled clinical study. Food & function. 2017;8:424-8.

[15] Vuorio AF, Miettinen TA, Turtola H, Oksanen H, Gylling H. Cholesterol metabolism in normal and heterozygous familial hypercholesterolemic newborns. J Lab Clin Med. 2002;140:35-42.

[16] Bindl L, Lutjohann D, Buderus S, Lentze MJ, v Bergmann K. High plasma levels of phytosterols in patients on parenteral nutrition: a marker of liver dysfunction. J Pediatr Gastroenterol Nutr. 2000;31:313-6.

[17] Zaloga GP. Phytosterols, Lipid Administration, and Liver Disease During Parenteral Nutrition. JPEN J Parenter Enteral Nutr. 2015;39:39S-60S.

[18] Satrom K, Gourley G. Cholestasis in Preterm Infants. Clin Perinatol. 2016;43:355-73.

[19] Savini S, Correani A, Pupillo D, D'Ascenzo R, Biagetti C, Pompilio A, et al. Phytosterol Esterification is Markedly Decreased in Preterm Infants Receiving Routine Parenteral Nutrition. Lipids. 2016;51:1353-61.

[20] Pupillo D, Simonato M, Cogo PE, Lapillonne A, Carnielli VP. Short-Term Stability of Whole Blood Polyunsaturated Fatty Acid Content on Filter Paper During Storage at -28 degrees C. Lipids. 2016;51:193-8.

[21] Clayton PT, Bowron A, Mills KA, Massoud A, Casteels M, Milla PJ. Phytosterolemia in children with parenteral nutrition-associated cholestatic liver disease. Gastroenterology. 1993;105:1806-13.

[22] Carter BA, Taylor OA, Prendergast DR, Zimmerman TL, Von Furstenberg R, Moore DD, et al. Stigmasterol, a soy lipid-derived phytosterol, is an antagonist of the bile acid nuclear receptor FXR. Pediatr Res. 2007;62:301-6.

[23] Mellies MJ, Ishikawa TT, Glueck CJ, Bove K, Morrison J. Phytosterols in aortic tissue in adults and infants. J Lab Clin Med. 1976;88:914-21.

[24] Pianese P, Salvia G, Campanozzi A, D'Apolito O, Dello Russo A, Pettoello-Mantovani M, et al. Sterol profiling in red blood cell membranes and plasma of newborns receiving total parenteral nutrition. J Pediatr Gastroenterol Nutr. 2008;47:645-51.

[25] Neary RH, Kilby MD, Kumpatula P, Game FL, Bhatnagar D, Durrington PN, et al. Fetal and maternal lipoprotein metabolism in human pregnancy. Clinical science (London, England : 1979). 1995;88:311-8.

[26] Lin DS, Steiner RD, Merkens LS, Pappu AS, Connor WE. The effects of sterol structure upon sterol esterification. Atherosclerosis. 2010;208:155-60.

[27] Whitelaw MJ. THE SERUM CHOLESTEROL LEVEL OF THE PREMATURELY BORN INFANT AND ITS MOTHER. The Journal of Clinical Investigation. 1948;27:260-2.

[28] Bersohn I, Wayburne S. Serum cholesterol concentration in new-born African and European infants and their mothers. Am J Clin Nutr. 1956;4:117-23.

[29] Robertson AF, Sprecher H. A review of human placental lipid metabolism and transport. Acta paediatrica Scandinavica. 1968:Suppl 183:3-18.

[30] Bell FP. Diazepam inhibits cholesterol esterification by arterial ACAT and plasma LCAT, in vitro. Atherosclerosis. 1984;50:345-52.

[31] Bell FP. The effect of local anesthetics on arterial lipid metabolism. Inhibition of sterol esterification in vitro. Atherosclerosis. 1981;38:81-8.

[32] Bell FP, Hubert EV. Membrane-active agents. Effect of various anesthetics and chlorpromazine on arterial lipid metabolism. Atherosclerosis. 1981;39:517-25.

[33] Kempen HJ, de Knijff P, Boomsma DI, van der Voort HA, Gevers Leuven JA, Havekes L. Plasma levels of lathosterol and phytosterols in relation to age, sex, anthropometric parameters, plasma lipids, and apolipoprotein E phenotype, in 160 Dutch families. Metabolism. 1991;40:604-11.

Chapter 3

Phytosterol Esterification is Markedly Decreased in Preterm Infants Receiving Routine Parenteral Nutrition

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Summary

Several studies reported the association between total plasma phytosterol concentrations and the parenteral nutrition-associated cholestasis (PNAC). To date, no data are available on phytosterol esterification in animals and in humans during parenteral nutrition (PN).

We measured free and esterified sterols (cholesterol, campesterol, stigmasterol and sitosterol) plasma concentrations during PN in 16 preterm infants (500-1249 g of birth weight; Preterm-PN), in 11 term infants (Term-PN) and in 12 adults (Adult-PN). Gas-chromatography-mass spectrometry was used for measurements.

Plasma concentrations of free cholesterol (Free-CHO), free phytosterols (Free-PHY) and esterified phytosterols (Ester-PHY) were not different among the 3 PN groups. Esterified cholesterol (Ester-CHO) was statistically lower in Preterm-PN than Adult-PN. Preterm-PN had significantly higher Free-CHO/Ester-CHO and Free-PHY/Ester-PHY ratios than Adult-PN (Free-CHO/Ester-CHO: 1.1±0.7 vs 0.6±0.2; Free-PHY/Ester-PHY: 4.1±2.6 vs 1.3±0.8; *P<0.05). Free-CHO/Ester-CHO and Free-PHY/Ester-PHY ratios of Term-PN (Free-CHO/Ester-CHO: 1.1±0.4; Free-PHY/Ester-PHY: 2.9±1.7) were not different neither from Preterm-PN nor from Adult-PN. Plasma Free-CHO/Ester-CHO and Free-PHY/Ester-PHY were unchanged after 24-hrs on fat free PN both in Preterm-PN and in Adult-PN. Free-PHY/Ester-PHY did not correlate with phytosterol intake in Preterm-PN. Free-PHY/Ester-PHY of Preterm-PN was positively correlated with the Free-CHO/Ester-CHO and negatively correlated with gestational age and birth weight. In conclusion, PHY were esterified to a lesser extent than CHO in all study groups; the esterification was markedly decreased in Preterm-PN compared to Adult-PN. The clinical consequences of these findings warrant further investigations.

Abbreviations: ACAT: Acyl-CoA cholesterol acyltransferase; Adult-PN: adults on PN; CAMP: campesterol; CHO: cholesterol; Ester: esterified; IV: intravenous; LCAT: lecithin cholesterol acyltransferase; LE: lipid emulsion; NICU: Neonatal Intensive Care Unit; PHY: phytosterols; PN: parenteral nutrition; PNAC: parenteral nutrition-associated cholestasis; Preterm-PN: preterm infants on PN; SITO: sitosterol; SO: soybean oil; STIGM: stigmasterol; Term-PN: term infants on PN; Δ: difference.

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INTRODUCTION

The association between plasma phytosterol concentrations and the severity of parenteral nutrition-associated cholestasis (PNAC) has been reported in several studies [1-7]. Published studies provided data on plasma total phytosterols (Total-PHY) [3, 8, 9], but no information is available on plasma free and esterified phytosterols (Free-PHY and Ester-PHY) neither in animals nor in humans during parenteral nutrition (PN). To the best of our knowledge plasma Free-PHY and Ester-PHY were measured in healthy subjects and in patients with hypertriglyceridemia and sitosterolemia [10-12].

During PN large amount of PHY, mainly campesterol (CAMP), stigmasterol (STIGM) and sitosterol (SITO), are given intravenously as they are part of the vegetable oil-based intravenous lipid emulsions (IV LE) [13-15]. Information on the metabolism of IV PHY in humans is limited. IV Free-PHY likely undergo the same esterification process as dietary or endogenous cholesterol (CHO). CHO is esterified in humans by two enzymes: lecithin cholesterol acyltransferase (LCAT; EC 2.3.1.43) and Acyl-CoA cholesterol acyltransferase (ACAT; EC 2.3.1.26). LCAT is present in higher concentrations in plasma than in tissues, and it mainly transfers fatty acids from the 2-position of lecithin to CHO [16-18]. ACAT is an integral membrane protein that catalyzes a reaction in which the fatty acid of an acyl-CoA molecule is esterified to CHO [19]. The specificity of LCAT and ACAT toward the acyl acceptor (sterol substrate) has been reported in different studies [20-23].

We are interested in studying whether patients with poorer esterification are more prone to liver damage and/or if preterm infants with PNAC exhibit altered sterol esterification.

The aim of this pilot study was to measure the CAMP, STIGM, SITO and CHO esterification during PN in preterm infants (Preterm-PN) and compare them with term infants on PN (Term-PN) and human adults on PN (Adult-PN).

MATERIALS AND METHODS

Preterm infants

Neonates with a birth weight of 500 to 1249 g, who routinely receive PN from the first hour of life, were enrolled at the Neonatal Intensive Care Unit (NICU) of “G. Salesi” Children's Hospital, Ancona, Italy, between January 2008 and December 2012. Both genders were included. Exclusion criteria were severe malformations, inborn errors of metabolism and severe congenital sepsis. Preterm infants were started on PN with glucose, amino acids and lipids at about 1 hour after birth, according to the local NICU guidelines.

All preterm infants in this study (n=16) were chosen from a previous study done by our group [13] based on the availability of sufficient amount of left-over plasma for the determination of free and esterified sterols. Study infants received one of the 2 IV LE as part of their routine PN: MS [Lipofundin MCT®, B.Braun; 50% medium-chain triglycerides and 50% SO] or MSF [Lipidem®, B.Braun; 50% medium-chain triglycerides, 40% SO, and 10% fish oil] (Table 1). The IV LE were infused at dose of 1, 1.5, 2, 2.5, and 3 g·kg-1·d-1 from postnatal day 0 to day 5 respectively and were then kept constant from day 5 to day 7, when PN tapering was begun, until day 21, when it was stopped. Minimal enteral feeding with human milk was provided from day 0 to day 7, the maximum amount supplied being 8 mL·kg-1·d-1 from day 1 to day 4, and 16 mL·kg-1·d-1 from day 5 to day 8. Enteral feeding was gradually increased from day 9 to reach full oral feeding on day 18. The gestational age of preterm infants was 197±16 days, the birth weight was 950±173 g and they received PN for 20±9 days.

The clinical characteristics of the 16 preterm infants from whom a sufficient amount of plasma was available were not different from those of 144 preterm infants studied in our previous publication (ALP, U/L: 1067.8±314.5; AST, U/L: 31.9±19.5; ALT, U/L: 10.7±7.1; GGT, U/L: 73.3±62.5; total bilirubin, mg/dL: 2.57±1.71; conjugated bilirubin, mg/dL: 0.56±0.24) [13].

Term infants and Human adults

Eleven term infants on PN (Term-PN) for surgical diseases and 12 human adults on PN (Adult-PN) for malnutrition or intestinal failure were studied. The gestational age of Term-PN was 268±10 days, birth weight was 2840±630 g and their blood was collected after 1 week from the PN start (6±2 days). The age range of Adult-PN was 43-79 years and the PN duration at the time of sampling was 23±11 days. Both genders were included and all patients had normal liver function tests.
All Term-PN and Adult-PN received the same lipid emulsion (MS: Lipofundin MCT®, B.Braun) containing 50% medium-chain triglycerides and 50% SO. Term-PN received the same IV lipid intake as the Preterm-PN. Adult-PN patients received a mean IV lipid intake of 1 g·kg-1·d-1 throughout the study.

Sample handling

In all preterm infants, we collected 0.5 mL EDTA-treated blood samples after 1 week of lipid infusion (7±1 days), when they were on full PN and enteral feeding was less than 16 mL·kg-1·d-1. In 8 preterm infants a second blood sample was obtained also after 24-hrs on fat free PN. In Term-PN and Adult-PN groups blood sample was collected during PN infusion, and in 10 Adult-PN patients also after 24-hrs from the end of lipid infusion. Blood was immediately centrifuged at 2800 rpm and pyrogallol was added to plasma before storage at -20°C.

Reagents

All the reagents were obtained from Steraloids (Newport, USA). Cholest-5-en-3β-ol (cholesterol), 5α-cholestane (IS for esterified sterols), 5β-cholestan-3α-ol (epicoprostanol; IS for free sterols), 24α-methylcholest-5-en-3β-ol (campesterol), 3β-hydroxy-24-ethyl-5,22-cholestadiene (stigmasterol) 24α-ethylcholest-5-en-3β-ol (β-sitosterol) had a purity level of 99%, 98%, 98%, 65%, 95% and 71%, respectively.

Analytic methods

Stock solutions of 5α-cholestane and epicoprostanol in chloroform were prepared at a concentration of 2.50 mg/ml. Solutions containing 0.25 mg/mL of 5α-cholestane and epicoprostanol were prepared as working standards and 20 µl of both were added to 200 µl of plasma before the total lipids extraction.
Lipid extraction was performed using a modified version of the method by Folch et al. [24]. The lipid extracts were separated on silica gel thin-layer chromatography plates (TLC) using heptane/diisopropylether/acetic acid glacial (60:40:3, by vol) as developing solvent. Free and esterified sterols were collected by scraping the respective band on TLC plates and they were then extracted with Folch (chloroform/methanol, 2:1 by vol). Sterol esters were saponified with potassium hydroxide/methanol (5M) and heated at 60°C for 1 hour. Sterols were extracted from the hydroalcoholic phase by means of liquid-liquid extraction with an equal volume of hexane for 15 minutes under shaking and after centrifugation. This operation was repeated a second time adding diethyl ether instead of hexane. Hexane and diethyl ether were removed under a gentle stream of nitrogen. Free and esterified sterols were derivatized by bis (trimethylsilyl)trifluoro-acetamide in pyridine and extracted in 200 µl of hexane.

A 50 µl aliquot of this solution containing TMS-derivates of free sterols were added to 100 µl of pure hexane, while those of esterified sterols were added to 200 µl of pure hexane. This approach did not allow column overload caused by high cholesterol concentration in plasma. One microliter of both diluted solutions was analyzed by gas chromatography-mass spectrometry [1, 4].
Coefficient of variation of Free-CHO/Ester-CHO and Free-PHY/Ester-PHY with our method was always less than 10% (Data not shown).
Gas chromatography-mass spectrometry analysis was performed by an Agilent Technologies apparatus (model GC7890A/MD5975) controlled by a work station using Agilent ChemStation as software.
A capillary column with non-polar stationary phase (HP-5MS, 30m x 0.25mm x 0.25µm film thickness) was used for chromatographic separation. One microliter of samples was injected, by pulsed split-less mode, and the injector temperature was set at 270°C. The initial column temperature of 200°C was held for 1 minute, then programmed at 10°C/min to 275°C, after raised to 280°C at a rate of 0.1°C/min and finally increased to 300°C at a rate of 10°C/min that was held for 5 minutes. The mass spectrometer operated in electron impact mode at ionization voltage of 70 eV, with an ion source temperature of 230°C and quadrupole analyzer temperature of 150°C. Quantitative analysis was performed by single ion monitoring mode. The ions at 357 m/z, 355 m/z, 458 m/z, 472 m/z, 484 m/z and 486 m/z were used to detect 5α-cholestane, epicoprostanol, CHO, CAMP, STIGM and SITO, respectively.
Sterol concentrations (mg/l) were obtained from the peak area ratios of each compound compared with standard, corrected by their relative response factors calculated on the basis of calibration curves obtained with variable amounts of CHO, CAMP, STIGM and SITO and fixed amounts of 5α-cholestane and epicoprostanol [25].
Total-CHO, Total-CAMP, Total-STIGM and Total-SITO were calculated as the sum of Free-CHO and Ester-CHO, Free-CAMP and Ester-CAMP, Free-STIGM and Ester-STIGM, Free-SITO and Ester-SITO, respectively. Free-PHY were calculated as the sum of Free-CAMP, Free-STIGM and Free-SITO and the Ester-PHY as the sum of Ester-CAMP, Ester-STIGM and Ester-SITO.

Ethics

The study was conducted in accordance with the principles of the Helsinki Declaration as revised in Edinburgh 2000 and was reviewed and approved by the local ethics committee and institutional review board (No. 469/DG). Written informed consent was obtained from both parents for term and preterm infants and prior to enrollment for adults.

Statistical Analysis

Phytosterol and cholesterol plasma concentrations were the primary endpoint. Data were expressed as Mean±SD and were analyzed by one-way independent ANOVA.
Significant differences between groups were analyzed by analysis of variance with Bonferroni correction. Repeated measures ANOVA and paired t-test were used to identify significant differences into groups. A P value <0.05 was considered significant. A simple linear regression analysis was used to study the correlations between variables. All statistical analyses were performed using SPSS (v 19.0; SPSS Inc, Chicago, Illinois) and Microsoft EXCEL (v 2000; Microsoft Corp Redmond, Washington).

RESULTS

The concentration of PHY (MS LE: 266.1±23.1, MSF LE: 245.9±16.2 mg/L) and CHO (MS LE: 70.8±4.8, MSF LE: 156.4±10.2 mg/L) in the IV LE are shown in **Table 1**.

Table 1. Composition and sterol content of IV LE used in this study[1,2]

Variable	MS	MSF
Medium Chain Triglycerides oil (g/L)	100	100
Soybean oil (g/L)	100	80
Fish oil (g/L)	–	20
Egg yolk phospholipids (g/L)	12	12
Glycerol (g/L)	25	25
Total-Cholesterol (mg/L)	70.8±4.8	156.4±10.2
Free-CHO/Ester-CHO	7.7±0.3	0.7±0.1
Total-Campesterol (mg/L)	42.8±3.2	32.2±1.6
Free-CAMP/Ester-CAMP	5.2±0.6	5.3±0.3
Total-Stigmasterol (mg/L)	58.5±5.3	50.2±2.9
Free-STIGM/Ester-STIGM	5.6±0.6	6.0±0.3
Total-Sitosterol (mg/L)	164.8±14.7	163.5±15.4
Free-SITO/Ester-SITO	2.2±0.2	2.8±0.4
Total-Phytosterols (mg/L)	266.1±23.1	245.9±16.2
Free-PHY/Ester-PHY	2.9±0.2	3.4±0.3

[1] MS: Lipofundin MCT®, B.Braun - 50% Medium Chain Triglycerides oil and 50% SO; MSF: Lipidem®, B.Braun - 50% Medium Chain Triglycerides oil, 40% SO and 10% fish oil; CHO: cholesterol; Ester: esterified; CAMP: campesterol; STIGM: stigmasterol; SITO: sitosterol; PHY: phytosterols.
[2] Analyzed by gas chromatography-mass spectrometry in our laboratory. All experiments were conducted in triplicate, and data is shown as mean±SD; LE analyzed are representative of only a single Lot number.

Plasma Free-CHO, Ester-CHO, Free-PHY and Ester-PHY and their ratios are reported in Table 2. Plasma Free-CHO and Total-CHO were not different among the 3 PN groups. Plasma Ester-CHO of Preterm-PN was lower than Adult-PN while Ester-CHO of Term-PN was intermediate and not different from Preterm-PN and also not from Adult-PN (**Table 2**).
Free and esterified CAMP, STIGM and SITO and PHY were not different among Preterm-PN, Term-PN and Adult-PN. Total-STIGM was higher in Preterm-PN than in Adult-PN but it was not different from Term-PN. Total-STIGM was not different between Term-PN and Adult-PN. Total-CAMP, Total-SITO and Total-PHY of Preterm-PN were not different from Term-PN and Adult-PN.
Preterm-PN had a significantly higher Free-CHO/Ester-CHO ratio than Adult-PN while the ratio was intermediate in Term-PN. The same result was found for Free-CAMP/Ester-CAMP, Free-SITO/Ester-SITO and Free-PHY/Ester-PHY ratios. Free-STIGM/Ester-STIGM of Preterm-PN was significantly higher than in the other study groups. There were no significant differences in any of sterol ratios between the 6 preterm infants who received MSF and the 10 preterm infants who received the MS LE (Data not shown).

Table 2. Plasma sterol concentrations and free/esterified ratios[1,2]

Variable	Preterm-PN (n=16)	Term-PN (n=11)	Adult-PN (n=12)	*P ANOVA
Cholesterol (mg/L)				
Free-CHO	613.4±170.9	601.6±153.9	518.7±162.2	0.293
Ester-CHO	647.1±240.5[a]	642.7±304.8[a,b]	944.3±387.2[b]	0.030
Total-CHO	1260.5±330.6	1244.3±374.3	1463.0±533.0	0.355
Free-CHO/Ester-CHO	1.1±0.7[a]	1.1±0.4[a,b]	0.6±0.2[b]	0.026
Campesterol (mg/L)				
Free-CAMP	3.0±1.1	2.3±1.2	2.1±1.4	0.131
Ester-CAMP	1.5±0.8	1.6±1.5	2.7±2.4	0.129
Total-CAMP	4.5±1.6	3.8±2.6	4.8±3.7	0.694
Free-CAMP/Ester-CAMP	2.5±1.6[a]	2.0±1.1[a,b]	1.0±0.4[b]	0.006
Stigmasterol (mg/L)				
Free-STIGM	3.6±1.3[a]	2.3±1.7[a,b]	1.7±1.1[b]	0.003
Ester-STIGM	0.2±0.1	0.4±0.4	0.5±0.4	0.066
Total-STIGM	3.8±1.4[a]	2.7±1.9[a,b]	2.2±1.5[b]	0.026
Free-STIGM/Ester-STIGM	20.3±10.6[a]	11.3±7.8[b]	4.8±3.4[b]	0.000
Sitosterol (mg/L)				
Free-SITO	9.3±4.0	10.6±7.5	6.2±4.5	0.131
Ester-SITO	3.4±2.0	5.1±4.1	7.5±6.7	0.062
Total-SITO	12.7±5.3	15.7±10.6	13.8±11.0	0.689
Free-SITO/Ester-SITO	3.7±2.4[a]	2.7±1.5[a,b]	1.2±0.7[b]	0.004
Phytosterols (mg/L)				
Free-PHY	15.8±6.0	15.1±8.8	9.9±6.7	0.085
Ester-PHY	5.1±2.8	7.1±5.6	10.8±9.3	0.069
Total-PHY	20.9±7.9	22.2±13.2	20.7±15.7	0.949
Free-PHY/Ester-PHY	4.1±2.6[a]	2.9±1.7[a,b]	1.3±0.8[b]	0.003

[1] Preterm-PN: preterm infants on parenteral nutrition; Term-PN: term infants on parenteral nutrition; Adult-PN: adults on parenteral nutrition; CHO: cholesterol; Ester: esterified; CAMP: campesterol; STIGM: stigmasterol; SITO: sitosterol; PHY: phytosterols.
[2] Data are expressed as Mean±SD. Values of *P<0.05 by one-way independent ANOVA were considered significant. Different superscripts indicate significant differences between groups by Bonferroni test.

Free-CHO/Ester-CHO, Free-CAMP/Ester-CAMP and Free-SITO/Ester-SITO were lower than Free-STIGM/Ester-STIGM ratio in all PN groups. Free-CHO/Ester-CHO of Preterm-PN, Term-PN and Adult-PN was significantly lower than any of phytosterol ratios (*P<0.05). Free-SITO/Ester-SITO of Preterm-PN and Term-PN was higher than Free-CAMP/Ester-CAMP. No difference was found between Free-CAMP/Ester-CAMP and Free-SITO/Ester-SITO in Adult-PN. The Free-CHO/Ester-CHO was significantly lower than Free-PHY/Ester-PHY in all PN groups (1.1±0.7 vs 4.1±2.6 in Preterm-PN respectively, *P=0.000; 1.1±0.4 vs 2.9±1.7 in Term-PN respectively, *P=0.003; 0.6±0.2 vs 1.3±0.8 in Adult-PN respectively, *P=0.004).

We calculated the differences of the sterol ratios between IV LE and plasma of each study patients (**Table 3**). The differences of the ratios were significantly higher in Preterm-PN than in Adult-PN. Term-PN were not different from the other groups. All plasma phytosterol ratios in Preterm-PN were statistically different from the respective values in IV LE. Significant differences were also found in CAMP and STIGM ratios of Term-PN and in CAMP and SITO ratios of Adult-PN (*P<0.05).

Table 3. Differences of sterol ratios between IV LE and plasma values[1,2]

Variable	Preterm-PN (n=16)	Term-PN (n=11)	Adult-PN (n=12)	*P ANOVA
Cholesterol				
Δ of Free-CHO/Ester-CHO	-4.8±3.4[a]	-6.6±0.4[a,b]	-7.1±0.2[b]	0.022
Campesterol				
Δ of Free-CAMP/Ester-CAMP	-2.7±1.6[a]	-3.2±1.1[a,b]	-4.2±0.4[b]	0.007
Stigmasterol				
Δ of Free-STIGM/Ester-STIGM	14.6±10.5[a]	5.7±7.8[b]	-0.8±3.4[b]	0.000
Sitosterol				
Δ of Free-SITO/Ester-SITO	1.3±2.4[a]	0.5±1.5[a,b]	-1.0±0.7[b]	0.005

[1] Preterm-PN: preterm infants on parenteral nutrition; Term-PN: term infants on parenteral nutrition; Adult-PN: adults on parenteral nutrition; Δ: difference; CHO: cholesterol; Ester: esterified; CAMP: campesterol; STIGM: stigmasterol; SITO: sitosterol; PHY: phytosterols.
[2] Data are expressed as Mean±SD. Values of *P<0.05 by one-way ANOVA were considered significant. Different superscripts indicate significant differences between groups by Bonferroni test.

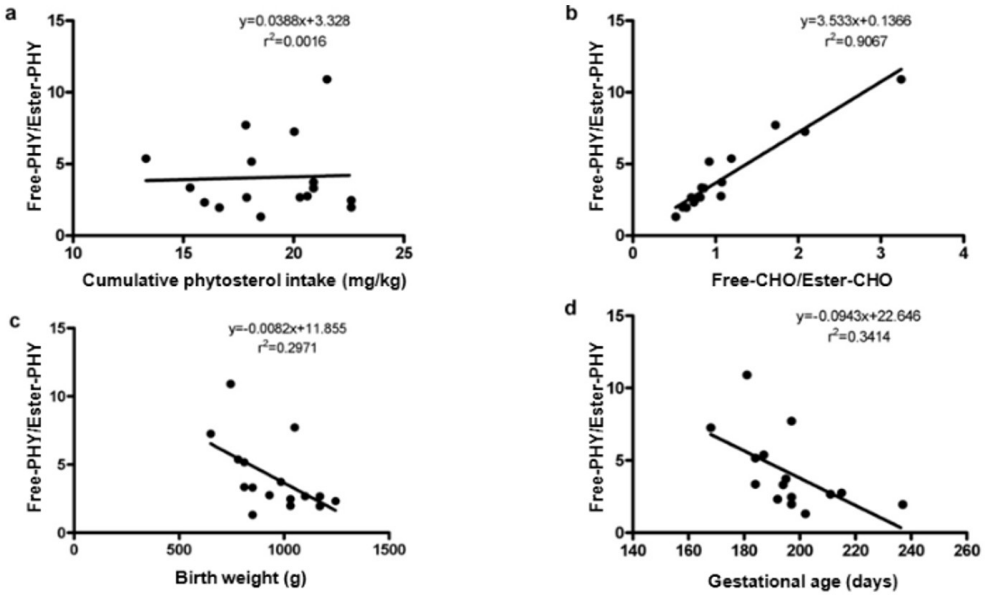
Free/esterified sterol ratios before and after stopping the lipid infusion are reported in **Table 4**. No significant differences were found neither in preterm infants nor in adults.
In preterm infants, plasma Free-PHY/Ester-PHY did not correlated with cumulative (birth to sampling) phytosterol intake (r=0.040, *P=0.882; **Fig.1**, **panel a**).
A significant positive correlation was found between Free-PHY/Ester-PHY and Free-CHO/Ester-CHO (r=0.952; *P=0.000; Fig.1, panel b). Free-PHY/Ester-PHY was found to be negatively correlated with birth weight and with gestational age (r=-0.545; *P=0.029 and r= -0.584; *P=0.017, respectively; **Fig.1**, **panel c** and **d**).

Table 4. Plasma free and esterified sterols ratio in Preterm-PN and in Adult-PN during the continuous infusion of PN and 24-hrs after a fat free PN[1,2]

Variable	Preterm-PN			Adult-PN		
	With IVfat (n=8)	Free IV fat (n=8)	*P t-test	With IV fat (n=10)	Free IVfat (n=10)	*P t-test
Free-CHO/Ester-CHO	1.0±0.5	0.9±0.3	0.802	0.6±0.2	0.6±0.3	0.08
Free-CAMP/Ester-CAMP	1.9±1.1	1.7±0.7	0.744	1.1±0.5	0.9±0.4	0.08
Free-STIGM/Ester-STIGM	14.6±7.8	12.9±5.5	0.949	5.7±3.4	5.0±3.1	0.09
Free-SITO/Ester-SITO	2.9±1.9	2.6±1.3	0.674	1.3±0.7	1.0±0.5	0.06
Free-PHY/Ester-PHY	3.1±1.9	2.8±1.3	0.706	1.4±0.8	1.1±0.7	0.09

[1] Preterm-PN: preterm infants on PN; Adult-PN: adults on PN; CHO: cholesterol; Ester: esterified; CAMP: campesterol; STIGM: stigmasterol; SITO: sitosterol; PHY: phytosterols; IV: intravenous. [2] Data are expressed as Mean±SD; Values of *P<0.05 by paired t-test were considered significant.

Fig.1 Linear regression analysis between Free-PHY/Ester-PHY ratio and several features of study Preterm-PN (cumulative phytosterol intake, Free-CHO/Ester-CHO ratio, gestational age and birth weight)[1,2]. [1] PHY: phytosterol; Ester: esterified; CHO: cholesterol; Preterm-PN: preterm infants on parenteral nutrition (n=16). [2] Panel a shows a positive correlation between Free-PHY/Ester-PHY and cumulative phytosterol intake but it was not significant (r=0.040, *P=0.882); Panel b shows a positive correlation between Free-PHY/Ester-PHY and Free-CHO/Ester-CHO that it was statistically significant (r=0.952, *P=0.000); Panel c and d show negative and significant correlations between Free-PHY/Ester-PHY and birth weight and gestational age (r=-0.545, *P =0.029 and r=-0.584, *P=0.017, respectively).



DISCUSSION

To the best of our knowledge, this is the first report on plasma Free-PHY/Ester-PHY concentration in Preterm-PN. The Free-PHY/Ester-PHY in Preterm-PN was about 3-fold higher than in Adult-PN while Free-CHO/Ester-CHO of Preterm-PN was twice as high as Adult-PN. Free and esterified sterol ratios in Adult-PN were higher than those reported for adults on a standard diet [11, 26]. Term-PN exhibited intermediate values between Adult-PN and Preterm-PN. In all study groups, PHY were esterified to a lesser CHO. These results show that the esterification of IV PHY is much lower than that of CHO at all ages.

Decreased rate of PHY esterification compared to that of CHO was reported in hypertriglyceridemic subjects, healthy adults and phytosterolemic patients on a standard diet. This finding was explained by the lower intestinal absorption of PHY compared to CHO due to different ABCG5/ABCG8 affinity to sterols [27]. In our study, we demonstrated that this also happens in patients on PN with SO-based LE. All our patients received negligible amounts of enteral feeding, thus we can reasonably exclude the previous interpretation of Vahouny et al [27]. In our view, the different sterol esterification likely lies in the lower affinity of LCAT and ACAT for PHY as suggested by Lin et al. in sitosterolemic patients [10].

Of note, the limited ability of preterm infants to esterify PHY did not correlate with the cumulative phytosterol intake during PN. Decreased phytosterol esterification in preterm infants was not affected by the kind of LE used. Furthermore, the ratios were not different before and after stopping IV LE for 24-hrs. These observations together with the age-related changes in esterification strongly support the notion that this phenomenon depends on the development/maturation of the metabolic pathway. An age-dependent effect on sterol esterification is well described for plasma cholesterol [28, 29].

The consequences of such a marked decrease in esterification are unknown. Unfortunately, the limited number of patients in this pilot study prevented us to study the possible correlations with liver function and/or PNAC. In the IV LE, the Free-SITO/Ester-SITO ratio was nearly half of both CAMP and STIGM. In plasma, the free and esterified sterol ratios of CAMP and SITO were significantly lower than that STIGM in all study groups. A lower ratio in plasma than in IV LE (negative values) could be indicative of active phytosterol esterification in the body. A higher ratio in plasma than in IV LE (positive values) could suggest poor esterification or enhanced clearance of phytosterol esters compare to free sterols. We found negative values of CAMP and SITO or values lesser positive than STIGM from the comparison of plasma and IV LE phytosterol ratios in all study groups.

Furthermore, Preterm-PN exhibited a markedly and significantly higher ratios compared to Term-PN and Adult-PN. Similar findings were reported by Lin et al. in sitosterolemic patients (adolescents to the 40-years-olds) on a standard diet [10]. These results could be explained by the different effect of phytosterol structure upon LCAT and ACAT activity resulting in different esterification rates. However, a faster incorporation of Ester-PHY into membranes cannot be excluded, even if all study patients received IV lipids for a quite long period of time before sampling and thus likely on steady-state [30]. In vitro reconstitution of sterol transfer by G5 and G8 in a cell-free system showed a better sterol free ABCG5/ABCG8-mediate transport than that of esterified sterols [31]. Our study in human adults and infants cannot provide information on the potential different clearance of free and esterified sterols.

Of interest, plasma phytosterol concentrations in all our study patients on PN were up to 3-4 folds higher than in healthy adult controls taken from our laboratory (unpublished data) and also from the literature [32]. Elevated plasma phytosterol concentrations were linked to severe liver dysfunction both in animals and in humans [33-38]. Thus, vegetable oil-lipid exposed

patients may have a high risk for developing acute liver failure. Whether or not liver failure is associated with a decreased sterol esterification is not established yet. We are interested in studying if individuals with a decreased phytosterol esterification are at higher risk of developing liver failure. Our clinical pilot study is clearly unable to address all these above-mentioned issues. We are planning to measure free and esterified phytosterols in tissues and red blood cell membranes.

Liver is the sole organ responsible for the final elimination of sterols from the body either as free sterols or bile acids. High density lipoprotein-derived sterols are the major source of biliary sterols and represents a mechanism for the removal of CHO and PHY from peripheral tissues including artery wall-associated macrophage foam cells. Via selective uptake through scavenger receptor BI, high density lipoprotein CHO and PHY enter in the hepatic metabolic pool and sterol esters is hydrolyzed prior to its storage. In hepatocytes, free sterol secretion is mainly performed by ABCG5/G8 transporters and scavenger receptors BI. More than 80% of sterols associated with high density lipoprotein is present as sterol esters thus sterol esterification plays a pivotal role in liver function [39].

The finding that preterm infants who exhibited a higher cholesterol esterification had also higher phytosterol esterification strongly support the hypothesis that high free phytosterol plasma concentration is related to the low LCAT and ACAT activity rather than to the low ABCG5/ABCG8 or scavenger receptor BI transport activity in hepatocytes. Supportive of this interpretation, there is also the inverse significant correlations with birth weight and gestational age.

Unfortunately, our data does not permit to identify any association between free and esterified phytosterol ratio and PNAC in preterm infants on PN. A large number of preterm infants with PNAC should be investigated.

In conclusion, we provided novel data on the markedly decreased phytosterol esterification in Preterm-PN compared to Adult-PN. We also demonstrated that plasma Free-PHY were esterified to a lesser extent than CHO in Preterm-PN, Term-PN and in Adult-PN and this phenomenon was independent from the IV lipid intake. Additionally, we found that preterm infants who exhibited the highest CHO esterification were also better at esterifying PHY. This effect correlated with birth weight and gestational age. The clinical relevance of our findings prompts further studies.

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There are no conflicts of interest to declare.

There were 12 authors, who contributed to the work. We report below the contribution of each author:

• VPC was responsible for the design of the study;
• SS, AC, DP, MS and AP contributed to data collection and analysis;
• RD, GV, CB, MT and AN contributed to subject recruitment and data collection;
• PC was in charge of the statistical analysis.

All authors read and approved the final manuscript.

REFERENCES

1. Kaufman SS, Gondolesi GE, and Fishbein TM (2003) Parenteral nutrition associated liver disease. Semin Neonatol 8 : 375-381

2. La Scala GC, Le Coultre C, Roche BG, Bugmann P, and Belli DC (1993) The addition of lipids increases the total parenteral nutrition-associated cholestasis in the rat. Eur J Pediatr Surg 3 : 224-227

3. Clayton PT, Bowron A, Mills KA, Massoud A, Casteels M, and Milla PJ (1993) Phytosterolemia in children with parenteral nutrition-associated cholestatic liver disease. Gastroenterology 105 : 1806-1813

4. Whitfield PD, Clayton PT, and Muller DP (2000) Effect of intravenous lipid emulsions on hepatic cholesterol metabolism. J Pediatr Gastroenterol Nutr 30 : 538-546

5. Llop JM, Virgili N, Moreno-Villares JM, Garcia-Peris P, Serrano T, Forga M, Solanich J, and Pita AM (2008) Phytosterolemia in parenteral nutrition patients: implications for liver disease development. Nutrition 24 : 1145-1152

6. Bindl L, Lutjohann D, Buderus S, Lentze MJ, and v Bergmann K (2000) High plasma levels of phytosterols in patients on parenteral nutrition: a marker of liver dysfunction. J Pediatr Gastroenterol Nutr 31 : 313-316

7. Iyer KR, Spitz L, and Clayton P (1998) BAPS prize lecture: New insight into mechanisms of parenteral nutrition-associated cholestasis: role of plant sterols. British Association of Paediatric Surgeons. J Pediatr Surg 33 : 1-6

8. Pianese P, Salvia G, Campanozzi A, D'Apolito O, Dello Russo A, Pettoello-Mantovani M, and Corso G (2008) Sterol profiling in red blood cell membranes and plasma of newborns receiving total parenteral nutrition. J Pediatr Gastroenterol Nutr 47 : 645-651

9. Ellegard L, Sunesson A, and Bosaeus I (2005) High serum phytosterol levels in short bowel patients on parenteral nutrition support. Clin Nutr 24 : 415-420

10. Lin DS, Steiner RD, Merkens LS, Pappu AS, and Connor WE (2010) The effects of sterol structure upon sterol esterification. Atherosclerosis 208 : 155-160

11. Lembcke J, Ceglarek U, Fiedler GM, Baumann S, Leichtle A, and Thiery J (2005) Rapid quantification of free and esterified phytosterols in human serum using APPI-LC-MS/MS. J Lipid Res 46 : 21-26

12. Tilvis RS, and Miettinen TA (1986) Serum plant sterols and their relation to cholesterol absorption. Am J Clin Nutr 43 : 92-97

13. Savini S, D'Ascenzo R, Biagetti C, Serpentini G, Pompilio A, Bartoli A, Cogo PE, and Carnielli VP (2013) The effect of 5 intravenous lipid emulsions on plasma phytosterols in preterm infants receiving parenteral nutrition: a randomized clinical trial. Am J Clin Nutr 98 : 312-318

14. Xu Z, Harvey KA, Pavlina T, Dutot G, Hise M, Zaloga GP, and Siddiqui RA (2012) Steroidal compounds in commercial parenteral lipid emulsions. Nutrients 4 : 904-921

15. Forchielli ML, Bersani G, Tala S, Grossi G, Puggioli C, and Masi M (2010) The spectrum of plant and animal sterols in different oil-derived intravenous emulsions. Lipids 45 : 63-71

16. Sperry WM (1935) Cholesterol esterase in blood. J Biol Chem 111 : 467-478

17. Glomset JA (1968) The plasma lecithins:cholesterol acyltransferase reaction. J Lipid Res 9 : 155-167

18. Sperry WM, and Stoyanoff VA (1938) The enzymatic synthesis and hydrolysis of cholesterol esters in blood serum. J Biol Chem 126 : 77-89

19. Lee RG, Kelley KL, Sawyer JK, Farese RV, Jr., Parks JS, and Rudel LL (2004) Plasma cholesteryl esters provided by lecithin:cholesterol acyltransferase and acyl-coenzyme a:cholesterol acyltransferase 2 have opposite atherosclerotic potential. Circ Res 95 : 998-1004

20. Piran U, and Nishida T (1979) Utilization of various sterols by lecithin-cholesterol acyltransferase as acyl acceptors. Lipids 14 : 478-482

21. Macauley SK, Billheimer JT, and Ritter KS (1986) Sterol substrate specificity of acyl coenzyme A:cholesterol acyltransferase from the corn earworm, Heliothis zea. J Lipid Res 27 : 64-71

22. Tavani DM, Nes WR, and Billheimer JT (1982) The sterol substrate specificity of acyl CoA:cholesterol acyltransferase from rat liver. J Lipid Res 23 : 774-781

23. Lee RG, Shah R, Sawyer JK, Hamilton RL, Parks JS, and Rudel LL (2005) ACAT2 contributes cholesteryl esters to newly secreted VLDL, whereas LCAT adds cholesteryl ester to LDL in mice. J Lipid Res 46 : 1205-1212

24. Folch J, Lees M, and Sloane Stanley GH (1957) A simple method for the isolation and purification of total lipides from animal tissues. J Biol Chem 226 : 497-509

25. Corso G, Rossi M, De BD, Rossi I, Parenti G, and Dello RA (2002) Effects of sample storage on 7- and 8-dehydrocholesterol levels analysed on whole blood spots by gas chromatography-mass spectrometry-selected ion monitoring. J Chromatogr B Analyt Technol Biomed Life Sci 766 : 365-370

26. Bhattacharyya AK, and Connor WE (1974) Beta-sitosterolemia and xanthomatosis. A newly described lipid storage disease in two sisters. J Clin Invest 53 : 1033-1043

27. Vahouny GV, Connor WE, Subramaniam S, Lin DS, and Gallo LL (1983) Comparative lymphatic absorption of sitosterol, stigmasterol, and fucosterol and differential inhibition of cholesterol absorption. Am J Clin Nutr 37 : 805-809

28. Papadopoulos A, Hamosh M, Chowdhry P, Scanlon JW, and Hamosh P (1988) Lecithin-cholesterol acyltransferase in newborn infants: low activity level in preterm infants. J Pediatr 113 : 896-898

29. Jain SK (1985) Prematurity and lecithin-cholesterol acyltransferase deficiency in newborn infants. Pediatr Res 19 : 58-60

30. Nghiem-Rao TH, Tunc I, Mavis AM, Cao Y, Polzin EM, Firary MF, Wang X, Simpson PM, and Patel SB (2015) Kinetics of phytosterol metabolism in neonates receiving parenteral nutrition. Pediatr Res 78 : 181-189

31. Wang J, Sun F, Zhang DW, Ma Y, Xu F, Belani JD, Cohen JC, Hobbs HH, and Xie XS (2006) Sterol transfer by ABCG5 and ABCG8: in vitro assay and reconstitution. J Biol Chem 281 : 27894-27904

32. Chan YM, Varady KA, Lin Y, Trautwein E, Mensink RP, Plat J, and Jones PJ (2006) Plasma concentrations of plant sterols: physiology and relationship with coronary heart disease. Nutr Rev 64 : 385-402

33. Hukkinen M, Mutanen A, Nissinen M, Merras-Salmio L, Gylling H, and Pakarinen MP (2016) Parenteral Plant Sterols Accumulate in the Liver Reflecting Their Increased Serum Levels and Portal Inflammation in Children With Intestinal Failure. JPEN J Parenter Enteral Nutr

34. Karim C. El Kasmi ALA, Michael W. Devereaux,1 Padade M. Vue, Wujuan Zhang, Kenneth D. R. Setchell, Saul J. Karpen, Ronald J. Sokol (2013) Phytosterols promote liver injury and Kupffer cell activation in parenteral nutrition-associated liver disease. Sci Transl Med 5 : 1-10

35. Clayton PT, Whitfield P, and Iyer K (1998) The role of phytosterols in the pathogenesis of liver complications of pediatric parenteral nutrition. Nutrition 14 : 158-164

36. Vlaardingerbroek H, Ng K, Stoll B, Benight N, Chacko S, Kluijtmans LA, Kulik W, Squires EJ, Olutoye O, Schady D, Finegold ML, van Goudoever JB, and Burrin DG (2014) New generation lipid emulsions prevent PNALD in chronic parenterally fed preterm pigs. J Lipid Res 55 : 466-477

37. Zaloga GP (2015) Phytosterols, Lipid Administration, and Liver Disease During Parenteral Nutrition. JPEN J Parenter Enteral Nutr 39 : 39S-60S

38. Carter BA, Taylor OA, Prendergast DR, Zimmerman TL, Von Furstenberg R, Moore DD, and Karpen SJ (2007) Stigmasterol, a soy lipid-derived phytosterol, is an antagonist of the bile acid nuclear receptor FXR. Pediatr Res 62 : 301-306

39. Yuan Q, Bie J, Wang J, Ghosh SS, and Ghosh S (2013) Cooperation between hepatic cholesteryl ester hydrolase and scavenger receptor BI for hydrolysis of HDL-CE. J Lipid Res 54 : 3078-3084

Chapter 4

Half-life of Plasma Phytosterols in Very Low Birth Weight Preterm Infants on Routine Parenteral Nutrition with Vegetable Oil-Based Lipid Emulsions

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Summary

Background: Phytosterols in vegetable oil (VO)-based lipid emulsions (LE) likely contribute to parenteral nutrition-associated cholestasis (PNAC) in preterm infants. No characterization of plasma phytosterol half-lives has been done in very low birth weight (VLBW) preterm infants receiving parenteral nutrition (PN) with LE.

Methods: In a prospective cohort study, 45 VLBW preterm infants who received PN underwent serial blood sample measurements of sitosterol (SITO), campesterol (CAMP), and stigmasterol (STIGM). Plasma phytosterol half-lives were calculated from the phytosterol concentrations-decay curves by using a single-compartment model.

Results: After the stop of the intravenous LE, study infants had significantly lower plasma total CAMP, STIGM and SITO concentrations. The decay of plasma phytosterol concentrations was monoexponential. Half-life of plasma total CAMP, STIGM and SITO was 13.5±6.9, 10.3±4.5 and 10.3±4.0 days, respectively. Plasma phytosterol half-lives did not correlate with gestational age, birth weight, cumulative phytosterol intakes and plasma conjugated bilirubin.

Conclusion: VLBW preterm infants on PN with LE had rather long plasma phytosterol half-lives similar to hypercholesterolemic adults and phytosterolemic homozygotes patients. We speculate that the accumulation of phytosterols could contribute to their vulnerability to PNAC.

Clinical trial registry: The Ethics Committee of Marche-Italy (DG/469); www.clinicaltrials.gov (identification number NCT02758834).

Abbreviations: CAMP: campesterol; EN: enteral nutrition; LE: lipid emulsion; PHY: phytosterols; PN: parenteral nutrition; PNAC: parenteral nutrition-associated cholestasis; SITO: sitosterol; STIGM: stigmasterol; VLBW: very low birth weight; VO: vegetable oil.

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INTRODUCTION

Phytosterols in vegetable oil (VO)-based lipid emulsions (LE) may contribute to parenteral nutrition-associated cholestasis (PNAC) in infants receiving parenteral nutrition (PN). Accumulating scientific evidence suggests a definite association between high plasma phytosterol concentrations and the onset and severity of PNAC. Phytosterol accumulation in the body, associated with the use of intravenous LE, is caused by the bypass of the protective mechanisms occurring in enteral nutrition (EN). Under normal conditions, phytosterol elimination occurs by intestinal and hepatic ABCG5/G8 transporters. Dietary phytosterols are excreted into the intestinal lumen by intestinal ABCG5/G8 transporters and this prevents the entry into the bloodstream of more than 95% of the ingested phytosterols. The small amounts of phytosterols absorbed by the gut are then excreted by the hepatic ABCG5/G8 into the bile [1]. During PN administration, the direct entry of phytosterols into the bloodstream may expose the liver to these potential toxins and challenge the normal hepatic excretion. In animal and in vitro models, phytosterols induce cholestasis, cause direct hepatocyte damage and antagonize nuclear receptors critical for hepatoprotection against bile acid injury [2-4]. In humans, the “toxic” effects of high plasma phytosterol concentrations were described in phytosterolemic patients, who develop xanthoma, atherosclerosis, thrombocytopenia, and hemolytic anemia [5, 6]. Despite of potential phytosterol toxicity, VO-based intravenous LE are the only lipid emulsions approved by the US Food and Drug Administration and the European Medicines Evaluation Agency, thus they are routinely administered to preterm infants needing PN. To date, information on phytosterol metabolism in preterm infants receiving PN is limited. Nghiem-Rao et al. found that phytosterols in preterm infants on PN reach steady median plasma concentrations 3-5 days after the start of LE. Furthermore, very preterm infants (gestational age < 28 weeks) had higher exposure to phytosterols than the more mature infants (gestational age ≥ 28 weeks) [7]. In healthy subjects on a normal diet [1, 8, 9] and in phytosterolemic homozygotes patients [10-13], half-life of plasma phytosterols was about 4 and 10 days, respectively.

The ability of preterm infants on PN to metabolize phytosterols is still unclear. To date, the contribution of phytosterol toxicity to PNAC is also not well established and the sequelae of phytosterol accumulation are unknown and deserve further studies.

The aim of this prospective pilot cohort study was to measure the plasma phytosterol half-life in VLBW preterm infants receiving PN with VO-based LE.

METHODS

The study was conducted in accordance with the principles of the Helsinki Declaration as revised in Edinburgh 2000 and was approved by the the local ethics committee and institutional review board (No. 469/DG). Written informed consent was obtained from the parents or legal guardians of all study patients.

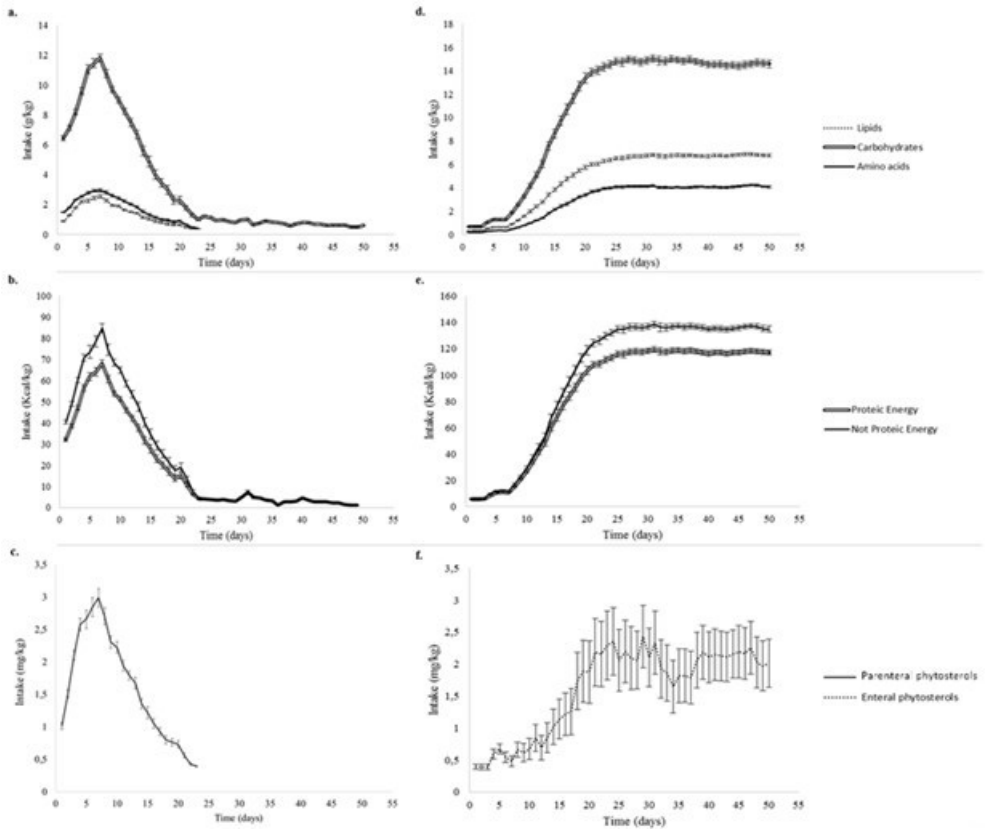
Between January 2014 and March 2016, preterm infants admitted to the “G. Salesi” Children's Hospital of Ancona Neonatal Intensive Care Unit with a birth weight of 500-1249 g, a gestational age under 32 weeks and who received VO-based intravenous LE from the first hour of life were screened for participation in this prospective pilot study. Both genders were included. Neonates with known primary liver disease, anatomic liver abnormalities, severe congenital sepsis, metabolic inborn errors, or known genetic or chromosomal defects were excluded. Liver dysfunction during PN (plasma conjugated bilirubin > 1 mg/dl), death in the first week of life and PN duration longer than 24 days, considered a marker of unidentified

metabolic disorder or feeding intolerance, were also exclusion criteria at the time of the data analysis.

All study patients received PN in an all-in-one mixture. At birth, the pharmacist selected the LE by a sealed-envelope method, as part of the routine PN. Three LE were used for this study: two containing fish oil (MSF, Lipidem® 20%, B.Braun - 50% medium chain triglycerides, 40% soybean oil, and 10% fish oil; MSOF, SMOFlipid® 20%, Fresenius-Kabi - 30% medium chain triglycerides, 30% soybean oil, 25% olive oil and 15% fish oil) and the third one not containing fish oil (MS; Lipofundin MCT® 20%, B.Braun; 50% medium-chain triglycerides and 50% soybean oil).

According to the local NICU protocol, lipids were infused at dose of 1, 1.5, 2, 2.5, and 3 g·kg-1·d-1 from postnatal day 0 to day 5 respectively and were then kept constant from day 5 to day 7. PN was tapered from day 8 until day 21, when it was usually stopped. Minimal enteral feeding with human milk was provided from day 0 to day 8, the maximum amount supplied being 8 mL·kg-1·d-1 from day 1 to day 4, and 16 mL·kg-1·d-1 from day 5 to day 8. EN was gradually increased from day 9 to reach full oral feeding on day 18. Additional information on PN and EN of study infants are reported in **Table 1** and in **Figure 1**.

Figure 1. Parenteral and enteral nutrition schedules of study infants (n=46). Lipids, phytosterols, carbohydrates, amino acids and energy provided during parenteral (a, b, c) and enteral nutrition (d, e, f) are shown. Data are presented as Mean±SE (mg/Kg or Kcal/kg).



Enteral intake of phytosterols was calculated from the phytosterol concentrations in infant formulas (PreNidina, Nestlé, Italy; Aptamil pre, Milupa, Italy; Mellin 0, Mellin, Italy) and in premature human milk measured in our laboratory.
EDTA-treated blood samples remaining after completion of clinical laboratories were weekly collected from 1st to 7th week of life for measurements of sitosterol (SITO), campesterol (CAMP) and stigmasterol (STIGM). Blood samples were immediately centrifuged at 2800 rpm (1358 g) and plasma was stored in pyrogallol added-tubes at -20°C until analysis. Samples of VO-based intravenous LE given to preterm infants were also obtained for phytosterol analysis.

Analytic method

All the reagents were obtained from Steraloids (Newport, USA). Cholest-5-en-3β-ol (cholesterol), 5α-cholestane (internal standard), 24α-methylcholest-5-en-3β-ol (campesterol), 3β-hydroxy-24-ethyl-5,22-cholestadiene (stigmasterol) 24α-ethylcholest-5-en-3β-ol (β-sitosterol) had a purity level of 99%, 97%, 65%, 95% and 71%, respectively.
A stock solution of 5α-cholestane in chloroform was preparated at a concentration of 2.50 mg/ml. A solution containing 0.25 mg/mL of 5α-cholestane was used as working standards and 20 µl of this were added to 50 µl of plasma before saponification reaction. In brief, plasma samples were saponified with potassium hydroxide/methanol (5M) and heated at 60°C for 1 hour. Sterols were extracted from the hydroalcoholic phase by means of liquid-liquid extraction with an equal volume of hexane for 15 minutes under shaking and after centrifugation. A second extraction was performed adding diethyl ether instead of hexane. Hexane and diethyl ether were removed under a gentle stream of nitrogen. Free sterols were extracted with 200 µl of pure hexane and derivatized by bis(trimethylsilyl)trifluoro-acetamide in pyridine. One microliter of this solution was analyzed by gas chromatography-mass spectrometry [14].
The VO-based intravenous LE, infant formulas and human milk were diluted 40, 10 and 10 times, respectively, and were analyzed with the same method used for plasma samples. Coefficient of variation of plasma total CAMP, STIGM and SITO concentrations was always less than 10% (Data not shown). Gas chromatography-mass spectrometry analysis was performed by using the same method previously published [14]. Plasma total concentration of phytosterols (PHY) was calculated as the sum of total CAMP, STIGM and SITO concentrations.

Pharmacokinetic Models

Graphs of plasma phytosterol concentrations as a function of time after the stop of lipid infusion were used to evaluate the kinetics of phytosterol elimination. According to Relas at al. [8], serial plasma phytosterol concentrations had monoexponetial decay in all study patients when lipids were stopped. Logarithm of plasma phytosterol concentrations versus time was linear. The constant of decay (K) was obtained from the following equation:

$$\ln C = -Kt + \ln C0$$

where “lnC” is natural logarithm of plasma phytosterol concentration at different time, “t” is the sampling time and “lnC0” is natural logarithm of plasma phytosterol concentration at first sampling time after stopping lipids that was imposed at x=0. Plasma phytosterol half-lives (t1/2) were calculated using the following equation:

$$t1/2 = \ln 2/K.$$

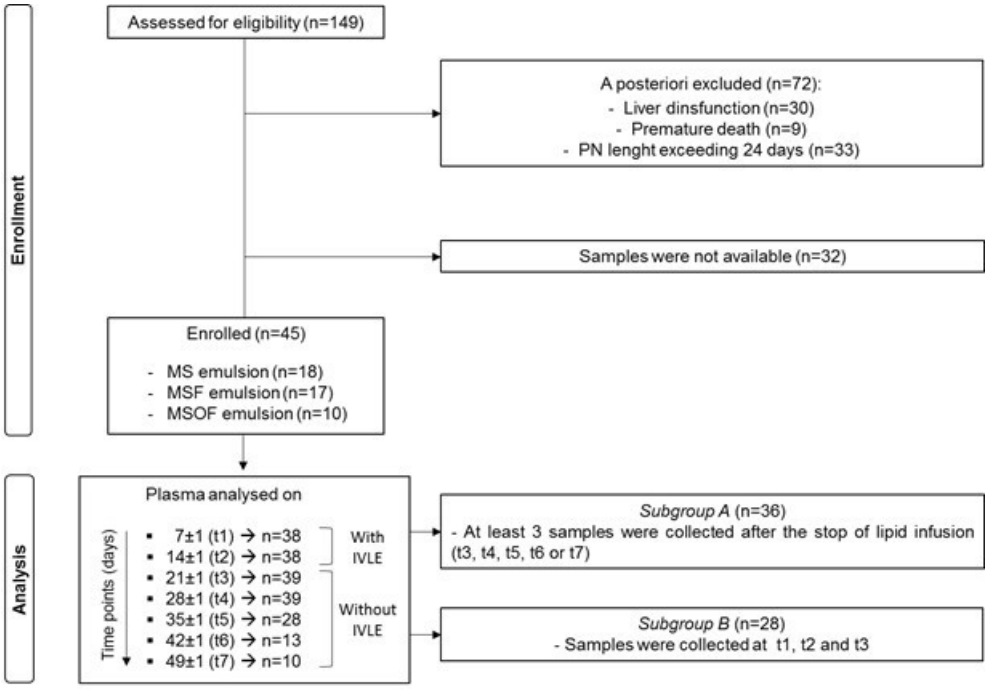
Statistical Analysis

The primary outcome was the temporal change of plasma phytosterol concentrations. Elimination was assessed using plasma phytosterol concentrations obtained after the stop of lipid infusion. Significant differences of plasma phytosterol half-lives among groups were analyzed by repeated measures ANOVA with Bonferroni test. A *P-value<0.05 was considered significant. Plasma phytosterol concentrations at each time points were presented as Mean±SD (mg/L) and were analyzed by repeat measured ANOVA with Bonferroni test. Independent t-test and Chi-square were used to analyze demographic and clinical characteristics of study patients. A simple linear regression analysis was used to study the relationship between variables. All statistical analyses were performed using SPSS (v 19.0; SPSS Inc, Chicago, Illinois) and Microsoft EXCEL (v 2010; Microsoft Corp Redmond, Washington).

RESULTS

One hundred forty-nine VLBW preterm infants were screened for the study from January 2014 to March 2016. Seventy-two patients were excluded: 30 for liver dysfunction during PN, 9 died before the first week of life and 33 had a PN duration longer than 24 days. Forty-five of the 77 remaining neonates had enough leftover plasma for phytosterol analysis: 38 samples were available at 7±1 (d7), 38 at 14±1 (d14), 39 at 21±1 (d21), 39 at 28±1 (d28), 28 at 35±1 (d35), 13 at 42±1 (d42) and 10 at 49±1 (d49) days (**Figure 2**).

Figure 2. Flow diagram of the study's progress, detailing participant numbers during enrollment and analytical approach.



Lipid infusion was stopped before 21±1 (d21) days in all study infants. In 36 patients (subgroup A) we collected 3 or more blood samples after the stop of LE for phytosterol half-life. In 44 infants we had at least one sample from day 7 (d7) to 21 (d21) and in 28 infants samples were available at all time-points (subgroup B).
Eight-teen patients received non-fish oil-containing LE and 27 preterm infants received LE with fish oil (17 received MSF and 10 MSOF LE).
Eight of the study infants were fed exclusively human milk, 3 infant formulas and the rest received mixed oral feedings. No differences were found between subgroup A and subgroup B for PN and EN of lipids, carbohydrates, amino acids and energy (**Table 1**).

Table 1. Information on parenteral and enteral nutrition of study infants[1]

	All study infants (n=45)	Subgroup A (n=36)	Subgroup B (n=28)	P-Value
Parenteral nutrition				
Lipids (mg/Kg) -	28.3±0.7	28.2±0.8	28.4±1.0	0.86
Phytosterols (mg/Kg)	34.7±1.4	33.9±1.6	33.9±1.8	0.91
Amino acids (mg/Kg)	36.5±1.1	36.5±1.3	36.6±1.4	0.96
Glucids (mg/Kg)	144.0±3.7	143.2±4.4	145.0±4.5	0.79
Protein energy (Kcal/Kg)	983.6±24.8	984.5±29.5	982.6±29.0	0.97
Not-Protein energy (Kcal/Kg)	779.1±19.4	780.0±23.3	778.0±22.6	0.95
Enteral nutrition				
Lipids (mg/Kg)	244.2±3.9	245.9±4.3	242.0±5.0	0.57
Phytosterols (mg/Kg)	77.1±14.1	76.5±16.0	98.3±20.2	0.40
Amino acids (mg/Kg)	145.9±2.2	145.6±2.7	146.3±2.6	0.86
Glucids (mg/Kg)	538.3±10.3	529.0±11.7	545.5±12.8	0.37
Protein energy (Kcal/Kg)	4876.7±73.0	4969.8±80.4	4756.1±90.6	0.09
Not-Protein energy (Kcal/Kg)	4277.1±70.3	4319.6±77.5	4222.0±90.8	0.43

[1] Data are presented as Mean±SE. P-value<0.05 by independent t-test was considered significant.

Demographic and clinical characteristics of study infants are shown in **Table 2**.
Phytosterol concentrations in VO-based intravenous LE and in plasma of study infants at different time points are shown in **Table 3** and in **Table 4**, respectively. Phytosterol concentrations in infant formulas were: PreNidina, Nestlé, Italy – total CAMP: 12.4±1.8 mg/L, total STIGM: 2.4±0.4 mg/L, total SITO: 22.8±5.6 mg/L, total PHY: 37.6±4.9 mg/L; Aptamil pre, Milupa, Italy – total CAMP: 12.5±1.1 mg/L, total STIGM: 6.9±0.5 mg/L, total SITO: 36.2±6.5 mg/L, total PHY: 55.6±7.8 mg/L; Mellin 0, Mellin, Italy – total CAMP: 14.7±1.6 mg/L, total STIGM: 7.6±0.9 mg/L, total SITO: 40.4±4.3 mg/L, total PHY: 62.7±5.6 mg/L. Phytosterols in premature human milk were obtained from 10 samples from different mothers at 30±10 days post-partum (total CAMP: 0.2±0.1 mg/L, total STIGM: 0.2±0.1 mg/L, total SITO: 0.7±0.2 mg/L, total PHY: 1.1±0.3 mg/L).

Table 2. Demographic and clinical characteristics of study infants[1,2]

	All study infants (n=45)	Subgroup A (n=36)	Subgroup B (n=28)	P-Value
Gestational age (days)	196±14	195±14	192±13	0.77
Birth weight (g)	919±182	909±183	895±182	0.30
Sex (%)				
Male	38% (17/45)	39% (14/36)	50% (14/28)	0.37
Diagnosis (%)				
SGA	7% (3/45)	6% (2/38)	4% (1/28)	0.72
RDS	11% (5/45)	11% (4/36)	11% (3/28)	0.97
MMI	69% (31/45)	67% (24/36)	75% (21/28)	0.42
PDA	49% (22/45)	47% (17/36)	46% (13/28)	0.80
Sepsis	31% (14/45)	31% (11/36)	21% (6/28)	0.68
BPD	24% (11/45)	28% (10/36)	29% (8/28)	0.93
ROP I-II	38% (17/45)	33% (12/36)	46% (13/28)	0.27
ROP plus	7% (3/45)	8% (3/36)	7% (2/28)	0.87
IVH I-II	20% (9/45)	17% (6/36)	18% (5/28)	0.51
IVH >III	2% (1/45)	0% (0/36)	4% (1/28)	0.25
LPV I	18% (8/45)	19% (7/36)	25% (7/28)	0.58
LPV >I	2% (1/45)	0% (0/36)	0% (0/28)	-
Cholestasis	0% (0/45)	0% (0/36)	0% (0/28)	-
Parenteral nutrition (days)				
Day of life at initiation	1	1	1	-
Length of PN	18±3	18±3	18±3	0.92
Length of stay (days)	75±29	80±28	78±27	0.87
VO-based IV LE (%)				
MS	40% (18/45)	44% (16/36)	39% (11/28)	0.63
MSF	38% (17/45)	31% (11/36)	39% (11/28)	0.47
MSOF	22% (10/45)	25% (9/36)	21% (6/28)	0.74

[1] SGA, small for gestational age; RDS, respiratory distress syndrome; MMI, hyaline membrane disease; PDA, patent ductus arteriosus; BPD, bronchopulmonary dysplasia, ROP, retinopathy of prematurity; IVH, intraventricular hemorrhage; LPV, periventricular leukomalacia. [2] Data are presented as Mean±SD or n (%). P-value<0.05 by independent t-test or Chi-square were considered significant.

Plasma phytosterols were not different between d7 and d14, while they were significantly higher than on d21 (subgroup B). These concentrations were not different between the LE (**Figure 3**). The decay of plasma CAMP, STIGM and SITO from the day 21(d21) to 49 (d49) was log linear in subgroup A (**Figure 4**). Half-life of plasma CAMP was significantly longer than STIGM and SITO and this was not different among the 3 LE (**Table 5**). In our study population, we found no significant correlation between plasma phytosterol half-lives and cumulative phytosterol intakes (from birth to the stop of LE), gestational age, birth weight and conjugated bilirubin.

Table 3. Phytosterol concentrations (mg/L) in the study VO-based IV LE[1]

	MS	MSF	MOSF
Total-CAMP	42.8±3.2	32.2±1.6	25.8±2.4
Total-STIGM	58.5±5.3	50.2±2.9	35.6±1.5
Total-SITO	164.8±14.7	143.5±15.4	134.3±9.5
Total-PHY	266.1±23.1	225.9±16.2	195.7±9.8

[1] Analyzed by gas chromatography-mass spectrometry in our laboratory. All experiments were conducted in triplicate, and data is shown as mean±SD (mg/L); LE analyzed are representative of only a single Lot number.

Table 4. Plasma phytosterol concentrations at definite time points in preterm infants during and after routine parenteral nutrition[1]

	d7 (n=38)	d14 (n=38)	d21 (n=39)	d28 (n=39)	d35 (n=28)	d42 (n=13)	d49 (n=10)
Total-CAMP	2.9±1.7	2.9±1.6	1.6±1.2	0.9±0.6	0.6±0.4	0.4±0.1	0.4±0.3
Total-STIGM	2.4±1.5	1.8±0.9	0.9±0.7	0.4±0.3	0.2±0.1	0.2±0.1	0.2±0.1
Total-SITO	8.8±4.8	8.2±3.6	4.4±2.9	2.4±1.5	1.3±0.9	0.9±0.4	1.0±0.7
Total-PHY	14.1±7.5	12.9±5.5	6.9±4.3	3.7±2.1	2.1±1.3	1.4±0.5	1.5±1.1

[1] Data are presented as Mean±SD (mg/L).

Table 5. Half-life of plasma total CAMP, STIGM and SITO (days) in preterm infants from whom at least 3 blood sample were available after the stop of lipid infusion (subgroup A)[1]

	Total-CAMP	Total-STIGM	Total-SITO	*P
VO-based IV LE without FO (n=16)	14.7±8.9*	11.1±4.9§	11.2±4.5§	0.02
VO-based IV LE with FO (n=20)	12.5±4.6*	9.8±4.3*,§	9.5±3.5§	0.00
Subgroup A of study infants (n=36)	13.5±6.9*	10.3±4.5§	10.3±4.0§	0.00

[1] Data are presented as Mean±SD (days). P-value<0.05 by repeated measures ANOVA were considered significant. Different superscripts (*, §) indicate significant differences between groups by Bonferroni test.

Figure 3. Total CAMP, STIGM, SITO and PHY concentrations (mg/L) at the 7th, 14th and 21st days of life in subgroup B of study patients (n=28)[1]. [1] Data on plasma phytosterol concentrations of study infants receiving LE with (b) and without fish oil (a) are presented as Mean±SD (mg/L). P-value<0.05 by repeated measures ANOVA were considered significant. Different superscripts (*, §) indicate significant differences between groups by Bonferroni test.

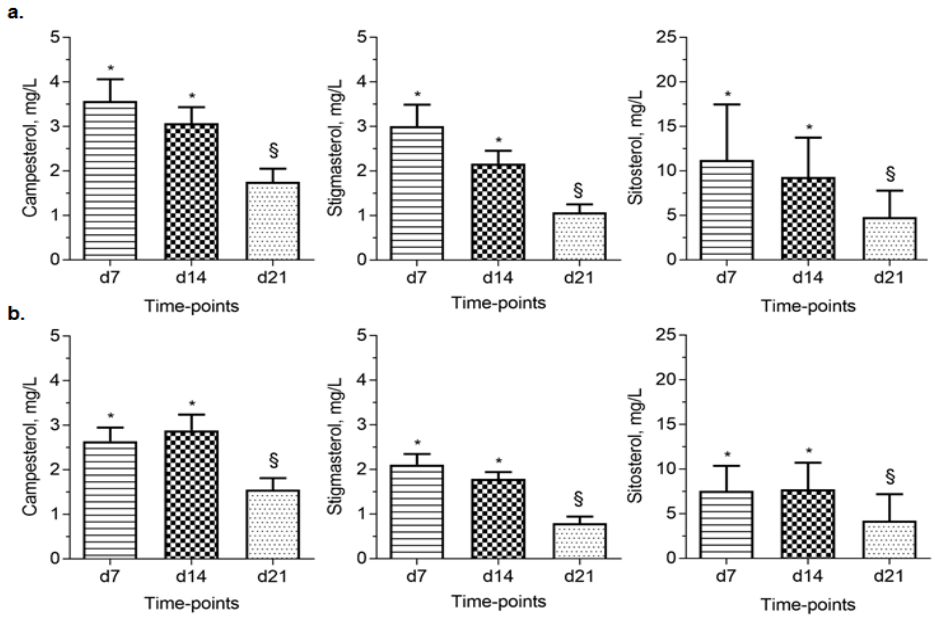
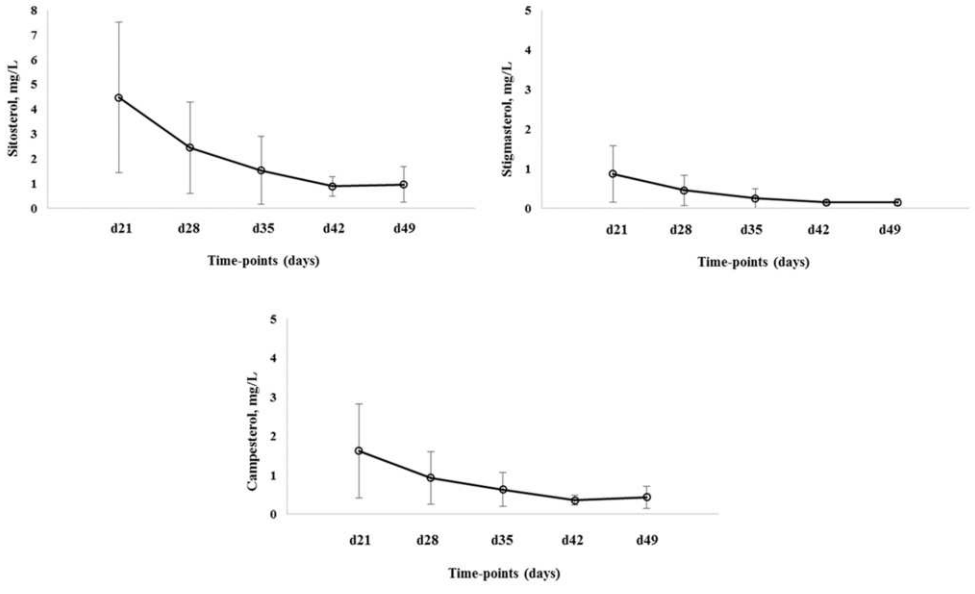


Figure 4. Total CAMP, STIGM, SITO and PHY concentrations (mg/L) over time in subgroup A of study patients (n=36) from day 21 (d21) to 49 (d49)[1]. [1] Data are presented as Mean±SD (mg/L).



DISCUSSION

To the best of our knowledge, this is the first report on the plasma phytosterol half-life in VLBW preterm infants receiving intravenous LE. We demonstrated that neonates receiving intravenous LE rapidly accumulate phytosterols after the start of PN and that the half-life was about 10 days.

Our data show that even a short duration of LE is enough to lead a high plasma concentration of phytosterols in preterm infants [15-17]. Plasma phytosterol concentrations in our preterm infants at 7 and 14 days of PN were similar to those previously reported by Pianese et al., Savini et al. and Nghiem-Rao et al. [7, 18, 19]. These concentrations were similar to those reported for hypercholesterolemic adults and phytosterolemic heterozygotes patients [11, 20]. The implications for the very high concentration of phytosterols in humans is not fully understood, but support for toxicity comes from patients with phytosterolemia where loss of ABCG5/G8 function lead to toxic accumulation of phytosterols in the body that can lead to premature cardiovascular disease, hematological disorders, endocrine disruption, and possible liver cirrhosis [5, 6, 21].

After the stop of LE, plasma total CAMP, STIGM and SITO concentrations decreased very slowly suggesting a very slow metabolism and/or a slow elimination by hepatic ABCG5/G8 transporters and scavenger receptor BI and/or possibly by tissue incorporation. Phytosterol accumulation in tissues was described in patients with phytosterolemia and xanthomatosis, in ABCG5/G8 knockout mice fed a high-phytosterol diet and in neonatal piglets involved the daily injection of phytosterols [22-24]. It is plausible that because of similar structure, phytosterols could substitute cholesterol in cell membranes and this may interfere with membrane function, signal transduction or membrane-bound enzyme activity [18, 24]. Displacement of cholesterol by phytosterols could also decrease deformability and so increase fragility of red blood cell membranes [25, 26]. To date, limited information is available on tissue incorporation of phytosterols in growing preterm infants [18]. Biliary excretion by hepatic ABCG5/G8 transporters and scavenger receptor BI are likely to be involved in phytosterol clearance and could explain the lowering plasma phytosterol concentrations after the stop of LE in VLBW preterm infants [10, 12, 27]. In vitro reconstruction of sterol transfer by G5 and G8 in a cell-free system showed a better free sterol ABCG5/ABCG8-mediate transport than that of esterified sterols [28]. A different sterol esterification could thus result in a variable excretion into the biliary tract. Of interest, Lin et al. reported a higher esterification of CAMP than STIGM and SITO by lecithin:cholesterol acyltransferase and acyl-coenzyme A:cholesterol acyltransferase enzymes [29].

Half-life of plasma phytosterols in preterm infants on PN was about 10 days and this was similar to that reported by Salen et al. and Bhattacharyya et al. in phytosterolemic homozygotes patients [10, 12], and by Lin et al. in a patient with coexisting phytosterolemia and cholestanolemia [11]. Of note, the plasma phytosterol half-life in our study infants was nearly twice of that reported for plasma cholesterol in healthy men and in patients with sitosterolemia and xanthomatosis [10, 30]. To the best of our knowledge, to date, no information is available on plasma cholesterol half-life in preterm infants even if, preliminary results from our laboratory, suggest a similar half-life between preterm infants and human adults (Data not published).

Plasma CAMP half-life was longer than that of other phytosterols in all study infants. Relas at al. reported similar findings in six healthy adults who received a single intravenous bolus of VO-based LE [8]. Sudhop et al. studied six male healthy volunteers (mean age 25) and found a lower biliary sterol excretion and a lower hepatic sterol clearance of CAMP compared to

SITO. A longer half-life of plasma CAMP could be due to a lower biliary excretion and/or a higher tissue incorporation and/or delayed released in comparison with other phytosterols.
Surprisingly gestational age, birth weight and lower values of conjugated bilirubin did not correlate with plasma phytosterol half-lives.
This study has limitations. The limited samples available did not allow us to apply two-exponential approach in this study. However, the high mean r-square (0.9±0.1) found for each phytosterol suggest that no improvement could be achieved by using more complex models. We cannot also exclude the effect of oral phytosterols with human milk or infant formulas on phytosterol half-life. However, plasma phytosterol half-lives were not different between infants who received more than 75% of the enteral feeding as human milk with contained very low levels of phytosterols or phytosterol-rich infant formulas (Data not shown). Since dietary phytosterols is poorly absorbed into the blood stream from the gut, plasma phytosterols concentrations would hardly be affected by the amount of enteral phytosterols. Furthermore, the accuracy of our kinetic models is limited by the few blood samples available. This is often a problem with the most pharmacokinetic studies in infancy.
It is important to underline that this was a pilot study and it was not powered to detect differences in phytosterol half-life according to the patient's clinical characteristics, to the associated diseases or to the LE type. A prospective study is currently underway to define plasma phytosterol half-lives in neonates with severe liver dysfunction.
In conclusion, this is the first report of the plasma phytosterol half-lives in VLBW preterm infants receiving VO-based intravenous LE. Our results demonstrate that all phytosterols in general, and campesterol in particular, were eliminated very slowly after the stop of intravenous LE. A rapid accumulation of phytosterols during PN with VO-based LE and a very slow elimination from the body, strongly support the view that preterm infants have poorly developed mechanisms for the phytosterol clearance. Further investigations are needed to extend these findings to infants with PNAC.

CONFLICT OF INTEREST

There are no conflicts of interest to declare.

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Contribution of each author:

- VPC was responsible for the design of the study;
- DP, AC, MS contributed to data collection and analysis;
- RD, CB, GV contributed to subject recruitment and data collection;
- MBLR was responsible for pharmacokinetic analysis;
- PC was in charge of the statistical analysis.

All authors read and approved the final manuscript.

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REFERENCES

[1] Ostlund RE, Jr., McGill JB, Zeng CM, Covey DF, Stearns J, Stenson WF, et al. Gastrointestinal absorption and plasma kinetics of soy Delta(5)-phytosterols and phytostanols in humans. Am J Physiol Endocrinol Metab. 2002;282:E911-6.

[2] Clayton PT, Whitfield P, Iyer K. The role of phytosterols in the pathogenesis of liver complications of pediatric parenteral nutrition. Nutrition. 1998;14:158-64.

[3] Carter BA, Taylor OA, Prendergast DR, Zimmerman TL, Von Furstenberg R, Moore DD, et al. Stigmasterol, a soy lipid-derived phytosterol, is an antagonist of the bile acid nuclear receptor FXR. Pediatr Res. 2007;62:301-6.

[4] El Kasmi KC, Anderson AL, Devereaux MW, Vue PM, Zhang W, Setchell KD, et al. Phytosterols promote liver injury and Kupffer cell activation in parenteral nutrition-associated liver disease. Sci Transl Med. 2013;5:206ra137.

[5] Shulman RS, Bhattacharyya AK, Connor WE, Fredrickson DS. Beta-sitosterolemia and xanthomatosis. N Engl J Med. 1976;294:482-3.

[6] Shefer S, Salen G, Nguyen L, Batta AK, Packin V, Tint GS, et al. Competitive inhibition of bile acid synthesis by endogenous cholestanol and sitosterol in sitosterolemia with xanthomatosis. Effect on cholesterol 7 alpha-hydroxylase. J Clin Invest. 1988;82:1833-9.

[7] Nghiem-Rao TH, Tunc I, Mavis AM, Cao Y, Polzin EM, Firary MF, et al. Kinetics of phytosterol metabolism in neonates receiving parenteral nutrition. Pediatr Res. 2015;78:181-9.

[8] Relas H, Gylling H, Miettinen TA. Fate of intravenously administered squalene and plant sterols in human subjects. J Lipid Res. 2001;42:988-94.

[9] Salen G, Ahrens EH, Jr., Grundy SM. Metabolism of beta-sitosterol in man. J Clin Invest. 1970;49:952-67.

[10] Bhattacharyya AK, Connor WE, Lin DS, McMurry MM, Shulman RS. Sluggish sitosterol turnover and hepatic failure to excrete sitosterol into bile cause expansion of body pool of sitosterol in patients with sitosterolemia and xanthomatosis. Arterioscler Thromb. 1991;11:1287-94.

[11] Lin HJ, Wang C, Salen G, Lam KC, Chan TK. Sitosterol and cholesterol metabolism in a patient with coexisting phytosterolemia and cholestanolemia. Metabolism. 1983;32:126-33.

[12] Salen G, Shore V, Tint GS, Forte T, Shefer S, Horak I, et al. Increased sitosterol absorption, decreased removal, and expanded body pools compensate for reduced cholesterol synthesis in sitosterolemia with xanthomatosis. J Lipid Res. 1989;30:1319-30.

[13] Salen G, Tint GS, Shefer S, Shore V, Nguyen L. Increased sitosterol absorption is offset by rapid elimination to prevent accumulation in heterozygotes with sitosterolemia. Arterioscler Thromb. 1992;12:563-8.

[14] Savini S, Correani A, Pupillo D, D'Ascenzo R, Biagetti C, Pompilio A, et al. Phytosterol Esterification is Markedly Decreased in Preterm Infants Receiving Routine Parenteral Nutrition. Lipids. 2016.

[15] Kempen HJ, de Knijff P, Boomsma DI, van der Voort HA, Gevers Leuven JA, Havekes L. Plasma levels of lathosterol and phytosterols in relation to age, sex, anthropometric parameters, plasma lipids, and apolipoprotein E phenotype, in 160 Dutch families. Metabolism. 1991;40:604-11.

[16] Normen AL, Brants HA, Voorrips LE, Andersson HA, van den Brandt PA, Goldbohm RA. Plant sterol intakes and colorectal cancer risk in the Netherlands Cohort Study on Diet and Cancer. Am J Clin Nutr. 2001;74:141-8.

[17] Valsta LM, Lemstrom A, Ovaskainen ML, Lampi AM, Toivo J, Korhonen T, et al. Estimation of plant sterol and cholesterol intake in Finland: quality of new values and their effect on intake. Br J Nutr. 2004;92:671-8.

[18] Pianese P, Salvia G, Campanozzi A, D'Apolito O, Dello Russo A, Pettoello-Mantovani M, et al. Sterol profiling in red blood cell membranes and plasma of newborns receiving total parenteral nutrition. J Pediatr Gastroenterol Nutr. 2008;47:645-51.

[19] Savini S, D'Ascenzo R, Biagetti C, Serpentini G, Pompilio A, Bartoli A, et al. The effect of 5 intravenous lipid emulsions on plasma phytosterols in preterm infants receiving parenteral nutrition: a randomized clinical trial. Am J Clin Nutr. 2013;98:312-8.

[20] Hidaka H, Nakamura T, Aoki T, Kojima H, Nakajima Y, Kosugi K, et al. Increased plasma plant sterol levels in heterozygotes with sitosterolemia and xanthomatosis. J Lipid Res. 1990;31:881-8.

[21] Lu K, Lee MH, Hazard S, Brooks-Wilson A, Hidaka H, Kojima H, et al. Two genes that map to the STSL locus cause sitosterolemia: genomic structure and spectrum of mutations involving sterolin-1 and sterolin-2, encoded by ABCG5 and ABCG8, respectively. Am J Hum Genet. 2001;69:278-90.

[22] Solca C, Tint GS, Patel SB. Dietary xenosterols lead to infertility and loss of abdominal adipose tissue in sterolin-deficient mice. J Lipid Res. 2013;54:397-409.

[23] McDaniel AL, Alger HM, Sawyer JK, Kelley KL, Kock ND, Brown JM, et al. Phytosterol feeding causes toxicity in ABCG5/G8 knockout mice. The American journal of pathology. 2013;182:1131-8.

[24] Iyer KR, Spitz L, Clayton P. BAPS prize lecture: New insight into mechanisms of parenteral nutrition-associated cholestasis: role of plant sterols. British Association of Paediatric Surgeons. J Pediatr Surg. 1998;33:1-6.

[25] Ratnayake WM, L'Abbe MR, Mueller R, Hayward S, Plouffe L, Hollywood R, et al. Vegetable oils high in phytosterols make erythrocytes less deformable and shorten the life span of stroke-prone spontaneously hypertensive rats. The Journal of nutrition. 2000;130:1166-78.

[26] Ratnayake WM, Plouffe L, L'Abbe MR, Trick K, Mueller R, Hayward S. Comparative health effects of margarines fortified with plant sterols and stanols on a rat model for hemorrhagic stroke. Lipids. 2003;38:1237-47.

[27] Sudhop T, Sahin Y, Lindenthal B, Hahn C, Luers C, Berthold HK, et al. Comparison of the hepatic clearances of campesterol, sitosterol, and cholesterol in healthy subjects suggests that efflux transporters controlling intestinal sterol absorption also regulate biliary secretion. Gut. 2002;51:860-3.

[28] Wang J, Sun F, Zhang DW, Ma Y, Xu F, Belani JD, et al. Sterol transfer by ABCG5 and ABCG8: in vitro assay and reconstitution. J Biol Chem. 2006;281:27894-904.

[29] Lin DS, Steiner RD, Merkens LS, Pappu AS, Connor WE. The effects of sterol structure upon sterol esterification. Atherosclerosis. 2010;208:155-60.

[30] Goodman DS, Noble RP. Turnover of plasma cholesterol in man. J Clin Invest. 1968;47:231-41.

Chapter 5

Plasma Phytosterol Half-Life and Levels Are Increased in Very Low Birth Weight Preterm Infants with Parenteral Nutrition-Associated Cholestasis

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Summary

Parenteral nutrition-associated cholestasis (PNAC) has been linked to plasma accumulation of phytosterols in infants receiving vegetable oil-based lipid emulsions (LE). To date, information on the ability of infants with PNAC to metabolize intravenous (IV) phytosterols is very limited. We characterized plasma phytosterol half-life in very low birth weight (VLBW) preterm infants with PNAC. As part of a prospective cohort study, VLBW infants with PNAC underwent serial blood sample measurements of sitosterol (Sito), campesterol (Camp), and stigmasterol (Stigma). Infants without PNAC served as controls (CTRL). Thirty-seven PNAC infants and 14 CTRL were studied. On PN day 7 and PN day 14, PNAC infants had higher plasma phytosterol concentrations compared to those of CTRL ($p<0.05$). A significant and positive correlation was found between plasma Camp, Stigma, and Sito concentrations and IV phytosterol intake from birth to PN day 7 ($p=0.001$, $p=0.001$, and $p=0.005$, respectively). Stigma concentration was positively correlated with conjugated bilirubin on PN day 7 ($p=0.012$). After stopping IV LE, half-lives of Camp, Stigma and Sito were significantly longer in PNAC infants than in CTRL (Camp: 18.8±6.2 vs 11.8±3.0 days, $p=0.001$; Stigma: 13.8±5.8 vs 9.4±3.4 days, $p=0.023$; Sito: 15.3±5.0 vs 9.8±3.0 days, $p=0.002$). In conclusion, phytosterols increased earlier during PN and were eliminated slower after stopping IV LE in PNAC infants than in CTRL. Stigma concentration on PN day 7 could represent an early marker of cholestasis. Our results provide additional evidence on the relationship between IV phytosterols and PNAC.

Abbreviations: BW, birth weight; Camp, campesterol; CTRL: control infants; EN, enteral nutrition; GA, gestational age; IV, intravenous; LE, lipid emulsion; MCT, medium chain triglycerides; PN, parenteral nutrition; PNAC, parenteral nutrition-associated cholestasis; Sito, sitosterol; SO, soybean oil; Stigma, stigmasterol; VLBW, very low birth weight; VO, vegetable oil.

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INTRODUCTION

Cholestasis is one of the most common complications associated with the administration of PN in neonates [1, 2]. The etiology of parenteral nutrition-associated cholestasis (PNAC) is unclear but is thought to result from multifactorial causes related to genetic and dietary factors. Intravenous (IV) intake of vegetable oil (VO)-based lipid emulsions (LE) containing phytosterols is felt to be a major contributing factor [3, 4]. Phytosterols may cause cholestasis by interfering with the expression and function of transporters for sterols and bile acids within the hepatobiliary system [5]. Several animal and human studies have associated lipid and phytosterol intake with the development of cholestasis, but a causal relationship has not been established yet [6-8]. Support for phytosterol toxicity comes from patients with phytosterolemia where the toxic accumulation of phytosterols can lead to premature cardiovascular disease, hematological disorders, endocrine disruption, and possible liver cirrhosis [9]. Additionally, ABCG5/G8 knockout mice fed a high-phytosterol diet and neonatal piglets receiving IV phytosterols exhibit hepatic, cardiac, and hematologic dysfunctions as well as growth failure and premature death [10-12].

Despite the potential phytosterol toxicity, LE containing VO are the only approved lipid formulations for PN used by the US Food and Drug Administration and the European Medicines Evaluation Agency.

Thus, in our view, a comprehensive understanding of phytosterol metabolism would be important to provide a mechanistic insight into the vulnerability of preterm infants to cholestasis.

To date, information on phytosterol metabolism in preterm infants receiving PN is limited. Savini et al. reported a lower esterification of IV phytosterols in very low birth weight (VLBW) preterm infants compared to term infants and human adults [13]. Pianese et al. observed a significant phytosterol accumulation in red blood cell membrane of preterm infants after 10 days of PN [8]. Nghiem-Rao et al. found that phytosterols in preterm infants on PN reached steady median plasma concentrations within 3-5 days after the start of LE. Moreover, in PNAC infants, changes in conjugated bilirubin closely resembled those of plasma phytosterols [14]. Pupillo et al. reported that preterm infants without PNAC had a plasma phytosterol half-life of about 10 days, which was similar to that reported in phytosterolemic adult patients [15-17].

In the present study, we measured the plasma phytosterol half-life in VLBW preterm infants on PN who developed cholestasis. Preterm infants without PNAC were used as controls (CTRL).

SUBJECTS AND METHODS

Study design

In a prospective cohort study, preterm infants were recruited from the Neonatal Intensive Care Unit (NICU) of “G. Salesi” Children's Hospital, Ancona, Italy, between January 2014 and August 2016. The consecutively admitted neonates with a birth weight (BW) of 500-1249 g and a gestational age (GA) below 32 weeks, received full PN from the first hour of life as part of the routine care given to this group of infants. Study Infants received one of the three IV LE as part of their routine PN: MSF (Lipidem® 20%, B.Braun - 50% Medium chain triglycerides, 40% Soybean oil, and 10% Fish oil) or MSOF (SMOFlipid® 20%, Fresenius-Kabi - 30% Medium chain triglycerides, 30% Soybean oil, 25% Olive oil and 15% Fish oil) or MS (Lipofundin MCT® 20%, B.Braun; 50% Medium-chain triglycerides and 50% Soybean oil).

At our institution, the neonatologist prescribes IV lipids and more than one LE is always available at the hospital pharmacy.

Neonates with known primary liver disease, anatomic liver abnormalities, severe congenital sepsis, metabolic inborn errors, or known genetic or chromosomal defects were excluded.
Liver-function tests were obtained as part of routine care at 7 days and 6 weeks' postnatal age, according to NICU policy. Additional biochemistry was performed at the discretion of the attending physician. PNAC was defined as a conjugated bilirubin > 1.0 mg/dL.
Data on CTRL born from January 2014 to March 2016 has been recently published elsewhere [17].

Nutrition protocol management

Full PN with lipids was started at about 1 hour after birth, according to the NICU protocol. LE were infused at dose of 1.0, 1.5, 2.0, 2.5, and 3.0 g·kg-1·d-1 from postnatal day 0 to day 5 respectively and were then kept constant from day 5 to day 7. PN was tapered from day 8 until day 21, when it was usually stopped. Minimal enteral feeding with human milk was provided from day 0 to day 8, the maximum amount supplied being 8 mL·kg-1·d-1 from day 1 to day 4, and 16 mL·kg-1·d-1 from day 5 to day 8. Enteral nutrition was gradually increased from day 9 to reach full oral feeding on day 18.

Sample collection and handling

EDTA-treated blood samples remaining after completion of routine biochemistry analysis (scavenged blood) were weekly collected for the first 3 months of life. Blood samples were centrifuged at 2800 rpm (1358 g) and plasma was stored in pyrogallol-added tubes at -20°C until analysis.

Primary outcome

The primary outcome was defined as plasma phytosterol half-life as calculated using the kinetic model previously described by our group [17].
Secondary outcomes: clinical data
Body weight was measured daily according to a standard NICU procedure by using the same scale (precision: 5 gr). Phytosterol intakes were computed by using the daily amount of LE, preterm human milk or infant formula (PreNidina, Nestlé, Italy; Aptamil pre, Milupa, Italy; Mellin 0, Mellin, Italy), and their phytosterol concentrations as previously measured by our laboratory [17]. The major complications of prematurity, according to predefined criteria [18-21], were collected for all study patients.

Analytical methods

All the reagents were obtained from Steraloids Inc. (Newport, USA). 5α-cholestane (internal standard), 24α-methylcholest-5-en-3β-ol (campesterol), 3β-hydroxy-24-ethyl-5,22-cholestadiene (stigmasterol) 24α-ethylcholest-5-en-3β-ol (β-sitosterol) had a purity level of 97%, 65%, 95% and 71%, respectively. Plasma analysis of phytosterols (Camp, Stigma, and Sito) was evaluated by gas chromatography-mass spectrometry as previously described [1, 17]. In brief, 5α-cholestane was added as an internal standard to plasma (50 µL), saponified in methanolic potassium hydroxide (5M) for one hour. Then, 1.5 mL water with sodium chloride was added and sterols extracted with two sequential portions of 3 mL each of hexane and diethyl ether, pooled and dried. Dried sterol samples were derivatized as trimethylsilylethers, dissolved in 200 µL hexane and 1 µL samples injected in splitless mode into an Agilent Technologies apparatus (model GC7890A/MD5975), using a capillary column with a non-polar stationary phase (HP-5MS, 30m x 0.25mm x 0.25mm film thickness; Agilent Technologies). Sterols were identified by selected ion monitoring (SIM). The ions at 357, 382, 484 and 486 m/z were used to detect 5α-cholestane, Camp, Stigma and Sito, respectively.

The appropriate calibration curve was used for the quantitation of each studied sterol. Coefficient of variation of plasma Camp, Stigma and Sito concentrations was always less than 10% (Data not shown).

Ethics

The study was conducted in accordance with the principles of the Helsinki Declaration as revised in Fortaleza (Brasil) 2013 and was approved by the local ethics committee and institutional review board (No. 469DG). Written informed consent was obtained from the parents or legal guardians of all study patients.

Statistical analysis

This study was exploratory in nature as the half-life of plasma phytosterols in PNAC preterm infants was unknown at the time this study was done; a formal power calculation was therefore not performed. Chi-square and independent t-test were used to compare demographic and clinical characteristics, and phytosterol half-lives. Phytosterol concentrations both on PN d7 and PN d14 were compared by using paired t-test (within groups) and independent t-test (between groups). Repeated measures ANOVA and post-hoc Bonferroni test were used to compare the Camp, Stigma, and Sito half-life both in PNAC infants and CTRL. A simple linear regression analysis was used when there was a significant correlation between variables. A p value < 0.05 was considered significant. All statistical analyses were performed by using SPSS (v 19.0; SPSS Inc, Chicago, Illinois).

RESULTS

Study patients

Forty of the 168 screened VLBW preterm infants developed PNAC, and 37 had enough leftover plasma for phytosterol analysis (**Figure 1**): 22 samples were available at 7±1 (d7), 19 at 14±1 (d14), 17 at 21±1 (d21), 19 at 28±1 (d28), 19 at 35±1 (d35), 14 at 42±1 (d42), 13 at 49±1 (d49), 10 at 56±2 (d56), 19 at 65±2 (d65), 7 at 71±2 (d71), 8 at 80±2 (d80) and 6 at 89±3 (d89) days. Seventeen PNAC infants had samples on both d7 and d14 (PNAC infants$^{PN\text{-}on}$), whereas 16 infants had 3 or more blood samples after stopping IV LE to measure phytosterol half-life (PNAC infants$^{PN\text{-}off}$).

Fourteen infants without PNAC were also studied as controls (CTRL): 14 samples were available at d7, 12 at d14, 14 at d21, 13 at d28, 10 at d35, 8 at d42, and 4 at d49. All study CTRL had enough blood samples to calculate phytosterol half-life (CTRL$^{PN\text{-}off}$), and 12 of them had samples on both d7 and d14 (CTRL$^{PN\text{-}on}$). Fourteen infants without PNAC were also studied as controls (CTRL): 14 samples were available at d7, 12 at d14, 14 at d21, 13 at d28, 10 at d35, 8 at d42, and 4 at d49. All study CTRL had enough blood samples to calculate phytosterol half-life (CTRL$^{PN\text{-}off}$), and 12 of them had samples on both d7 and d14 (CTRL$^{PN\text{-}on}$). None of the CTRL received MSF, while PNAC infants received all IV LE (**Table 1**). CTRL received PN no longer than d21, whereas about two-third of PNAC infants for a longer period. IV phytosterol intakes from birth to 3 months of life were statistically different between PNAC infants and CTRL, whereas no differences were found from birth to PN d7 and from birth to PN d14. Twenty PNAC infants had a value of conjugated bilirubin on PN day 7(±1): 12 infants below 1 mg/dL, and 8 infants greater than 1 mg/dL. Conjugated bilirubin on d7 were available for 8 CTRL.

Figure 1. Flow diagram of the study's progress, detailing the participant numbers and the analytical approach (CTRL: control infants; IV: intravenous; LE: lipid emulsion; n.a: not available; PN: parenteral nutrition; PNAC: parenteral nutrition-associated cholestasis; VLBW: very low birth weight).

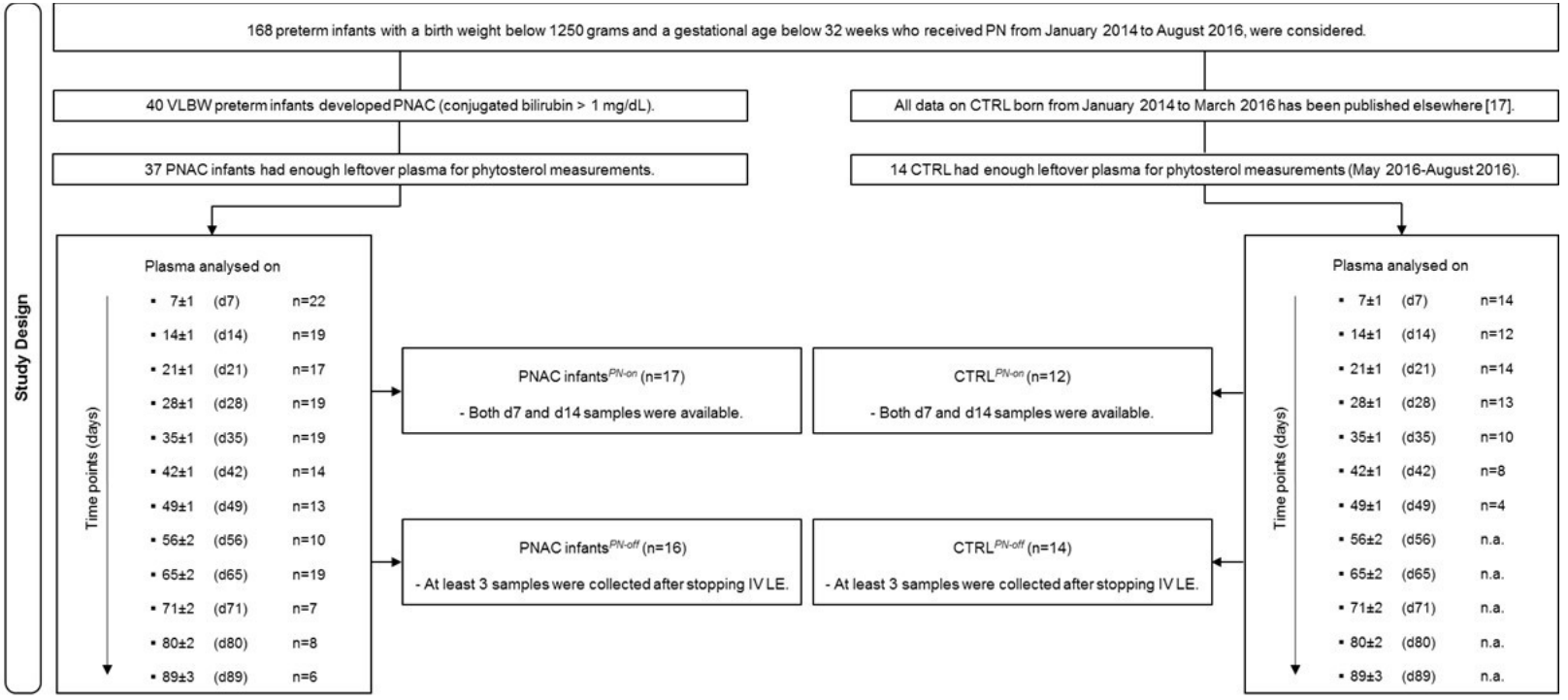


Table 1. Demographic and clinical characteristics of study VLBW preterm infants[a]

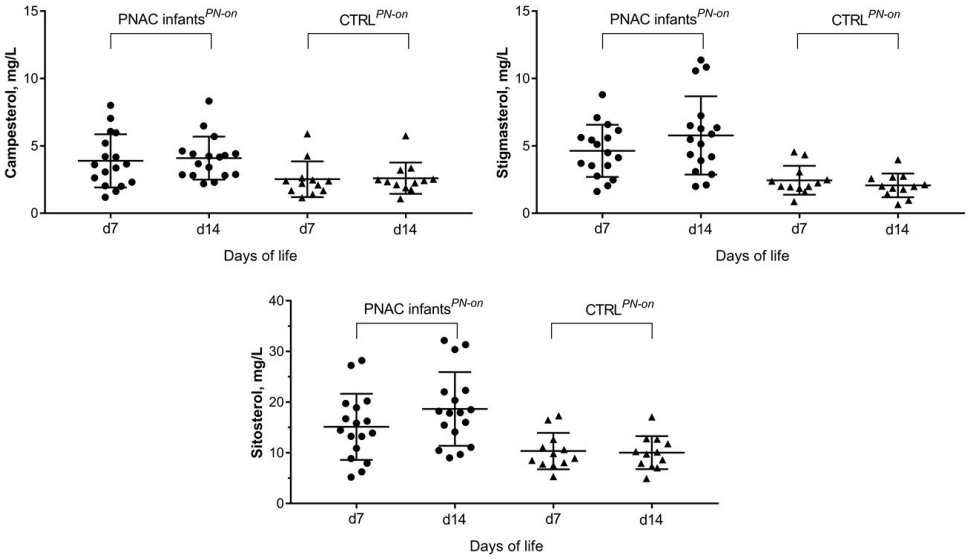
.	PNAC infants^{PNon} (n=17)	CTRL^{PNon} (n=12)	p-value	PNAC infants^{PNoff} (n=16)	CTRL^{PNoff} (n=14)	p-value
GA, days	193±19	189±13	0.52	200±18	190±14	0.11
BW, g	814±200	894±205	0.30	838±198	899±191	0.40
Gender (M/F)	10/7	5/7	0.36	11/5	5/9	0.070
Diagnosis, no. (%)						
NEC						
Small for gestational age	4 (23)	0 (0)	0.070	5 (31)	0 (0)	0.018
Patent ductus arteriosus	12 (70)	8 (67)	0.82	9 (56)	8 (57)	0.88
Necrotizing enterocolitis	2 (11)	0 (0)	0.22	2 (13)	0 (0)	0.16
Sepsis	11 (64)	6 (50)	0.43	11 (69)	7 (50)	0.20
Bronchopulmonary dysplasia	9 (53)	4 (33)	0.30	8 (50)	4 (29)	0.18
PN duration, no. (%)						
Below 22 days	5 (29)	12 (100)	<0.001	6 (38)	14 (100)	<0.001
Between 23-43 days	6 (35)	0 (0)	0.021	10 (63)	0 (0)	<0.001
Over 43 days	6 (35)	0 (0)	0.021	0 (0)	0 (0)	-
Hospital stay, days	116±46	95±26	0.17	122±48	92±25	0.045
VO-based IV LE, no. (%)						
MS LE	8 (47)	6 (50)	0.88	8 (50)	7 (50)	1.00
MSF LE	9 (53)	0 (0)	0.002	6 (38)	0 (0)	0.010
MSOF LE	0 (0)	6 (50)	0.001	2 (13)	7 (50)	0.025
Human milk, no. (%)	7 (41)	6 (50)	0.64	7 (44)	7 (50)	0.73
Infant formulas, no. (%)	2 (12)	2 (17)	0.71	3 (19)	2 (14)	0.74
Human milk and infant formulas, no. (%)	8 (47)	4 (33)	0.46	6 (38)	5 (35)	0.91
IV phytosterol intakes, mg/Kg						
from birth to 3 months	104±86	32±8	0.003	53±21	31±7	0.002
from birth to PN d7	15±3	14±4	0.57	16±3	14±4	0.15
from birth to PN d14	31±5	28±6	0.13	31±7	27±6	0.17
EN phytosterol intakes, mg/Kg						
from birth to 3 months	233±395	245±288	0.93	274±390	248±284	0.84
from birth to PN d7	1±2	1±2	0.81	2±2	1±2	0.81
from birth to PN d14	4±8	8±8	0.21	5±12	8±9	0.54

[a] Data are presented as mean±SD or no. (%). p value<0.05 by independent t-test or Chi-square were considered significant. BW: birth weight; CTRL: control infants; d7: day 7±1; d14: day 14±1; EN: enteral nutrition; GA: gestational age; IV: intravenous; LE: lipid emulsion; MS (Lipofundin MCT® 20%, B.Braun; 50% medium-chain triglycerides and 50% soybean oil); MSF (Lipidem® 20%, B.Braun - 50% medium chain triglycerides, 40% soybean oil, and 10% fish oil); MSOF (SMOFlipid® 20%, Fresenius-Kabi - 30% medium chain triglycerides, 30% soybean oil, 25% olive oil and 15% fish oil); PN: parenteral nutrition; PNAC: parenteral nutrition-associated cholestasis; VLBW: very low birth weight; VO: vegetable oil.

Plasma phytosterols

Plasma Camp, Stigma, and Sito concentrations of PNAC infants$^{PN\text{-}on}$ on d7 were not statistically different from those on d14 (Camp: 3.9±2.0 vs 4.1±1.6 mg/L, p=0.69; Stigma: 4.6±1.9 vs 5.8±2.9 mg/L, p=0.11, 15.1±6.5 vs 18.7±7.3 mg/L, p=0.084, respectively). Phytosterol concentrations were not different between d7 and d14 in CTRL$^{PN\text{-}on}$ (Camp: 2.5±1.3 vs 2.6±1.1 mg/L, p=0.64; Stigma: 2.5±1.1 vs 2.1±0.8 mg/L, p=0.071, Sito: 10.4±3.6 vs 10.0±3.3 mg/L, p=0.57, respectively; **Figure 2**).

Figure 2. Plasma Camp, Stigma, and Sito concentrations on PN d7 and PN d14 in infants with and without PNAC (PNAC infants$^{PN\text{-}on}$: n=17; CTRL$^{PN\text{-}on}$: n=12). Data are presented as mean±SD (mg/L). CTRL: control infants; d7: day 7±1; d14: day 14±1; PNAC: parenteral nutrition-associated cholestasis.



Camp, Stigma, and Sito concentrations on both d7 and d14 were statistically different between PNAC infants$^{PN\text{-}on}$ and CTRL$^{PN\text{-}on}$ (d7: p=0.047, p=0.001, p=0.029; d14: p=0.010, p<0.001, p=0.001; respectively). Plasma phytosterol concentrations after stopping IV LE were used for phytosterol half-life in both PNAC infants$^{PN\text{-}off}$ and CTRL$^{PN\text{-}off}$ (**Figure 3**).

Phytosterol half-life

The decay of plasma Camp, Stigma and Sito concentrations after stopping IV LE was log linear both in PNAC infants$^{PN\text{-}off}$ and in CTRLPN-off. Plasma phytosterol half-lives were significantly longer in PNAC infants$^{PN\text{-}off}$ than CTRL$^{PN\text{-}off}$ (**Table 2**).

Table 2. Half-life of plasma Camp, Stigma and Sito (days) in infants with and without PNAC[a]

Phytosterols	PNAC infantsPN-off n=16	CTRLPN-off n=14	p-value
Camp	18.8±6.2	11.8±3.0	0.001
Stigma	13.8±5.8	9.4±3.4	0.023
Sito	15.3±5.0	9.8±3.0	0.002

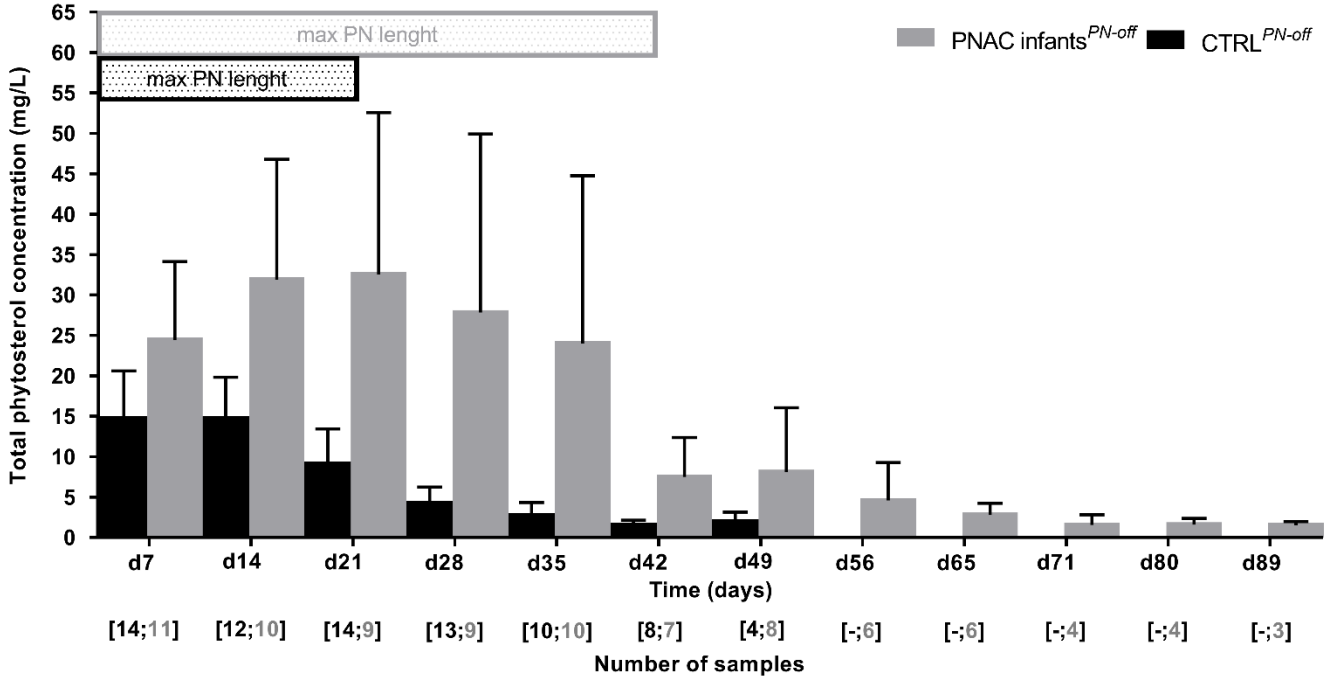
[a] Data are presented as mean±SD (days). p-values<0.05 by independent t-test were considered significant.

Both in PNAC infants$^{PN\text{-}off}$ and in CTRL$^{PN\text{-}off}$, half-life of plasma Camp was significantly longer than other phytosterols who had similar values (repeated-measures ANOVA: p=0.008 and p=0.011, respectively).
PNAC infants$^{PN\text{-}off}$ who received MS LE (n=8) had longer phytosterol half-lives than CTRL$^{PN\text{-}off}$ (n=7): Camp, 18.9±4.9 vs 11.0±3.6 mg/L, p=0.004; Stigma, 16.8±6.8 vs 9.0±2.5 mg/L, p=0.013; Sito, 17.1±5.5 vs 9.8±3.5 mg/L, p=0.011. No differences of phytosterol half-lives were found between PNAC infants$^{PN\text{-}off}$ and CTRL$^{PN\text{-}off}$ who received MSF or MSOF (n=8 and n=7, respectively): Camp, 18.7±7.5 vs 12.8±2.0 mg/L, p=0.067; Stigma, 10.8±2.5 vs 9.9±4.4 mg/L, p=0.62; Sito, 13.5±4.0 vs 9.8±2.5 mg/L, p=0.070.Stigma half-life of PNAC infants$^{PN\text{-}off}$ who received MSF or MSOF were statistically lower than those who received MS: PNAC infants$^{PN\text{-}off}$ – Camp, 18.9±4.9 vs 18.7±7.5 days, p=0.95; Stigma, 16.8±6.8 vs 10.8±2.5 days, p=0.044; Sito, 17.1±5.5 vs 13.5±4.0 days, p=0.17; CTRL$^{PN\text{-}off}$ – Camp, 11.0±3.6 vs12.8±2.0 days, p=0.30; Stigma, 9.0±2.5 vs 9.9±4.4 days, p=0.65; Sito, 9.8±3.5 vs 9.8±2.5 days, p=0.99.

Correlation analyses

We found positive and significant correlations between IV phytosterol intakes from birth to d7 and plasma phytosterol concentrations on PN d7 (Camp: r=0.59, y=1.948x-0.951, p=0.001; Stigma: r=0.57, y=1.392x-0.840, p=0.001; Sito: r=0.51, y=1.575x-1.962, p=0.005). Stigma concentration on PN d7 and conjugated bilirubin on d7 were also positively and significantly correlated (r=0.54, y=0.191x+0.217, p=0.012). None of phytosterol half-lives correlated with cumulative EN and PN phytosterol intakes. Neither in PNAC infants$^{PN\text{-}off}$ nor in CTRL$^{PN\text{-}off}$ there were significant correlations between PN duration and phytosterol half-lives.

Figure 3. Total plasma phytosterol concentration (Camp+Stigma+Sito) over the time both in PNAC infants$^{PN\text{-}off}$ (n=16; grey bars) and CTRL$^{PN\text{-}off}$ (n=14; black bars). Data are presented as mean±SD (mg/L). CTRL: control infants; d7: day 7±1; d14: day 14±1; d21: day 21±1; d28: day 28±1; d35: day 35±1; d42: day 42±1; d49: day 49±1; d56: day 56±2; d65: day 65±2; d71: day 71±2; d80: day 80±2; d89: day 89±3; PN: parenteral nutrition; PNAC: parenteral nutrition-associated cholestasis.



DISCUSSION

To the best of our knowledge, this is the first report on plasma phytosterol half-life in VLBW preterm infants with PNAC. In case of PNAC, we found that (a) plasma phytosterol concentrations on both PN d7 and d14 were significantly higher than CTRL, (b) Stigma concentration on PN d7 significantly correlated with conjugated bilirubin at d7, and (c) half-lives of IV phytosterols were markedly and significantly longer than in CTRL.

In our PNAC infants, plasma phytosterol concentrations on both PN d7 and d14 were up to 2-fold higher than CTRL, and, as reported in literature, slightly higher than those in patients with heterozygous phytosterolemia [6-8, 14, 17, 22, 23]. The high phytosterol concentrations observed in PNAC infants suggests that they may have poorly developed mechanisms of metabolizing phytosterols and that there might be a link between phytosterols and cholestasis [24, 25]. Growing evidence suggests a major role of Stigma on the onset of cholestasis in animals and in humans [5, 11, 12, 26]. In agreement with these studies, we found a positively significant correlation between Stigma concentration on PN d7 and conjugated bilirubin on d7. We speculate that VLBW preterm infants have a limited capacity to clear Stigma, which can contribute to increase vulnerability to PNAC. Exceeding safe threshold of Stigma, already from day 7, may lead to cholestasis in infants receiving PN [4, 6, 22, 27, 28]. We are now investigating if Stigma could be used as an early marker of cholestasis. Preliminary data from our laboratory suggest that cholestasis in preterm infants on PN can be predicted, with high sensitivity and specificity, by Stigma concentration ratio to IV Stigma intake (study in progress). On the other hand, the addition of Stigma, Camp and Sito to a LE containing 100% FO (Omegaven®) has not been associated with cholestasis in preterm pigs [29]. Further investigations are needed to define if phytosterols alone or in combination with other components of IV LE are responsible for neonatal PNAC.

It would be interesting to study if the high phytosterol concentrations of PNAC preterm infants could have long lasting effects. To date, information on long-term effects of high plasma phytosterol concentrations in humans is very limited. Of note, Mellies et al. reported a significant phytosterol accumulation in aortic tissues of VO formula-fed infants [30]. Miettinen et al. found phytosterols in atherosclerotic plaques of enterally fed human adults [31]. Recently, Hukkinen et al. associated cholestasis with the liver accumulation of phytosterols in children receiving VO-based LE [32]. In our opinion, additional studies are needed to clearly define the long-term side effects of high plasma phytosterol concentrations in infants.

As novel information, PNAC preterm infants had plasma phytosterol half-lives up to 2-fold longer than CTRL, suggesting a severe limitation to clear IV phytosterols in infants susceptible to cholestasis. Of note, the difference of phytosterol half-life between PNAC and CTRL was markedly higher in infants receiving IV LE without fish oil. The anti-inflammatory effect of fish oil might improve liver function in metabolizing phytosterols. However, it cannot be excluded that the lower phytosterol concentration in fish oil containing IV LE might be the responsible for the lower phytosterol half-life. Whether fish oil containing IV LE might reduce the incidence, the severity or the time for resolution of cholestasis, representing the best choice for PN in preterm infants, is still debated and our data cannot answer to this question.

Camp half-life was longer than that of Stigma and Sito in both PNAC infants and CTRL. This finding is in accordance with data on CTRL previously published by our group [17] and indicate that this difference is possibly related to the metabolism of the individual phytosterols rather than liver disease. A lower biliary excretion and/or a higher tissue incorporation and/or delayed release of Camp in comparison with other IV phytosterols may all be valid explanations for the different phytosterol half-life in humans [13, 17, 33, 34].

This study has limitations. The limited plasma samples available did not allow us to apply the two-exponential approach in this study. However, the high mean r-square (0.9□0.2) found for each phytosterol suggests that no improvement could be achieved by using more complex models. Phytosterol half-lives could be affected by using plasma concentrations at different time-points after stopping IV LE even though no correlations with PN duration and phytosterol intakes were found (data not shown).
In conclusion, this is the first report on plasma phytosterol half-life in VLBW preterm infants with PNAC. Our results demonstrate that: (i) IV phytosterols (Camp, Stigma and Sito) increased earlier and were eliminated slower in PNAC infants compared to CTRL, and (ii) Stigma might be possibly used as an early marker of cholestasis in preterm infants on PN. Further investigations are needed to clarify the role of phytosterols in the onset of neonatal PNAC.

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CONFLICT OF INTEREST
No conflicts of interests to declare.

AUTHORS' CONTRIBUTIONS
The authors' responsibilities were as follows:
• VPC designed the research project;
• AC, AP, LV analyzed data;
• LM, RD, CB, GV contributed to subject recruitment and samples collection;
• MBLR was responsible for pharmacokinetic analysis;
• PC performed statistical analysis.
All authors read and approved the final manuscript.

REFERENCES

1. Satrom K, Gourley G: Cholestasis in Preterm Infants. Clin Perinatol 2016, 43:355-373.

2. Beale EF, Nelson RM, Bucciarelli RL, Donnelly WH, Eitzman DV: Intrahepatic cholestasis associated with parenteral nutrition in premature infants. Pediatrics 1979, 64:342-347.

3. Zaloga GP: Phytosterols, Lipid Administration, and Liver Disease During Parenteral Nutrition. JPEN J Parenter Enteral Nutr 2015, 39:39S-60S.

4. Kurvinen A, Nissinen MJ, Andersson S, Korhonen P, Ruuska T, Taimisto M, Kalliomaki M, Lehtonen L, Sankilampi U, Arikoski P, et al: Parenteral plant sterols and intestinal failure-associated liver disease in neonates. J Pediatr Gastroenterol Nutr 2012, 54:803-811.

5. El Kasmi KC, Anderson AL, Devereaux MW, Vue PM, Zhang W, Setchell KD, Karpen SJ, Sokol RJ: Phytosterols promote liver injury and Kupffer cell activation in parenteral nutrition-associated liver disease. Sci Transl Med 2013, 5:206ra137.

6. Ellegard L, Sunesson A, Bosaeus I: High serum phytosterol levels in short bowel patients on parenteral nutrition support. Clin Nutr 2005, 24:415-420.

7. Savini S, D'Ascenzo R, Biagetti C, Serpentini G, Pompilio A, Bartoli A, Cogo PE, Carnielli VP: The effect of 5 intravenous lipid emulsions on plasma phytosterols in preterm infants receiving parenteral nutrition: a randomized clinical trial. Am J Clin Nutr 2013, 98:312-318.

8. Pianese P, Salvia G, Campanozzi A, D'Apolito O, Dello Russo A, Pettoello-Mantovani M, Corso G: Sterol profiling in red blood cell membranes and plasma of newborns receiving total parenteral nutrition. J Pediatr Gastroenterol Nutr 2008, 47:645-651.

9. Yoo EG: Sitosterolemia: a review and update of pathophysiology, clinical spectrum, diagnosis, and management. Ann Pediatr Endocrinol Metab 2016, 21:7-14.

10. Claumarchirant L, Matencio E, Sanchez-Siles LM, Alegria A, Lagarda MJ: Sterol Composition in Infant Formulas and Estimated Intake. J Agric Food Chem 2015, 63:7245-7251.

11. Jensen RG, Ferris AM, Lammi-Keefe CJ: Lipids in human milk and infant formulas. Annu Rev Nutr 1992, 12:417-441.

12. Harvey K, Xu Z, Walker C, Pavlina T, McGrath S, Zaloga G, Siddiqui R: Parenteral lipid emulsions in guinea pigs differentially influence plasma and tissue levels of fatty acids, squalene, cholesterol, and phytosterols. Lipids 2014, 49:777-793.

13. Savini S, Correani A, Pupillo D, D'Ascenzo R, Biagetti C, Pompilio A, Simonato M, Verlato G, Cogo P, Taus M, et al: Phytosterol Esterification is Markedly Decreased in Preterm Infants Receiving Routine Parenteral Nutrition. Lipids 2016, 51:1353-1361.

14. Nghiem-Rao TH, Tunc I, Mavis AM, Cao Y, Polzin EM, Firary MF, Wang X, Simpson PM, Patel SB: Kinetics of phytosterol metabolism in neonates receiving parenteral nutrition. Pediatr Res 2015, 78:181-189.

15. Bhattacharyya AK, Connor WE, Lin DS, McMurry MM, Shulman RS: Sluggish sitosterol turnover and hepatic failure to excrete sitosterol into bile cause expansion of body pool of sitosterol in patients with sitosterolemia and xanthomatosis. Arterioscler Thromb 1991, 11:1287-1294.

16. Lin HJ, Wang C, Salen G, Lam KC, Chan TK: Sitosterol and cholesterol metabolism in a patient with coexisting phytosterolemia and cholestanolemia. Metabolism 1983, 32:126-133.

17. Pupillo D, Correani A, Biagetti C, D'Ascenzo R, Simonato M, Verlato G, Cogo P, Rocchi MB, Carnielli VP: Half-life of plasma phytosterols in very low birth weight preterm infants on routine parenteral nutrition with vegetable oil-based lipid emulsions. Clin Nutr 2016.

18. Walsh MC, Yao Q, Gettner P, Hale E, Collins M, Hensman A, Everette R, Peters N, Miller N, Muran G, et al: Impact of a physiologic definition on bronchopulmonary dysplasia rates. Pediatrics 2004, 114:1305-1311.

19. Stoll BJ, Hansen N, Fanaroff AA, Wright LL, Carlo WA, Ehrenkranz RA, Lemons JA, Donovan EF, Stark AR, Tyson JE, et al: Late-onset sepsis in very low birth weight neonates: the experience of the NICHD Neonatal Research Network. Pediatrics 2002, 110:285-291.

20. Bell MJ, Ternberg JL, Feigin RD, Keating JP, Marshall R, Barton L, Brotherton T: Neonatal necrotizing enterocolitis. Therapeutic decisions based upon clinical staging. Ann Surg 1978, 187:1-7.

21. Horbar JD, Badger GJ, Carpenter JH, Fanaroff AA, Kilpatrick S, LaCorte M, Phibbs R, Soll RF: Trends in mortality and morbidity for very low birth weight infants, 1991-1999. Pediatrics 2002, 110:143-151.

22. Clayton PT, Bowron A, Mills KA, Massoud A, Casteels M, Milla PJ: Phytosterolemia in children with parenteral nutrition-associated cholestatic liver disease. Gastroenterology 1993, 105:1806-1813.

23. Wang J, Mitsche MA, Lutjohann D, Cohen JC, Xie XS, Hobbs HH: Relative roles of ABCG5/ABCG8 in liver and intestine. J Lipid Res 2015, 56:319-330.

24. Iyer KR, Spitz L, Clayton P: BAPS prize lecture: New insight into mechanisms of parenteral nutrition-associated cholestasis: role of plant sterols. British Association of Paediatric Surgeons. J Pediatr Surg 1998, 33:1-6.

25. Bindl L, Lutjohann D, Buderus S, Lentze MJ, v Bergmann K: High plasma levels of phytosterols in patients on parenteral nutrition: a marker of liver dysfunction. J Pediatr Gastroenterol Nutr 2000, 31:313-316.

26. Carter BA, Taylor OA, Prendergast DR, Zimmerman TL, Von Furstenberg R, Moore DD, Karpen SJ: Stigmasterol, a soy lipid-derived phytosterol, is an antagonist of the bile acid nuclear receptor FXR. Pediatr Res 2007, 62:301-306.

27. Clayton PT, Whitfield P, Iyer K: The role of phytosterols in the pathogenesis of liver complications of pediatric parenteral nutrition. Nutrition 1998, 14:158-164.

28. Mutanen A, Nissinen MJ, Lohi J, Heikkila P, Gylling H, Pakarinen MP: Serum plant sterols, cholestanol, and cholesterol precursors associate with histological liver injury in pediatric onset intestinal failure. Am J Clin Nutr 2014, 100:1085-1094.

29. Ng K, Stoll B, Chacko S, Saenz de Pipaon M, Lauridsen C, Gray M, Squires EJ, Marini J, Zamora IJ, Olutoye OO, Burrin DG: Vitamin E in New-Generation Lipid Emulsions Protects Against Parenteral Nutrition-Associated Liver Disease in Parenteral Nutrition-Fed Preterm Pigs. JPEN J Parenter Enteral Nutr 2016, 40:656-671.

30. Mellies MJ, Ishikawa TT, Glueck CJ, Bove K, Morrison J: Phytosterols in aortic tissue in adults and infants. J Lab Clin Med 1976, 88:914-921.

31. Miettinen TA, Railo M, Lepantalo M, Gylling H: Plant sterols in serum and in atherosclerotic plaques of patients undergoing carotid endarterectomy. J Am Coll Cardiol 2005, 45:1794-1801.

32. Hukkinen M, Kivisaari R, Merras-Salmio L, Koivusalo A, Pakarinen MP: Small Bowel Dilatation Predicts Prolonged Parenteral Nutrition and Decreased Survival in Pediatric Short Bowel Syndrome. Ann Surg 2016.

33. Relas H, Gylling H, Miettinen TA: Fate of intravenously administered squalene and plant sterols in human subjects. J Lipid Res 2001, 42:988-994.

34. Sudhop T, Sahin Y, Lindenthal B, Hahn C, Luers C, Berthold HK, von Bergmann K: Comparison of the hepatic clearances of campesterol, sitosterol, and cholesterol in healthy subjects suggests that efflux transporters controlling intestinal sterol absorption also regulate biliary secretion. Gut 2002, 51:860-863.

Chapter 6

Plasma and Dietary Phytosterols in Preterm and Term Infants Fed with Human Milk and Infant Formula

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Summary

It has long been established that diets rich in phytosterols (Phyto) lead to elevated plasma concentrations of Phyto and may cause adverse effects. Plasma Phyto concentrations in formula (IMF) fed infants are up to 20 times higher than those fed with human milk (HM). To our knowledge, limited data are now available on the intestinal Phyto absorption in infants. This information would be important to improve an adequate nutritional management especially of infants consuming vegetable oil-rich IMF.

We prospectively measured plasma concentrations and dietary intakes of Phyto in IMF- and HM-fed infants born prematurely and not: 10 preterm and 7 term infants exclusively fed with HM, and 12 preterm and 12 term infants who only received IMF were studied.

No differences in plasma Phyto concentrations were found between preterm and term infants fed with HM, whereas both campesterol (Camp) and sitosterol (Sito) were significantly lower in preterm than term infants fed with IMF (Camp: 427±193 vs 1136±615 µg/L, $p<0.001$; Sito: 520±324 vs 1072±502 µg/L, $p<0.001$; respectively). Both in IMF- and HM-fed infants, Phyto intakes after 10 days of enteral feeding were significantly greater in term than preterm infants (HM: 4.1±0.6 vs 10.8±2.7 mg, $p<0.001$; IMF: 182.8±29.9 vs 476.2±50.3 mg, $p<0.001$).

These findings suggested that a high oral/enteral phytosterol intake as it occurs in IMF-fed infants results in elevated plasma phytosterol levels both in preterm and term infants.

A low phytosterol diet should be preferred for infants. Further studies are needed to clarify consequences at medium- and long-term of Phyto accumulation in infants.

Abbreviations: BW, birth weight; Camp, campesterol; Cho, cholesterol; EN, enteral nutrition; GA, gestational age; HM, human milk; IMF, infant milk formula; Phyto, phytosterols; Sito, sitosterol; Stigma, stigmasterol; VLBW, very low birth weight.

INTRODUCTION

Human milk (HM) is the feed of choice for infant feeding, and, when not available, infant milk formulas (IMF) are the alternatives. Both HM and commercially available IMF contain phytosterols (Phyto) [1]. Phyto concentrations in IMF are up to 50 times higher than in HM, whereas it is the opposite for cholesterol (Cho) [2, 3]. Normally only small amount (<5%) of dietary Phyto enter into the bloodstream by intestinal ABCG5/G8 transporters. Among all Phyto, campesterol (Camp) is the most intestinally absorbed [4]. The small amount of Phyto absorbed are then excreted by the hepatic ABCG5/G8 into the biliary tract [5]. As result, plasma Phyto concentrations are low in enterally fed infants, especially those fed with HM: Mellies et al. reported that IMF feeding in infants aged from 1 to 12 mo. increased plasma Phyto concentrations up to 20 times [6,7].

It is generally assumed that preterm infants have increased intestinal permeability in comparison with term infants to enhance the prospects of growth and perhaps for proper metabolic programming in later life [8-10]. However, there is evidence on poor macromolecule uptake in response to premature birth as confirmed by a greater faecal excretion compared to term infants [11-13].

Previous studies reported that plasma Phyto concentrations are responsive to IMF or HM intakes in infants [6, 7]. To our knowledge, to date, no data are available on intestinal Phyto absorption in term and preterm infants. A comprehensive understanding of intestinal Phyto absorption in infants would be relevant for their nutritional management, especially in case of infants whose mothers decided to not provide HM. A high Phyto intake together with a high intestinal Phyto absorption could potentially result in early programming of higher endogenous Cho synthesis with a possible negative long-term effect on Cho metabolism and cardiovascular disease risk [15]. Other sequelae of Phyto accumulation are well-described in sitosterolemic patients and cannot be excluded [16,17].

In this study we measured plasma and dietary phytosterols of preterm and term infants fed with HM or IMF.

SUBJECTS AND METHODS

The study was conducted in accordance with the principles of the Helsinki Declaration as revised in Fortaleza (Brasil) 2013 and was approved by the local ethics committee and institutional review board. Written informed consent was obtained from the parents or legal guardians of all study patients.

Between November 2017 and December 2018, preterm infants admitted to the “G. Salesi” Children's Hospital of Ancona Neonatal Intensive Care Unit with gestational age (GA) < 36 weeks were enrolled. Exclusion criteria were: parenteral nutrition, exclusively HM or IMF feeding for less than 10 days, major congenital abnormalities, severe congenital sepsis, metabolic inborn errors, known genetic or chromosomal defects and liver dysfunction. Term infants admitted to Pediatrics and Infectious Disease Unit, G. Salesi Children's Hospital, Ancona with suspected infection were also studied. A duration of stay in unit less than 10 day and/or mixed feeding with HM and IMF were considered as exclusion criteria. Study infants were divided in 2 groups according to enteral feeding: HM or IMF.

EDTA-treated blood samples remaining after completion of clinical laboratories were collected for measurements of Camp, stigmasterol (Stigma) and sitosterol (Sito). Blood samples were immediately centrifuged at 2800 rpm (1358 g) and plasma was stored in pyrogallol added-tubes at -20°C until analysis.

Samples of HM and IMF given to infants were also obtained for Phyto analysis. Phyto concentrations both in preterm HM and IMF (Aptamil Pre®) were previously published by our group [14]. Term HM (Camp: 0.2±0.1, Stigma: 0.5±0.2, Sito: 1.0±0.1 mg/L) and term IMF (Aptamil1® - Camp: 26.0±1.6, Stigma: 8.2±0.8, Sito: 52.9±4.6 mg/L) were collected and measured. Analytical method used for sterol analysis was previously published elsewhere by our group [14].
The primary outcome was plasma Phyto concentration in term and preterm infants after 10 days of enteral feeding with HM and IMF.
This study was exploratory in nature as the plasma Phyto concentration in term and preterm infants were unknown at the time this study began. Data on Phyto concentrations and intakes were presented as mean ± SD. A p-value <0.05 was considered significant. All statistical analyses were performed by using SPSS (v 19.0; SPSS Inc, Chicago, Illinois).

PRELIMINARY RESULTS

From November 2017 to December 2018, 22 preterm and 19 term infants had enough leftover plasma for sterol analysis and were studied: 10 preterm and 7 term infants were exclusively fed with HM, and 12 preterm and 12 term infants only received IMF. Demographic and clinical characteristics of study infants are reported in **Table 1**.

Table 1. Demographic and clinical characteristics of study term and preterm infants.

	Preterm (n=22)	Term (n=19)	p-value
GA, days	220±8	273±9	**<0.001**
BW, g	1558±134	2951±454	**<0.001**
Gender (M/F)	6/16	13/6	**0.01**
Age at sampling, days	26±6	26±8	0.8

Data on plasma sterol concentrations in infants fed with HM and IMF are reported in **Table 2** and **Table 3**, respectively.

Table 2. Plasma Phyto concentrations in HM-fed infants[a]

	Preterm infants n=10	Term Infants n=7	Preterm compared to Term	p-value
Camp	321±127	260±199	=	0.4
Stigma	78±46	52±33	=	0.2
Sito	307±128	298±202	=	0.9

[a] Values are presented as mean±SD. Values are in µg/L for Camp, Stigma, and Sito. Independent t-test was used for the analysis. Statistical significance was set up at p<0.05.

Table 3. Plasma Phyto concentrations and in IMF-fed infants[a]

	Preterm infants n=12	Term Infants n=12	Preterm compared to Term	p-value
Camp	427±193	1136±615	**-**	**<0.001**
Stigma	70±48	72±28	=	0.9
Sito	520±324	1072±502	**-**	**<0.001**

[a] Values are presented as mean±SD. Values are in µg/L for Camp, Stigma, and Sito. Independent t-test was used for the analysis. Statistical significance was set up at $p<0.05$.

Both in IMF- and HM-fed infants, cumulative Phyto intakes after 10 days of enteral feeding were significantly greater in term than preterm infants (**Table 4**).

Table 4. Cumulative Phyto intake after 10 days of enteral feeding[a]

		Preterm infants	Term Infants	Preterm compared to Term	p-value
HM		n=10	n=7		
	Camp	0.8±0.1	1.3±0.3	**-**	**<0.001**
	Stigma	0.8±0.1	3.2±0.8	**-**	**<0.001**
	Sito	2.6±0.4	6.4±1.6	**-**	**<0.001**
IMF		n=12	n=12		
	Camp	41.1±6.7	130.5±35.4	**-**	**<0.001**
	Stigma	22.7±3.7	32.3±12.8	**-**	0.02
	Sito	119.0±19.5	257.5±48.8	**-**	**<0.001**

[a] Values are presented as mean±SD. Values are in mg for Camp, Stigma, and Sito. Independent t-test was used for the analysis. Statistical significance was set up at $p<0.05$.

GENERAL COMMENTS

We did not find differences in plasma Phyto concentrations between preterm and term infants fed with HM, whereas plasma Phyto were significantly higher in IMF-fed term infants than IMF-fed preterm infants. Both in HM- and IMF-infants, cumulative Phyto intake after 10 days of continuous enteral feeding was higher in terms than preterms. According to these preliminary data, plasma Phyto concentrations are responsive to IMF or HM intakes both in term and preterm infants. However, a larger number of infants should be studied to confirm this hypothesis.

As results, it is advisable a low Phyto diet for infants. Further studies are needed to clarify consequences at medium- and long-term of Phyto accumulation in infants.

REFERENCES

1. Jensen RG, Ferris AM, Lammi-Keefe CJ: Lipids in human milk and infant formulas. Annu Rev Nutr 1992, 12:417-441.

2. Hodgson PA, Ellefson RD, Elveback LR, Harris LE, Nelson RA, Weidman WH: Comparison of serum cholesterol in children fed high, moderate, or low cholesterol milk diets during neonatal period. Metabolism 1976, 25:739-746.

3. Claumarchirant L, Matencio E, Sanchez-Siles LM, Alegria A, Lagarda MJ: Sterol Composition in Infant Formulas and Estimated Intake. J Agric Food Chem 2015, 63:7245-7251.

4. Heinemann T, Axtmann G, von Bergmann K. Comparison of intestinal absorption of cholesterol with different plant sterols in man. Eur J Clin Invest. 1993 Dec;23(12):827-31. PubMed PMID: 8143759.

5. Wang J, Mitsche MA, Lutjohann D, Cohen JC, Xie XS, Hobbs HH: Relative roles of ABCG5/ABCG8 in liver and intestine. J Lipid Res 2015, 56:319-330.

6. Mellies M, Glueck CJ, Sweeney C, Fallat RW, Tsang RC, Ishikawa TT: Plasma and dietary phytosterols in children. Pediatrics 1976, 57:60-67.

7. Mellies MJ, Ishikawa TT, Gartside P, Burton K, MacGee J, Allen K, Steiner PM, Brady D, Glueck CJ: Effects of varying maternal dietary cholesterol and phytosterol in lactating women and their infants. Am J Clin Nutr 1978, 31:1347-1354.

8. Saleem B, Okogbule-Wonodi AC, Fasano A, Magder LS, Ravel J, Kapoor S, Viscardi RM: Intestinal Barrier Maturation in Very Low Birthweight Infants: Relationship to Feeding and Antibiotic Exposure. J Pediatr 2017, 183:31-36 e31.

9. Roberton DM, Paganelli R, Dinwiddie R, Levinsky RJ: Milk antigen absorption in the preterm and term neonate. Arch Dis Child 1982, 57:369-372.

10. Harit D, Faridi MM, Aggarwal A, Sharma SB: Lipid profile of term infants on exclusive breastfeeding and mixed feeding: a comparative study. Eur J Clin Nutr 2008, 62:203-209.

11. Hough RL, McCarthy FD, Thatcher CD, Kent HD, Eversole DE: Influence of glucocorticoid on macromolecular absorption and passive immunity in neonatal lambs. J Anim Sci 1990, 68:2459-2464.

12. Trahair JF, Sangild PT: Systemic and luminal influences on the perinatal development of the gut. Equine Vet J Suppl 1997:40-50.

13. Watkins JB: Mechanisms of fat absorption and the development of gastrointestinal function. Pediatr Clin North Am 1975, 22:721-730.

14. Pupillo D, Correani A, Biagetti C, D'Ascenzo R, Simonato M, Verlato G, Cogo P, Rocchi MB, Carnielli VP: Half-life of plasma phytosterols in very low birth weight preterm infants on routine parenteral nutrition with vegetable oil-based lipid emulsions. Clin Nutr 2016.

15. Owen CG, Whincup PH, Kaye SJ, Martin RM, Davey Smith G, Cook DG, Bergstrom E, Black S, Wadsworth ME, Fall CH, et al: Does initial breastfeeding lead to lower blood cholesterol in adult life? A quantitative review of the evidence. Am J Clin Nutr 2008, 88:305-314.

16. Yoo EG: Sitosterolemia: a review and update of pathophysiology, clinical spectrum, diagnosis, and management. Ann Pediatr Endocrinol Metab 2016, 21:7-14.

17. Clayton PT, Bowron A, Mills KA, Massoud A, Casteels M, Milla PJ: Phytosterolemia in children with parenteral nutrition-associated cholestatic liver disease. Gastroenterology 1993, 105:1806-1813.

Chapter 7

Hypertriglyceridemia in Very Low Birth Weight Infants on Routine Parenteral Nutrition: A Case-Control Study

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Summary

Introduction: Hypertriglyceridemia (HiTG) often occurs in infants on parenteral nutrition (PN), especially those with low birth weight (BW). In case of HiTG, the ESPGHAN/ESPEN/ESPR 2018 guidelines recommend an intravenous (IV) lipid (FAT) titration. The consequences of IV FAT titration in small infants are largely unknown.

Aim: To investigate the modifications of IV FAT, amino acids (AA), carbohydrates (CHO) and non-protein energy (NPE) intakes in infants with a BW less than 1250g on routine PN who developed HiTG (>250 mg/dL).

Materials and Methods: We retrospectively reviewed nutrition, growth and neurodevelopment of a cohort of infants with a BW<1250 g consecutively admitted to the "G. Salesi" Children's Hospital between Jan-2004 and Dec-2016 who received routine PN. Patients with and without HiTG were match-paired for BW and gestational age (GA).

Results: Six hundred and fifty-eight infants of the cohort were analysed and 196 (30%) had at least one HiTG episode in the first 10 days of life (DOL). One hundred and thirty-six HiTG patients were compared with 136 matched-controls (CNTRBW-GA). In the first 10 DOL, IV FAT and NPE, but not IV AA, were significantly lower in HiTG infants. The incidence of hyperglycaemia episodes associated with HiTG, was not significantly higher than in CNTRBW-GA (12% vs 7%, p=0.2). We found no differences between groups in the incidence of the main complications of prematurity. Anthropometry at 36 weeks (W) and at 24 months (Mo) corrected age (CA), and neurodevelopment at 24Mo CA were also not different.

Conclusion: At our institution, HiTG occurred in 30% of VLBW infants and IV FAT titration resulted in a significantly lower IV FAT and NPE intakes. HiTG infants on routine PN had similar growth and neurodevelopment than controls.

Abbreviations: AA= amino acids; BW= birth weight; CA= corrected age; CHO= carbohydrates; CNTR= controls; GA= gestational age; HiTG= hypertriglyceridemia; IV= intravenous; Mo= months; PN= parenteral nutrition; TG= triglyceride; VLBW= very low birth weight; W= weeks.

Submitted.

INTRODUCTION

Hypertriglyceridemia (HiTG) is a metabolic complication that often occurs in infants receiving parenteral nutrition (PN) [1]. Elevated triglycerides (TG) may precede the lipid overload syndrome with fever, thrombocytopenia, bleeding alteration, pulmonary hypertension, pancreatitis, liver failure and neurologic changes [2-4]. Current European guidelines in pediatrics recommend to check plasma TG within 1-2 days after initiation or adjustment of intravenous (IV) lipids (FAT) and thereafter from weekly to monthly [5]. Plasma TG concentrations should not exceed 265 mg/dL in infants on PN even if lower thresholds have been considered in the past (e.g. 150, 200 and 250 mg/dL) [6-9]. Suggested management of HiTG involves titration or halting the IV FAT infusion until plasma TG levels normalize. As it is not advisable to administer excessive carbohydrates (CHO), IV FAT titration may expose the small preterm infants to the risk of low non-protein energy (NPE) intakes whose consequences remain untested.

Adequate provision of amino acids (AA) and NPE intakes for the preterm infants is a primary goal of neonatal medicine, as inadequate nutrition and growth failure are associated with poor neurodevelopment [10, 11]. So far it is unclear the interplay between sickness and inadequate nutrition. Furthermore, it is still debated whether metabolic complications are caused by sickness or they simply reflect the poor metabolic tolerance to nutrients of preterm infants or both.

The purpose of our retrospective case-control study was to investigate modifications in the daily intake of IV macronutrients and energy intakes (AA, FAT, CHO and NPE) in a large cohort of very low birth weight (VLBW) infants who developed HiTG.

MATERIALS AND METHODS

Study infants

We retrospectively reviewed data of all infants consecutively admitted to the “G. Salesi” Children Hospital, Ancona, Italy, between January 2004 and December 2016. Neonates with a birth weight (BW) below 1250 g who routinely received an individualized all-in-one mixture PN from the first hours of life were evaluated in this study. Exclusion criteria were major congenital malformations, inborn errors of metabolism, admission to the NICU after 48 hours of life, death or transfer to another unit before 10 days of life (DOL) and missing clinical information. The study was approved by the local ethics committee (Prot. 2018 201).

Study design

The study infants were divided into two groups according to the plasma TG values: 1) HiTG if at least one of the TG measurements was >250 mg/dL during the first 10 DOL; 2) CNTR if none of the TG measurements were >250 mg/dL. Descriptive data of the study cohort was reported. Case-control analyses were carried out using gestational age (GA) ±2days and BW±80g as matching variables: 1) HiTG vs CNTR; 2) isolated HiTG vs CNTR without other metabolic complications during the study period.

Plasma TG and urea were measured by Refloton® dry chemistry system (Roche Diagnostics S.p.A, Monza, Italy), whereas blood glucose by Accu-Chek® (Roche Diagnostics S.p.A, Monza, Italy). Concomitant metabolic complications occurred within ±1 hour after the TG measurements. To calculate concomitant complications, a “fake HiTG episode” was simulated in CNTR infants at the same time when it occurred in the cases.

Nutrition protocol management

According to the local NICU Nutrition Schemes, IV FAT was prescribed at dose of 1.0-1.5 g/kg on the first day of life up to 2.5-3.5 g/kg/d on the 5th day of life. CHO were increased from 6-8 g/kg/d to 12-14 g/kg/d from day 1 to day 5. AA were prescribed at dose of 1.0-1.5 g/kg/d in the first day of life and increased up to 2.5-3.5 g/kg/d on the 5th day of life. Maximum IV FAT, AA and CHO intakes achieved were then kept constant from day 5 to day 7. Minimal enteral feeding with human milk or infant milk formula was provided from day 0 to day 7, the maximum amount supplied being 8 ml/kg/d from day 1 to day 4, and 16 ml/kg/d from day 5 to day 7. For each infant after day 7, PN was tapered and stopped on average on the 18th day of life while oral feeding was gradually increased to reach full feed and keep a maximum total fluid intake of 160 ml/kg/d.

Metabolic complications

During PN, HiTG was defined as at least a value of plasma TG> 250 mg/dL (severe HiTG: plasma TG >400 mg/dL) and elevated urea as a blood urea >100 mg/dL. A hyperglycaemia episode was defined as at least a value of blood glucose >175 mg/dL; the protocol provides for a second measurement to confirm the first episode (confirmed hyperglycaemia). At our institution, both plasma TG and urea were monitored on the 3°, 5°, and 7° day of life and then weekly until PN was stopped. Blood glucose was measured at least once daily. PN during HiTG, hyperglycaemia and elevated urea was handled according to the predefined algorithms: if TG were >250 mg/dL, the IV FAT intake was reduced by 1.0 g/kg/d and TG were checked again after 24 hours; if TG reached 400 mg/dL, the amount of IV FAT was lowered to the minimum intake of 0.5 g/kg/d. If plasma urea was >100 mg/dL, after excluding excessive weight loss, the IV AA intake was reduced by 1.0 g/kg/d if it was >120 mg/dL by 2.0 g/kg/d and values were checked again after 24 hours. Blood glucose cut-off level was set at 175 mg/dL above which glucose intake was lowered by 2 mg/kg/min steps until blood glucose value fell below 175 mg/dL and/or to a minimum glucose intake of 6 g/kg/d, whichever comes first. If hyperglycemia persisted at the minimum glucose intake, insulin infusion was started. Variations in blood sampling and intervention schemes might have occurred in selected cases at the discretion of the attending physician.

Data collection

Administered IV AA, FAT and CHO intakes (g/kg/d) were collected from electronic medical records and calculated using hourly PN infusion (ml/h) and daily weight (WT). IV NPE was obtained from the actual IV FAT and CHO intakes (9 and 4 kcal/g, respectively). Growth and other clinical information were prospectively recorded daily by a dedicated software (Neotools®; Interactive, Milan, Italy). Body WT (g) was measured daily using a digital infant scale; length (cm) and head circumference (cm) were measured at birth and weekly thereafter using a neonatal stadiometer and a flexible non-stretchable tape, respectively. Standard deviation scores (SDS) were computed using Italian growth chart. WT gain (g/kg/d) was calculated from birth to 36 weeks (W) corrected age (CA) and from the regained BW to 36W CA (mean of the day-by-day WT gain expressed in g/kg/d of each study patient). The major complications of prematurity were defined according to the Vermont-Oxford definitions. Cholestasis was defined as at least one plasma direct bilirubin concentration greater than 1.0 mg/dL. The 24Mo follow-up included medical history, physical examination, anthropometry and neurodevelopmental assessment (the Bayley scale of Infant and toddler development, third edition – Bayley III).

Primary and secondary outcomes

The primary outcome was the daily amount of administered IV macronutrient and energy intakes (AA, FAT, CHO in g/kg/d; and NPE in kcal/kg/d) in infants with and without HiTG from birth to DOL 10. Secondary outcomes were: perinatal characteristics, major neonatal diseases, in-hospital growth, anthropometry at 36W and at 24Mo CA and neurodevelopment assessment.

Statistical analysis

Due to the exploratory nature of the study, no formal sample size calculations were performed. Depending on the distribution, data were expressed as mean±SD or SE, as the median |25P 75P| or as a number (percentage). The clinical characteristics of the study groups were compared using the paired t-test, independent t-test, Wilcoxon test or McNemar test as appropriate. Statistical significance was set at *$p<0.05$. All statistical analyses and case-control matching were performed by using SPSS software (v 23.0; SPSS Inc, Chicago, Illinois).

RESULTS

PATIENTS: THE STUDY COHORT

From January 2004 to December 2016 all the records of 848 consecutively admitted infants with a BW below 1250g were screened for this study. One hundred and forty-seven patients were excluded because they did not meet the inclusion criteria: 41 had congenital malformations or inborn errors of metabolism, 24 were admitted to the NICU after 48 hours of life, 75 died or were transferred to another institution before 10 days of life and clinical information were not available for 7.
Seven hundred and one VLBW infants met the inclusion criteria, the TG measurements were available in 658 infants: 196 (30%) had at least one HiTG episode during the first 10 DOL and 462 (70%) had all the TG measurements <250 mg/dL threshold (**Figure 1**).

Compliance to TG monitoring protocol

Out of 701 VLBW infants who met the inclusion criteria, 658 (94%) had at least one TG measurement during the first 10 DOL and 43 (6%) received PN but no TG measurements were done. Sixty-six infants (10%) had only one TG measurement, 195 (30%) had two TG measurements and 397 (60%) had 3 or more TG measurements in the study period. Two hundred and eighty-three (43%) patients had TG measurements at DOL 3, 338 (51%) at DOL 5 and 309 (47%) at DOL 7 (**Figure 2**, **Panel A**).

TG concentrations over time and time to regain TG values≤250 mg/dL

The 196 HiTG infants had statistically greater plasma TG concentrations than CNTR until DOL 10 (**Figure 2**, **Panel B**). Severe HiTG was found in 63 infants (32%): 55 infants (87%) with only one TG value >400 mg/dL and 8 (13%) with two or more TG values >400 mg/dL.
In 94 HiTG infants (48%), the first TG value>250 mg/dL was returned under the HiTG threshold in ≤ 24 hours, in 56 (29%) between 24 and 48 hours, in 30 (15%) in a longer time frame (>48 hours) and in 16 (8%) no other TG values<250 mg/dL were available until DOL 10.

Figure 1. Flow diagram of the patients included in the study. [i]HiTG: at least one TG value >250 mg/dL; iiCNTR: infants without HiTG; *concomitant hyperglycaemia: at least one hyperglycaemia episode (blood glucose value>175 mg/dL) within ±1 hour after the HiTG measurements; **concomitant elevated urea: at least one plasma urea>100 mg/dL within ±1 hour after the HiTG measurements; **confirmed hyperglycaemia: two consecutive blood glucose values >175 mg/dL within 1 hour; ****elevated urea: plasma urea >100 mg/dL; ‡ no information on survival at 24Mo CA: 22/150 HiTG and 69/365 CNTR infants; ┼ COG (cognitive composite score - Bayley III) started in 2007; MOT (motor composite score - Bayley III) in 2009; CA: corrected age; Mo: months.

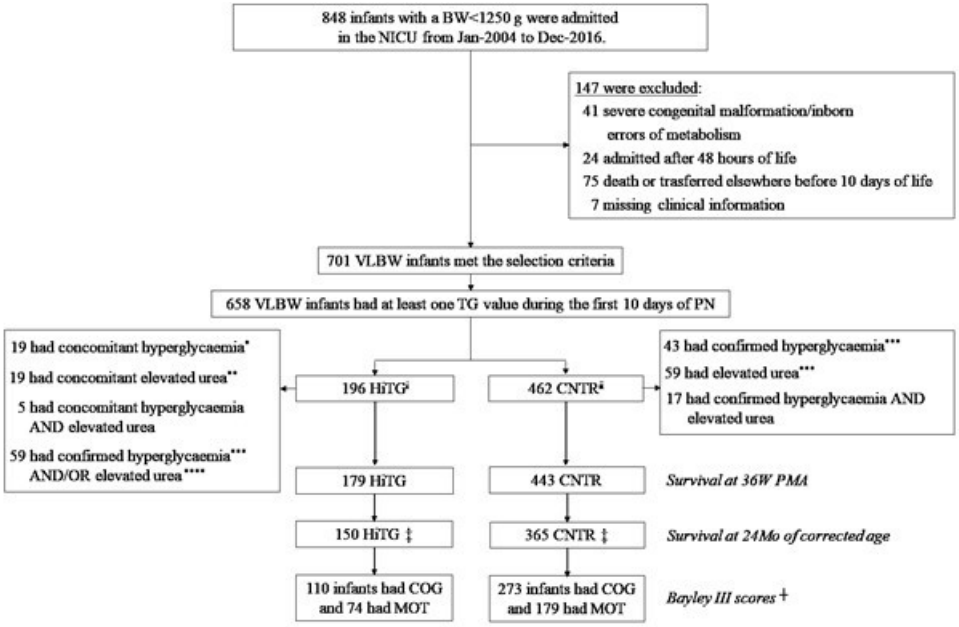
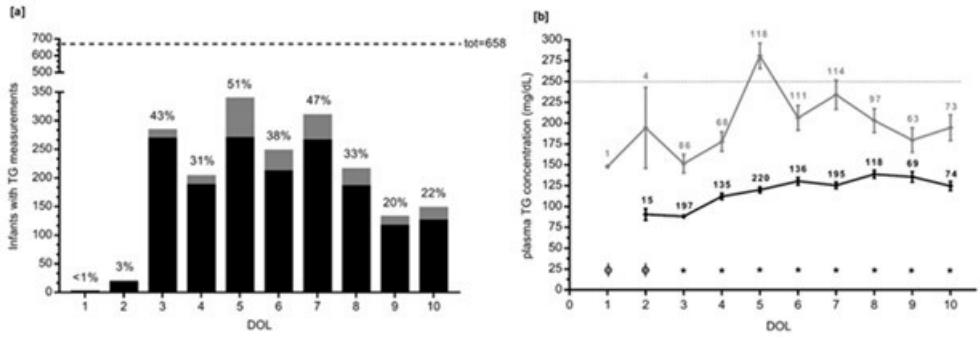


Figure 2. [a]. Number of infants with TG measurements per day of life (DOL). Black column: TG≤250 mg/dL; grey column: TG>250 mg/dL. Percentages describe the number of infants over the total number of the study patients which had at least one plasma TG value. [b]. Plasma TG concentrations (mean±SE) over time: HiTG (grey line) vs CNTR (black line) infants. Independent t-test was used for the statistical analysis (*p-value <0.05; Φ = statistical analysis was not performed due to low sample size). Numbers are referred to infants which had TG measurements in the study DOL.



PATIENTS: CASE-CONTROL ANALYSES

One hundred and thirty-six case-control matched pairs (HiTG vs CNTR) were obtained using BW and GA as matching variables. No differences in demographic and clinical characteristics at birth were found between the study matched case-control infants (**Table 1**).

Table 1. Demographic and clinical characteristics of HiTG and $CNTR^{BW\text{-}GA}$ infants.

	HiTG n=136	$CNTR^{bw\text{-}ga}$ n=136	Differences	p-value
Birth weight (BW) - g	875±173	877±170	-2±41	0.5
Gestational age (GA) - days	193±15	193±14	-0±1	0.9
Males, no. (%)	69 (51%)	64 (47%)	+5 (+4%)	0.5
BW - SDS	-0.59±1.02	-0.57±1.03	-0.03±0.35	0.4
Total Length at Birth (TL.B) - cm	34.7±2.7	34.4±2.8	+0.2±2.4	0.3
TL.B - SDS	-0.49±1.00	-0.57±1.02	+0.09±0.86	0.3
Head Circumference at Birth (HC.B) - cm	24.5±1.9	24.5±1.8	-0.1±1.5	0.6
HC.B - SDS	-0.43±1.08	-0.39±1.03	-0.04±1.07	0.6
SGA10°centile, n (%)	41 (30%)	38 (28%)	+3 (+2%)	0.3
SGA2SDS, n (%)	15 (11%)	13 (10%)	+2 (+1%)	0.5
Inborn - no. (%)	126 (93%)	120 (88%)	+6 (+4%)	0.2
Singleton birth - no. (%)	102 (75%)	103 (76%)	-1 (-1%)	0.9
Apgar5min – no.	8 |7 8|	8 |7 8|	0	0.4
Intubated 0-24 hours of life-no.(%)	101 (74%)	100 (74%)	+1 (+1%)	0.9
Surfactant therapy– no.(%)	97 (71%)	85 (63%)	+12 (+9%)	0.1
Perinatal steroid treatment–no.(%)	115 (88%)	117 (90%)	-2 (-2%)	0.7
Cesarean section– no. (%)	111 (85%)	113 (87%)	-2 (-2%)	0.7

Data are presented as mean±SD, median |25P 75P| or no. (%). Paired t-test, Wilcoxon test, or McNemar test were used for the statistical analysis. SDS: standard deviation score; SGA: small for gestational age.

PN duration was not different between HiTG and $CNTR^{BW\text{-}GA}$ (21 |18 26| vs 21 |18 25| days, respectively; p=0.4). Daily IV FAT and NPE intakes were significantly lower in HiTG than $CNTR^{BW\text{-}GA}$ infants. IV AA intakes were not different between the study groups. CHO intake at DOL 4 was significantly lower in cases than in controls (**Figure 3**, **Panel A**). Cumulative IV FAT intake in the first 10 DOL was 15.4±3.8 vs 18.2±3.1 g/kg, p<0.001 or -0.3±0.5 g/kg/d, p<0.0001, respectively. The mean reduction of IV FAT after an HiTG episode was -0.6±1.1 g/kg, compared to the IV FAT progression of +0.1±0.6 g/kg in controls (p<0.001). There were no differences between HiTG and $CNTR^{BW\text{-}GA}$ in cumulative PN and enteral AA and NPE intakes from birth to 36W (PN - AA: 53±35 vs 51±33 g/kg, p=0.6; NPE: 1091±652 vs 1141±766 kcal/kg, p=0.5; EN – AA: 150±65 vs 142±69 g/kg, p=0.3; NPE: 4614±1865 vs 4396±1979 kcal/kg, p=0.3).

The incidence of the major diagnoses of prematurity, anthropometry at 36W and at 24Mo CA and neurodevelopment were not different between the study groups (**Table 2-4**).

Table 2. The incidence of major diagnoses of prematurity from birth to 36W CA.

	HiTG n=136	CNTR^{bw-ga} n=136	Differences	p-value
EOS – no. (%)	8 (6%)	7 (5%)	+1 (+1%)	0.8
LOS – no. (%)	30 (22%)	36 (26%)	-6 (-4%)	0.4
NEC≥ grade II – no. (%)	8 (6%)	6 (4%)	+2 (+1%)	0.6
Cholestasis – no. (%)	13 (10%)	15 (11%)	-2 (-1%)	0.7
RDS and HMD – no. (%)	124 (91%)	123 (90%)	+1 (+1%)	0.8
BPD – no. (%)	34 (25%)	42 (31%)	-8 (-6%)	0.3
Asphyxia – no. (%)	8 (6%)	7 (5%)	+1 (+1%)	0.8
PDA – no. (%)	89 (65%)	95 (70%)	-6 (-4%)	0.4
PVL II-IV – no. (%)	3 (2%)	6 (4%)	-3 (-2%)	0.3
IVH ≥ grade III – no. (%)	12 (9%)	10 (7%)	+2 (+1%)	0.7
ROP ≥ grade III – no. (%)	2 (1%)	0 (0%)	+2 (+1%)	0.3

Data are presented as no. (%). McNemar test was used for the statistical analysis. BPD: bronchopulmonary dysplasia; EOS: early onset sepsis; IVH: intraventricular haemorrhage; LOS: late onset sepsis; HMD: hyaline membrane disease; NEC: necrotizing enterocolitis; PDA: patent ductus arteriosus; ROP: retinopathy of prematurity; PVL: periventricular leukomalacia; RDS: respiratory distress syndrome.

Table 3. Anthropometry and growth from birth to 36W CA.

	HiTG n=120	CNTR^{BW-GA} n=120	Differences	p-value
WT Nadir - g	761±162	759±150	+2±69	0.8
Age at Nadir - days	4.4±2.0	4.3±2.0	+0.1±2.9	0.6
Max WT Loss - %	13±6	13±6	-0±7	0.6
Time to Regain BW - days	12.5±5.2	11.8±4.9	+0.7±5.9	0.2
WT 36W - g	1861±354	1895±343	-34±283	0.2
WT SDS–36W - no.	-1.91±0.88	-1.81±0.86	-0.10±0.71	0.1
TL 36W - cm	42.6±2.5	42.7±2.5	-0.1±2.2	0.7
TL SDS-36W- no.	-1.87±0.99	-1.84±0.96	-0.04±0.85	0.7
HC 36W - cm	30.5±1.5	30.6±1.5	-0.1±1.9	0.5
HC SDS-36W - no.	-1.63±1.02	-1.53±1.08	-0.10±1.36	0.4
Δ WT (Birth-36W) - g	969±324	1000±322	-31±280	0.2
WT gain (Birth-36W) - g/kg/d	15.4±2.2	15.6±2.5	-0.2±3.3	0.5
WT gain (BW recovery-36W)- g/kg/d	16.7±2.8	16.6±2.9	+0.1±4.0	0.8

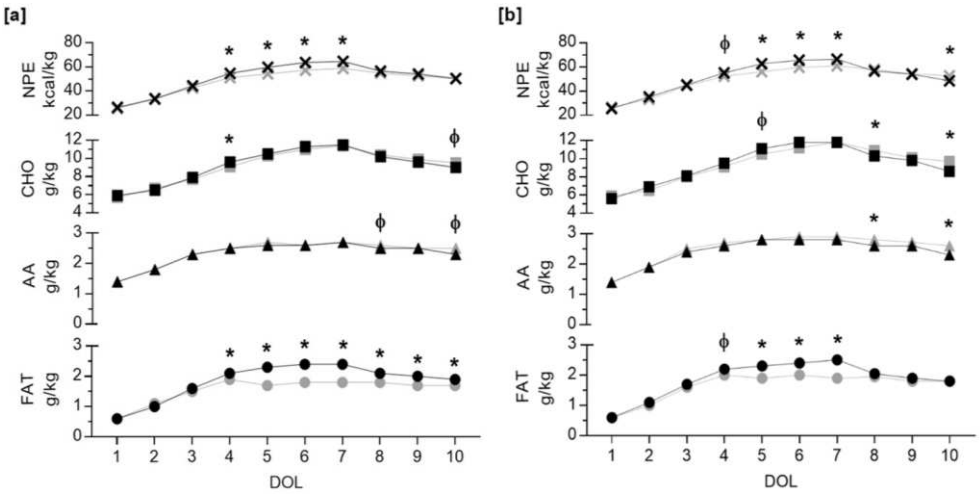
Data are presented as mean±SD. Paired t-test was used for the statistical analysis. HC: head circumference; SDS: standard deviation score; TL: total length; WT: weight; Δ WT (Birth-36W): difference in weight between birth and 36W CA.

Table 4. Anthropometry and neurodevelopment at 24Mo CA.

	HiTG n=43	CNTR^{bw-ga} n=43	Differences	p-value
WT at 24Mo - g,	11627±1759	11562±2184	+65±2564	0.9
WT SDS–24Mo - no	-0.29±1.30	-0.24±1.69	-0.05±1.90	0.9
TL at 24Mo - g	87.5±4.2	86.1±4.6	+1.4±6.0	0.1
TL SDS–24Mo - no	0.72±1.36	0.44±1.46	+0.28±1.82	0.3
HC at 24Mo - g	47.8±1.9	48.0±1.6	-0.2±2.2	0.6
HC SDS–24Mo - no	-1.05±1.56	-0.72±1.24	-0.33±1.78	0.2
COG score (Bayley III) – no., n=39/43	94±13	95±14	-2±18	0.6
MOT score (Bayley III) – no., n=22/43	100±14	101±11	-1±17	0.8

Data are presented as mean±SD. Paired t-test was used for the statistical analysis. COG: cognitive composite score; HC: head circumference; MOT: motor composite score; SDS: standard deviation score; TL: total length; WT: weight.

Figure 3. IV macronutrients (FAT, AA and CHO) and non-protein energy (NPE) intakes in matched cases (grey) and controls (black): FAT, g/kg (○); AA, g/kg (△); CHO, g/kg (□); NPE, kcal/kg (×). Data are presented as group means. Paired t-test was used for the statistical analysis. Significance was set-up at *p<0.05 (p-value between 0.05≤Φ≤0.1; *<0.05). [a]. HiTG vs CNTRBW-GA, n=136; [b]. Isolated HiTG vs CNTRBW-GA without other metabolic complications, n=68;



The incidence and mean glycaemic values(±SD) of concomitant hyperglycaemia episodes were not different between HiTG and CNTR^{BW-GA} infants: 16 (12%) vs 9 (7%), p=0.2; 128±61 vs 130±56 mg/dL, p=0.9, respectively. Insulin therapy was also not different between cases and controls: 15 (11%) vs 10 (7%), p=0.3, respectively.

To exclude the effect of hyperglycaemia and/or elevated urea on daily IV intake modifications, we matched 68 infants with isolated HiTG to 68 CNTR without metabolic complications during the first 10 DOL.

IV intakes of infants included in this case-control analysis are reported in **Figure 3**, **Panel B**. There were no differences in the perinatal characteristics, the incidence of major diagnoses of prematurity, cumulative PN and EN intakes (birth-36W), anthropometry at 36W and at 24Mo CA and neurodevelopment at 24Mo CA between the two groups (data not shown).

DISCUSSION

To our knowledge, this is the first study on the short and long-term consequences of HiTG in a large cohort of small preterm infants with a BW<1250g (n=848). Key results are: 1) elevated TG significantly affected daily PN intakes in the study infants; 2) HiTG appeared to be an isolated metabolic complication, and 3) HiTG patients did not show any growth or neurodevelopmental disadvantage when compared to matched-controls. We will comment these findings below.

The incidence of HiTG (30%) in this cohort of VLBW preterm infants on routine PN was in line with literature [9, 12-14]. To the best of our knowledge, none of the previous studies explored the consequences of HiTG and IV FAT titration on PN intakes, growth and neurodevelopment in small preterm infants. Monitoring HiTG was associated with a significantly reduction of IV FAT and NPE intakes in the first 10 DOL. This result can be a reason of concern as some physicians fear that intensive biochemical monitoring may lead to a marked reduction of PN intakes that in turn may result in poor growth and ultimately poor neurodevelopment. Our 24Mo follow-up data did not support this concern. IV FAT titration in our cohort resulted in a significant reduction of cumulative IV FAT intake of about 3.0 g/kg in the first 10 DOL corresponding to -0.3 g/kg/d. This difference albeit statistically significant did not lead to any difference in short term growth. We speculate that halting IV FAT for one or two days which may happen with the use of the standard PN bags may have no or very limited consequences on infant growth. However, further studies are required to confirm or refuse this hypothesis.

There are a few studies in preterm infants that reported significant associations between HiTG and disease/sickness [9, 14, 15]. Some physicians considered HiTG to be associated with illness, however our data did not support this notion. In our cohort of small preterm infants, we did not find any association between HiTG and the major complication of prematurity. We wish to discuss the association of HiTG with hyperglycaemia that is also considered as a marker of metabolic imbalance and it is reported to be higher during sickness [16, 17]. To our surprise, we detected a similar rate of hyperglycaemia episodes in HiTG and CNTR patients. This result suggests that hyperglycaemia in association with HiTG may represent an isolated metabolic complication rather than an expression of the severity of disease.

The most important finding of the present study, in our opinion, is that the reduction in IV FAT and NPE intakes observed in HiTG infants did not significantly affect neither in hospital nor late 24Mo CA growth. Perhaps even more importantly neurodevelopment outcome at 24Mo CA was not different. We do not have a clear explanation for these findings. We speculate that either compensatory mechanisms are in place even in small preterm infants or that the modest increase in IV AA and CHO intakes found in our HiTG patients (Figure 3) might have mitigated the potentially negative effects of reduced IV FAT intakes. Nowadays, IV FAT intakes of small preterm infants are set up to meet the target energy requirements during PN and to prevent essential fatty acid deficiency[1]. However, the optimal IV FAT intake of a VLBW infant during the first DOLs remains unknown. Some studies reported growth and neurodevelopment advantages in preterm infants receiving high IV FAT intakes from the first DOLs[18, 19], whereas other authors did not find any difference[13, 20].

Cochrane meta-analyses suggested no differences in growth of VLBW infants between “early” and “not early” (>2 DOL) initiation of IV FAT[21, 22]. Based on our results, we speculate that the recommended doses of IV FAT may still be in excess compared to those effectively required for adequate growth and metabolism of the small preterm infants. The benefits of the extra IV FAT during HiTG remain to be clarified in this group of infants.
This is a retrospective study and carries an inherent risk of bias. Patients were not matched for year of birth and little variations in the standard of care throughout the study period (2004-2016) might have occurred. We considered as HiTG infants those who had at least one TG value >250 mg/dL rather than 265 mg/dL as suggested by the current European guidelines. However, about 85% of the study infants with HiTG had plasma TG>265 mg/dL. Moreover, about 30% of both HiTG and isolated HiTG infants did not have a matched control and were not considered in the analysis: in the unmatched cases there was about a 20% excess in the incidence of SGA10centile diagnosis in comparison to unmatched controls, thus our case-control design (GA and BW used as matching variables) did not consider an important part of patients that according to previous studies are at high risk to develop HiTG [8, 15].
In conclusion, at our institution, HiTG occurred in 30% of VLBW infants and IV FAT titration resulted in a significantly lower IV FAT and NPE intakes. HiTG was not associated with reduced growth and poorer neurodevelopment. Information on the metabolic fate, utilization and impact on growth of early and high IV FAT intakes in small preterm infants remain undefined. The risk benefit ratio of IV FAT titration at plasma TG values higher than 250 mg/dL remains also undefined.

CONFLICT OF INTEREST STATEMENT AND FUNDING SOURCES
No conflicts of interest to declare. This study was partially supported by an unrestricted grant from Baxter International Corporation.

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AUTHORS' CONTRIBUTIONS
VPC was responsible for the design of the study;
AC, IG, CM, RD, CB contributed to data collection and analysis;
PC, LA were in charge of the statistical analysis.
All authors read and approved the final manuscript.

REFERENCES

[1] Salama GSA, Kaabneh MAF, Almasaeed MN, Alquran MIA. Intravenous Lipids for Preterm Infants: A Review. Clinical Medicine Insights Pediatrics. 2015;9:25-36.

[2] Kerner JA, Jr., Poole RL. The use of IV fat in neonates. Nutrition in clinical practice : official publication of the American Society for Parenteral and Enteral Nutrition. 2006;21:374-80.

[3] Rubin M, Naor N, Sirota L, Moser A, Pakula R, Harell D, et al. Are bilirubin and plasma lipid profiles of premature infants dependent on the lipid emulsion infused? Journal of pediatric gastroenterology and nutrition. 1995;21:25-30.

[4] Hojsak I, Kolacek S. Fat overload syndrome after the rapid infusion of SMOFlipid emulsion. JPEN Journal of parenteral and enteral nutrition. 2014;38:119-21.

[5] Lapillonne A, Fidler Mis N, Goulet O, van den Akker C, Wu J, Koletzko B, et al. ESPGHAN/ESPEN/ESPR guidelines on pediatric parenteral nutrition: Lipids. Clinical Nutrition. 2018.

[6] Koletzko B, Goulet O, Hunt J, Krohn K, Shamir R. 1. Guidelines on Paediatric Parenteral Nutrition of the European Society of Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) and the European Society for Clinical Nutrition and Metabolism (ESPEN), Supported by the European Society of Paediatric Research (ESPR). Journal of pediatric gastroenterology and nutrition. 2005;41 Suppl 2:S1-87.

[7] Calkins KL, Venick RS, Devaskar SU. Complications associated with parenteral nutrition in the neonate. Clinics in perinatology. 2014;41:331-45.

[8] Toce SS, Keenan WJ. Lipid intolerance in newborns is associated with hepatic dysfunction but not infection. Arch Pediatr Adolesc Med. 1995;149:1249-53.

[9] Holtrop P, Swails T, Riggs T. Hypertriglyceridemia in extremely low birth weight infants receiving lipid emulsions. Journal of neonatal-perinatal medicine. 2015;8:133-6.

[10] Keunen K, van Elburg RM, van Bel F, Benders MJNL. Impact of nutrition on brain development and its neuroprotective implications following preterm birth. Pediatric Research. 2015;77:148-55.

[11] Lee SM, Kim N, Namgung R, Park M, Park K, Jeon J. Prediction of Postnatal Growth Failure among Very Low Birth Weight Infants. Scientific Reports. 2018;8:3729.

[12] Vlaardingerbroek H, Vermeulen MJ, Rook D, van den Akker CH, Dorst K, Wattimena JL, et al. Safety and efficacy of early parenteral lipid and high-dose amino acid administration to very low birth weight infants. The Journal of pediatrics. 2013;163:638-44 e1-5.

[13] Drenckpohl D, McConnell C, Gaffney S, Niehaus M, Macwan KS. Randomized trial of very low birth weight infants receiving higher rates of infusion of intravenous fat emulsions during the first week of life. Pediatrics. 2008;122:743-51.

[14] Sinclair R, Schindler T, Lui K, Bolisetty S. Hypertriglyceridaemia in extremely preterm infants receiving parenteral lipid emulsions. BMC pediatrics. 2018;18:348.

[15] Lim MS, Choi CW, Kim BI, Yang HR. Clinical factors affecting lipid metabolism and optimal dose of heparin in preterm infants on parenteral nutrition. Pediatric gastroenterology, hepatology & nutrition. 2013;16:116-22.

[16] van der Lugt NM, Smits-Wintjens VE, van Zwieten PH, Walther FJ. Short and long term outcome of neonatal hyperglycemia in very preterm infants: a retrospective follow-up study. BMC pediatrics. 2010;10:52.

[17] Sinclair JC, Bottino M, Cowett RM. Interventions for prevention of neonatal hyperglycemia in very low birth weight infants. The Cochrane database of systematic reviews. 2011:CD007615.

[18] Tan MJ, Cooke RW. Improving head growth in very preterm infants--a randomised controlled trial I: neonatal outcomes. Archives of disease in childhood Fetal and neonatal edition. 2008;93:F337-41.

[19] Brandt I, Sticker EJ, Lentze MJ. Catch-up growth of head circumference of very low birth weight, small for gestational age preterm infants and mental development to adulthood. The Journal of pediatrics. 2003;142:463-8.

[20] Ibrahim HM, Jeroudi MA, Baier RJ, Dhanireddy R, Krouskop RW. Aggressive early total parental nutrition in low-birth-weight infants. Journal of perinatology : official journal of the California Perinatal Association. 2004;24:482-6.

[21] Vlaardingerbroek H, Veldhorst MA, Spronk S, van den Akker CH, van Goudoever JB. Parenteral lipid administration to very-low-birth-weight infants--early introduction of lipids and use of new lipid emulsions: a systematic review and meta-analysis. The American journal of clinical nutrition. 2012;96:255-68.

[22] Simmer K, Rao SC. Early introduction of lipids to parenterally-fed preterm infants. The Cochrane database of systematic reviews. 2005:CD005256.

Chapter 8

Does Intravenous Fish Oil Affect the Growth of Extremely Low Birth Weight Preterm Infants on Parenteral Nutrition?

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Summary

Background and aims: Long chain n-3 fatty acids (n-3 LCPUFA) play a pivotal role during central nervous system development and the provision of docosahexaenoic acid (DHA) is recommended for the preterm infant. However, there are concerns that oral fish oil, which is a good source of DHA, may adversely affect growth of preterm infants, as it decreases arachidonic acid (ARA). In recent years fish oil has been added to the fat blend of intravenous (IV) lipid emulsions (LE) but information on growth and other clinical outcomes of preterm infants is still scarce. We studied the effect of fish oil containing IV LE vs standard IV LE on growth in a large cohort of preterm infants who received routine parenteral nutrition (PN).

Methods: We retrospectively reviewed growth data of 546 preterm infants with a birth weight (BW)<1250g consecutively admitted to our NICU between Oct-2008 and Jun-2017 who received PN starting from the first day of life. Individual patients received only one of 5 commercially available IV LE. For the purpose of this study we grouped the patients who received the fish oil containing LE (IV-FO) and those who received conventional LE (CNTR). We compared PN and enteral nutrition (EN) intakes, and growth from birth to 36+0 weeks post-menstrual age (W PMA).

Results: Demographics, birth data and the incidence of the main complications of prematurity were similar between the two groups (IV-FO: n=240, Gestational age (GA) 197±16 d, BW 942±181 g; CNTR: n=237, GA 199±17 d, BW 960±197 g). No difference was found in PN and EN energy and macronutrient intakes from birth to 36+0W PMA, as well as in the proportion of human milk to infant milk formula. Weight gain from the regained BW to 36+0W PMA was slightly but significantly higher in IV-FO group: 17.3±2.8 and 16.8±2.7 g·kg-1·d-1, IV-FO and CNTR respectively (p=0.03). There was no difference in length gain and head growth nor in body size at 36+0W PMA between the two groups.

Conclusions: The use of IV fish oil did not negatively affect weight gain in a cohort of preterm infants. Large randomized controlled trials are needed to assess the effect of IV fish oil on the complication of prematurity and on selected domains of infant development.

Abbreviations: AA: amino acids; ARA: arachidonic acid; BW: birth weight; BPD: bronchopulmonary dysplasia; DHA: docosahexaenoic acid; ELBW: extremely low birth weight; EN: enteral nutrition; EOS: early onset sepsis; GA: gestational age; HC: head circumference; HM: human milk; IMF: infant milk formula; IQR: interquartile range; IV: intravenous; IVH: intraventricular hemorrhage; LE: lipid emulsion; L: length; LCPUFA: long chain polyunsaturated fatty acids; LOS: late onset sepsis; MCT: medium chain triglycerides; NEC: necrotizing enterocolitis; NICU: neonatal intensive care unit; NPE: non protein energy; PDA: patent ductus arteriosus; PMA: post menstrual age; PN: parenteral nutrition; PVL: periventricular leukomalacia; RCT: randomized controlled trial; RDS: respiratory distress syndrome; ROP: retinopathy of prematurity; SDS: standard deviation score; W: week; WT: weight.

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INTRODUCTION

Long-chain polyunsaturated fatty acids (LCPUFA), in particular docosahexaenoic acid (DHA) and arachidonic acid (ARA), are in high proportions in structural lipids of cell membranes and play a major role during central nervous system development. Their accretion primarily occurs during the third trimester of gestation, thus infants born prematurely are deprived of the trans-placental passage of these essential lipids (1). Human milk contains variable amounts of LCPUFA, depending on the mother's diet. Studies in preterm infants showed benefits of LCPUFA supplementation for retinal and cognitive development. Thus, the provision of DHA is now recommended for the preterm infant (2, 3).

There is still a concern that oral fish oil, which is often used as a source of DHA, could impair growth of preterm infants, as some studies in the early '90s showed that enteral supplementation of marine oil was associated with poorer growth than in control infants (4-9). The negative effect of oral fish oil on growth was attributed to the low plasma level of ARA of the marine oil supplemented infants (4). In the subsequent 20 years no new data became available to confirm or refute these findings, as a source of ARA was added to the fat blend together with fish oil to ensure an adequate omega 6/omega 3 LCPUFA ratio (2).

It has been about ten years since oil was added to the fat blend of the intravenous (IV) lipid emulsions (LE) used for parenteral nutrition (PN). Information on the impact of fish oil containing LE on growth and other clinical outcomes in preterm infants is still scarce. Our group showed that a fish oil containing LE in extremely low birth weight (ELBW) preterm infants receiving routine PN was associated with a statistically significant reduction not only of ARA but also of free cholesterol, cholesterol esters and phospholipids (10). In that study there was no difference in plasma triglycerides. In addition, a lipid lowering effect, mainly on plasma triglycerides, was reported in several adult studies where pharmaceutical doses of oral fish oil were given either for their lipid lowering effect and for the prevention of cardiovascular diseases (11). We recently studied cholesterol biosynthesis and lipogenesis in preterm infants on PN, randomized to receive 10% fish oil or a standard LE and found a marginal but measurable reduction of lipogenesis in the IV fish oil group in comparison with a control group who received a LE of similar fatty acid composition but without fish oil (12). In our view, if reducing plasma triglycerides/lipids in adults seems to be desirable, the same may not be true in the case of the small preterm infant. As plasma lipids probably reflect the trafficking/availability of the fatty acids, including essential LCPUFA, to the growing organs, the safety of any plasma lipid reducing intervention should be tested in follow up studies, including respiratory outcomes/bronchopulmonary dysplasia and brain growth/neurodevelopment.

A recent study, the N3RO trial, that aimed at giving to preterm infants enteral DHA at 60 mg/kg/day starting within 3 days of first enteral feed, showed an increased risk of bronchopulmonary dysplasia in the intervention group; this finding raised a renewed concern about the possible negative effect of marine oils in preterm infants (13).

To date, only few studies about the use of IV fish oil for the PN of the preterm infant reported growth data, mainly as secondary outcomes. A recent meta-analysis did not observe significant differences in growth between fish oil LE and controls, but evidence was too low grade to draw firm conclusions (14).

The aim of this study was to retrospectively review the growth of a rather large cohort of preterm infants on PN with different LE, with and without fish oil.

MATERIALS AND METHODS

Study design and participants

We retrospectively reviewed data on preterm infants admitted to the neonatal intensive care unit (NICU) of “G. Salesi” Children's Hospital, Ancona, Italy, between October 2008 and June 2017. Neonates with a birth weight (BW) of 400-1249 g and a gestational age (GA) at birth of at least 24+0 weeks (W) of postmenstrual age (PMA) and below 36+0 W PMA, in-born or out-born admitted before 24 hours of age, who routinely received PN from the first day of life, were evaluated in this study. Individual patients received only one of the 5 LE used, sometimes as part of clinical trials (RCT) conducted during this ten year period in the NICU, with randomization according to the trial protocol presented previously (12, 15, 16). Patients not enrolled in trials received nevertheless one of the 5 LE routinely acquired by the hospital pharmacy and assigned at birth by the neonatologist according to the pharmacy availability (more than one LE is always available at the hospital pharmacy). LE were: 1=MSF (40:50 medium chain triglycerides-MCT: Soybean oil, 10% fish oil-FO; Lipidem®,, B Braun), 2=MOSF (30:30:25 MCT:Soybean oil:olive oil, 15% FO; SMOFlipid®,, Fresenius Kabi), 3=S (100% soybean oil; Intralipid®, Fresenius Kabi), 4=MS (50%MCT and 50%Soybean oil; Lipofundin MCT®,, B Braun) and 5=OS (80% olive oil and 20% Soybean oil; Clinoleic®,, Baxter spa). PN was an individualized all-in-one mixture for all the preterm infants evaluated in this study and the PN bags containing the study LE were of the same size and were of identical appearance. Caregivers involved with data collection were unaware of the LE assignment. To the purpose of this study we grouped the patients who received the fish oil containing LE MSF and MOSF (IV-FO) and those without fish oil S, MS and OS (CNTR).

Exclusion criteria were severe malformations, inborn errors of metabolism, death in the first day of life without receiving PN, neonatal transfer in another NICU before 24 hours of life and start of PN after 24 hours of life. This retrospective study was approved by the local ethics committee (Prot. N. 2017 0503 OR; Det. N. 145/DG).

Nutrition Protocol Management

Patients started PN with glucose, amino acids, and lipids in the first day of life, according to the NICU Nutrition Schemes. The Nutrition Protocols of the different RCT since 2008 onward and Nutrition Schemes used in the NICU as guidelines for ELBW infants had similar daily progressive intakes. LE were infused at dose of 1-1.5 g·kg-1·d-1on the first day of life up to 2.5-3.5 g·kg-1·d-1 on the 5th day of life. Glucose was increased from 6-8 g·kg-1·d-1 to 12-14 g·kg-1·d-1 from day 1 to day 5. Amino acids were started at dose of 1-1.5 g·kg-1·d-1 in the first day of life and increased up to 2.5-3.5 g·kg-1·d-1on the 5th day of life. Maximum lipid, amino acid and glucose intakes achieved were then kept constant from day 5 to day 7. Minimal enteral feeding with human milk or infant milk formula was provided from day 0 to day 7, the maximum amount supplied being 8 mL·kg-1·d-1 from day 1 to day 4, and 16 mL·kg-1·d-1 from day 5 to day 7. For each infant after day 7, PN was tapered and stopped at a median age of 18 day of life while oral feeding was gradually increased to reach full feed and keep a maximum total fluid intake of 160 ml·kg-1·d-1.

Data collection and analysis

Detailed information on nutrition and growth was prospectively recorded daily with complete records of amount and type of enteral nutrition and PN, using a dedicated software (Neotools; Interactive, Milan, Italy). Body weight (WT) was measured daily using a digital infant scale; length (L) and head circumference (HC) were measured at birth and weekly thereafter using a neonatal stadiometer and a flexible non-stretchable tape respectively.

Standard deviation scores (SDS) were computed using Italian growth charts. WT gain (g·kg-1·d-1) was calculated from birth to 36+0W PMA and from the regained birth WT to 36+0W PMA (mean of the day-by-day WT gain expressed in g·kg-1·d-1 of each study patient). Moreover, we used an additional method to calculate patient's growth. The WT gain (g·d-1) from the 10th day of life (that is the estimated mean time to the regained BW in our cohort) until 36+0W PMA, the L gain (cm/w) and HC gain (cm/w) from birth and from the 5th day of life until 36+0W PMA were calculated by using a simple linear regression fitting model. The major diagnosis/complications of prematurity were defined according to the Vermont-Oxford definitions, and the physiological definition of bronchopulmonary dysplasia was used according to Walsh et al (17). Cholestasis was defined as plasma direct bilirubin concentration above 1 mg/dl.

Sample size was calculated according to data available about WT gain from the regained BW to 36+0W PMA in our cohort: patients admitted to our NICU who received PN with a conventional LE had a WT gain of 17.2 ± 3.02 g·kg-1·d-1. According to our database, about 550 ELBW patients were admitted to our unit in the last 10 years. Assuming a patient drop of about one third after checking for inclusion/exclusion criteria, and same variance in WT gain in both study patients and controls, we estimated a sample of 200 patients per group to detect a difference of at least 1 g·kg-1·d-1 with a 90% power at a significance level of 0.05.

Depending on the distribution, data were expressed as group means ± SD, as the median (interquartile range [IQR]) or as a number (percentage). The clinical characteristics of the two groups were compared using the Student's t-test, Mann-Whitney test or $\chi 2$ test as appropriate. Significance was set at $p<0.05$.

All statistical analyses were performed using SPSS (v 23.0; SPSS Inc, Chicago, Illinois) and Microsoft EXCEL (v 2016; Microsoft Corp, Redmond, Washington) software.

RESULTS

From October 2008 to June 2017 all the records of 546 consecutively preterm infants admitted to the neonatal intensive care unit were screened for this study. Sixty-nine patients were excluded because they did not meet inclusion criteria: 18 patients because admitted after 24 hours of life, 27 patients because of congenital malformations, 10 patients who died in the first day of life (without receiving PN), one patient transferred before 24 hours of life because of shortage of beds and 13 patients in whom PN was started after 24 hours of life. Four hundred seventy-seven preterm infants were studied of whom 240 received one of the two fish oil containing LE (IV-FO group) and 237 received one of the three conventional LE (CNTR group). Thirty-seven patients died before 36+0W PMA (IV-FO n=19, CNTR n=18). Four hundred forty patients were finally analyzed for the assessment of growth, nutritional data and outcomes at 36+0W PMA, 221 in the IV-FO group (MSF n=131, MOSF n=90) and 219 in the CNTR group (MS n=163, OS n=21, S n=35).

Anthropometry at birth was similar between the two groups. A slight but significant difference in prenatal exposure to steroids was found, with a greater proportion of patients in the IV-FO group that received antenatal betamethasone (**Table 1**).

Table 1. Demographics and clinical characteristics at birth.

	IV-FO (n=240)[a]	CNTR (n=237)[b]	p
M/F– no. (%)	121/119 (50/50)	110/127 (46/54)	0.4
GA (d w)	28 ± 2	28 ± 2	0.5
BW (g)	942 ± 181	960 ± 197	0.3
Z- score	-0.7 ± 1.0	-0.7 ± 1.1	0.8
Birth length (cm)	35.3 ± 2.8	35.5 ± 2.8	0.4
Z-score	-0.7 ± 1.0	-0.6 ± 1.0	0.7
Birth Head Circ. (cm)	25.2 ± 1.9	25.3 ± 1.9	0.6
Z-score	-0.5 ± 1.0	-0.4 ± 1.1	0.9
SGA 10p – no. (%)	93 (39)	91 (38)	0.9
SGA 2SDS – no. (%)	29 (12)	35 (15)	0.4
Exposure to antenatal glucocorticoids-no. (%)	227 (95)	212 (90)	0.04
APGAR at 5 minutes-median (IQR)	8 (7-9)	8 (7-9)	0.5

[a]MSF (Lipidem®; B Braun): 50% medium chain triglycerides(MCT), 40% soybean oil (SO), and 10% fish oil; MOSF (SMOFlipid®; Fresenius Kabi): 30:30:25 MCT:Soybean oil:olive oil, 15% FO.
[b]MS (Lipofundin MCT®; B Braun): 50% MCT and 50% SO; S (Intralipid®; Fresenius Kabi): 100% soybean oil; OS (Clinoleic®; Baxter spa): 80% olive oil and 20% Soybean oil.
Data are expressed as Mean ± SD, unless otherwise indicated, $p<0.05$ (Student's t-test, Mann-Whitney test or $\chi 2$ test, as appropriate).
GA: gestational age; BW: birth weight; SDS: standard deviation score; SGA: small for gestational age.

No significant differences were found in the incidence of the main complications of prematurity recorded from birth to 36+0W PMA (**Table 2**).
We found no differences in parenteral and enteral nutrition intakes between the two study groups from birth to 36+0W PMA. The proportion of human milk to the total enteral milk volume was also not different (**Table 3**).
The two groups were also similar in the incidence of surfactant therapy after birth (52% vs 54% in IV-FO and CNTR, p=0.7), number of days of respiratory support (32 d [5-59] vs 31 d [6-54] in IV-FO and CNTR, p=0.7) or duration of oxygen therapy (363 hours [3-1336] vs 301 hours [9-1189] in IV-FO and CNTR, p=0.8) from birth to 36+0W PMA.
There were no differences in WT loss, postnatal age at WT nadir and time to regain BW. Anthropometry at 36+0W PMA was not significantly different between the two study groups (**Table 4**). We found a statistically higher WT gain from birth to 36+0W PMA and from the regained BW to 36+0W PMA and a significant higher WT-SDS gain from birth to 36+0W PMA in the IV-FO group (**Table 4**). No differences were found in the absolute difference of gains of the other main anthropometric data. Growth calculated by a linear fitting model was not different between the two study groups: WT gain (10 days of life to 36+0W PMA): 23.3 ± 5.8 vs 22.4 ± 5.2 g·d-1 (p=0.1); L gain (birth to 36+0W PMA): 1.1 ± 0.4 vs 1.1 ± 0.4 c/week (p=0.7); HC gain (5 days of life to 36+0W PMA): 0.9 ± 0.2 vs 0.8 ± 0.2 (p=0.2), IV-FO group and CNTR respectively.

Table 2. Neonatal outcomes until 36+0 weeks postmenstrual age.

	IV-FO (n=221)[a]	CNTR (n=219)[b]	p
RDS (%)	174 (79)	185 (85)	0.1
PDA (%)	131 (59)	127 (58)	0.8
NEC≥ 2 (%)	9 (4)	10 (5)	0.8
IVH ≥ 3 (%)	14 (6)	21 (10)	0.2
Cystic PVL (%)	6 (3)	4 (2)	0.5
ROP ≥ 3 (%)	0 (0)	1 (1)	0.5
BPD (%)	59 (27)	45 (21)	0.1
EOS (%)	8 (4)	7 (3)	0.8
LOS (%)	43 (20)	36 (16)	0.4
Cholestasis (%)	31 (14)	31 (14)	1.0

[a]MSF (Lipidem®; B Braun): 50% medium chain triglycerides(MCT), 40% soybean oil (SO), and 10% fish oil; MOSF (SMOFlipid®; Fresenius Kabi): 30:30:25 MCT:Soybean oil:olive oil, 15% FO.
[b]MS (Lipofundin MCT®; B Braun): 50% MCT and 50% SO; S (Intralipid®; Fresenius Kabi): 100% soybean oil; OS (Clinoleic®; Baxter spa): 80% olive oil and 20% Soybean oil.
Data are expressed as no. (%), $p<0.05$ ($\chi 2$ test).
RDS: respiratory distress syndrome; PDA: patent ductus arteriosus; NEC: necrotizing enterocolitis; IVH: intraventricular hemorrhage; PVL: periventricular leukomalacia; ROP: retinopathy of prematurity; BPD: bronchopulmonary dysplasia; EOS: early onset sepsis; LOS: late onset sepsis.

Table 3. Cumulative and mean daily nutritional intakes until 36+0 weeks postmenstrual age.

	IV-FO (n=221)[a]	CNTR (n=219)[b]	p
PNCum AA (g/kg)	37 (32-49)	37 (32-48)	0.8
PNCum NPE (Kcal/kg)	768 (671-1020)	768 (669-980)	0.8
ENCum Protein (g/kg)	149 (97-190)	148 (100-188)	0.9
ENCum NPE (Kcal/kg)	4435 (3129-5567)	4354 (3189-5571)	0.8
FluidsCum (ml/kg)	8611 (6514-9968)	8195 (6267-9905)	0.4
HMCum (ml/kg)	4377 (1946-6235)	4132 (2071-6280)	0.8
IMFCum (ml/kg)	1442 (238-3858)	1407 (141-4387)	0.9

continued

	IV-FO (n=221)[a]	CNTR (n=219)[b]	p
PN AA (g/kg/d)	2.1 ± 0.4	2.1 ± 0.3	0.4
PN NPE (Kcal/kg/d)	45 ± 5.6	45 ± 5.5	0.8
EN Protein (g/kg/d)	2.8 ± 0.7	2.8 ± 0.6	0.8
EN NPE (Kcal/kg/d)	83 ± 17	83 ± 15	0.9
Fluids (ml/kg/d)	154 ± 8	154 ± 9	0.2

[a]MSF (Lipidem®; B Braun): 50% medium chain triglycerides(MCT), 40% soybean oil (SO), and 10% fish oil; MOSF (SMOFlipid®; Fresenius Kabi): 30:30:25 MCT:Soybean oil:olive oil, 15% FO. [b]MS (Lipofundin MCT®; B Braun): 50% MCT and 50% SO; S (Intralipid®; Fresenius Kabi): 100% soybean oil; OS (Clinoleic®; Baxter spa): 80% olive oil and 20% Soybean oil.
Data are expressed as Median (IQR) or mean (SD), $p<0.05$ (Mann-Whitney or Student t-test as appropriate). PNCum:parenteral nutrition cumulative intakes from birth to 36+0W PMA ; ENCum: enteral nutrition cumulative intakes from birth to 36+0W PMA; AA: amino acids; NPE: non-protein energy; HM: human milk; IMF: infant milk formula.

Table 4. Anthropometry and growth until 36+0 weeks postmenstrual age.

	IV-FO (n=221)[a]	CNTR (n=219)[b]	p
WT nadir (g)	827 ± 173	839 ± 181	0.5
Age at nadir (d)	4.4 ± 2.1	4.1 ± 1.6	0.1
WT loss at nadir (%)	12.3 ± 5.4	12.6 ± 5.5	0.6
Time to regain BW (d)	11.8 ± 5.1	11.9 ± 5.0	0.9
WT at 36+0W PMA (g)	1912 ± 367	1870 ± 351	0.2
Z- score	-1.8 ± 0.9	-1.9 ± 0.9	0.2
L at 36+0W PMA (cm)	42.5 ± 2.6	42.7 ± 2.4	0.4
Z-score	-1.9 ± 1.0	-1.8 ± 0.9	0.3
HC at 36+0W PMA (cm)	30.6 ± 1.5	30.4 ± 1.5	0.4
Z-score	-1.6 ± 1.1	-1.6 ± 1.0	0.5
WT gain (Birth-36+0W PMA) (g·kg-1·d-1)	16.0 ± 2.7	15.5 ± 2.7	0.03
WT gain (BW recovery-36+0W PMA) (g·kg-1·d-1)	17.3 ± 2.8	16.8 ± 2.7	0.03
Δ WT (Birth-36+0WPMA)	970 ± 353	910 ± 341	0.07
Δ L (Birth-36+0WPMA)	7.2 ± 2.9	7.2 ± 2.9	0.9

continued.

	IV-FO (n=221)[a]	CNTR (n=219)[b]	p
Δ HC (Birth-36+0WPMA)	5.3 ± 2.0	5.2 ± 2.0	0.3
Δ WT SDS (Birth-36+0WPMA)	-1.0 ± 0.6	-1.2 ± 0.6	0.02
Δ L SDS (Birth-36+0WPMA)	-1.3 ± 0.8	-1.2 ± 0.8	0.4
Δ HC SDS (Birth-36+0WPMA)	-1.1 ± 1.0	-1.2 ± 1.0	0.5
WT gain (1W) (g·kg-1·d-1)	-17.4 ± 13.5	-19.1 ± 13.8	0.2
WT gain (2W) (g·kg-1·d-1)	16.1 ± 10.2	16.6 ± 9.5	0.5
WT gain (3W) (g·kg-1·d-1)	15.2 ± 9.3	15.3 ± 8.7	0.9
WT gain (4W) (g·kg-1·d-1)	17.1 ± 8.3	16.6 ± 8.5	0.5

[a]MSF (Lipidem®; B Braun): 50% medium chain triglycerides(MCT), 40% soybean oil (SO), and 10% fish oil; MOSF (SMOFlipid®; Fresenius Kabi): 30:30:25 MCT:Soybean oil:olive oil,15% FO. [b]MS (Lipofundin MCT®; B Braun): 50% MCT and 50% SO; S (Intralipid®; Fresenius Kabi): 100% soybean oil; OS (Clinoleic®; Baxter spa): 80% olive oil and 20% Soybean oil. Data are expressed as Mean ± SD, $p<0.05$ (Student's t-test).
WT: weight; L: length; HC: head circumference; Δ WT (Birth-36+0WPMA): difference in weight between birth and 36 weeks postmenstrual age (g); Δ L (Birth-36+0WPMA): difference in length between birth and 36 weeks postmenstrual age (cm); Δ HC (Birth-36+0WPMA): difference in head circumference between birth and 36 weeks postmenstrual age (cm); Δ WT SDS (Birth-36WPMA): difference in SDS of weight between birth and 36 weeks postmenstrual age; Δ L SDS (Birth-36WPMA): difference in SDS of length between birth and 36 weeks postmenstrual age; Δ HC SDS (Birth-36WPMA): difference in SDS of head circumference between birth and 36 weeks postmenstrual age.

DISCUSSION

To our knowledge this is the first report on growth in a rather large cohort of preterm infants who received routine PN from the first day of life. We found that the use of LE containing 10 to 15% of fish oil as part of the routine PN of ELBW infants during the first 3 weeks of life was associated with a slightly but significantly higher WT gain until 36+0W PMA than controls. There was no difference in body size at 36+0W PMA. In addition we found no difference in the main complications of prematurity.
To date, data about IV fish oil and growth of preterm infants are scanty and not conclusive. Six randomized controlled trials compared MOSF (containing 15% fish oil) versus a conventional 100% SO based LE (S) (15, 16, 18-21). Two study reported data comparing a 10% fish oil LE (MSF) with SO LE (10, 16) and one study compared MOSF patients versus an olive oil containing LE (OS) group (22). Growth was a secondary outcome in all these studies, and none of them was powered to detect differences in growth.
MOSF and S were compared in three studies with no differences in growth, neither at the end of PN nor at hospital discharge. Of note, in two of these studies lipids were started at a variable length after birth (from the first four to seven days of life), the lipid infusion time ranged from 18 to 20 hours per day and the target dose was reached after several days from the PN start (18, 20). The study by Skouroliakou compared 14 study infants versus 18 controls and was clearly underpowered to detect growth differences (19).

D'Ascenzo et al. found a significantly greater postnatal WT loss and longer time to regain BW with MOSF LE in comparison with S (14.3±5.8% vs 11.1±5.7% and 13.4±5.6 d vs 10.5±5.1 d, MOSF and S groups respectively). WT gain until 36+0 W PMA was similar between the two groups (17.1 g·kg-1·d-1 vs 16.6 g·kg-1·d-1, MOSF and S groups respectively) (15). Savini et al. described weekly growth rates in 174 infants with clinical characteristics similar to the present study and found no differences between MOSF and S, MSF and S and MSF and MS (16). WT gain was 3.1 g·d-1 higher and HC z-score gain was 0.6 greater (both statistically significant) in 48 patients who received MOSF in comparison with 48 controls who received S LE in the study by Vlaardingerbroek et al (21).

There is only one study comparing MSF and MS; in this study the author states that no differences in growth were found, but no data were provided (10). Najm et al. in 2017 compared MOSF versus an olive oil containing LE (OS) in ELBW infants. IV lipids were started from the first day of life with daily increments up to 2 g·kg-1·d-1. Growth was not different between the study arms (22).

In the present study we report data from a rather homogenous population of preterm infants with a BW of less than 1250 g who all received routine PN starting in the first day of life. Demographics and clinical characteristics at birth were rather similar between the two groups, except for a statistically higher proportion of antenatal steroids treatment in the IV-FO group. We interpret this as a chance finding due to multiple comparisons. In addition, albeit statistically significant, the difference in our opinion is likely to be biologically negligible. Early postnatal growth parameters (first three weeks of life, Table 4) were not different between the two groups. However, WT gain until 36+0W PMA was significantly higher in the IV-FO group. Because of the study design, we are unable to answer the question if this difference should be attributed to PN, to enteral nutrition or both. The nutrient intake data reported in Table 3 clearly show that macronutrient and energy supplies were not different between the two groups, neither during PN nor during enteral nutrition. The two study groups did not differ also for the proportion of human milk to infant milk formula received. We are therefore unable to provide a solid interpretation for the statistically higher WT gain in the study group. In our view the higher WT gain in the IV-FO group although statistically significant, is biologically negligible; this view is also supported by the fact that we found no difference in length gain and in head growth. Moreover, complications of prematurity were not different in the two groups. These findings all together are in our view somewhat reassuring, as we feared (see introduction section) a potential negative effect of fish oil on the growth of preterm infants.

Even if this was not a randomized controlled trial, the large cohort of preterm infants we analyzed, the standardized nutritional protocols used during the period in our NICU, and the homogeneity of the preterm population admitted, confer some degree of strength to our results and we believe selection bias if any was negligible. Moreover, growth data are slightly in favor of the IV-FO group, so that we can reasonably say that the use of 10 to 15% fish oil containing LE does not negatively affect short-term growth of ELBW preterm infants on PN.

In a study by Groh-Wargo et al., preterm infants fed a DHA and ARA supplemented formula from the first enteral feed to 12 months corrected age had greater lean body mass and reduced fat mass, with no differences in overall WT in comparison to controls (23). This finding suggests a possible effect of LCPUFA supplementation on body composition, because of the net effect on hepatic and muscular cells metabolism. We therefore cannot exclude that even if anthropometry and growth were similar in patients who received IV fish oil and in controls, body composition could have been different.

In conclusion, this large retrospective study showed that administration of IV fish oil did not negatively affect short-term growth in preterm infants on PN, and add data about safety of its use in preterm infants. However, data are still of low grade evidence and no information is available about IV fish oil effects on the growth and development of selected organs, on body composition and on neurodevelopmental outcome. Further larger RCT are warranted to clarify these important clues of preterm infant care.

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STATEMENT OF AUTHORSHIP

Contribution of each author:

- VPC was responsible for the design of the study;
- CB, RD, and MPB contributed to data collection and analysis;
- AC and AP contributed to subject recruitment and data collection;
- PEC, PM and LA were in charge of the statistical analysis.

All authors read and approved the final manuscript.

CONFLICT OF INTEREST STATEMENT AND FUNDING SOURCES

There are no conflicts of interest to declare.

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REFERENCES

1.Clandinin MT, Chappell JE, Leong S, Heim T, Swyer PR, Chance GW. Intrauterine fatty acid accretion rates in human brain: implications for fatty acid requirements. Early Hum Dev. 1980 Jun;4(2):121-9. PubMed PMID: 7408742. Epub 1980/06/01. eng.

2.Agostoni C, Buonocore G, Carnielli VP, De Curtis M, Darmaun D, Decsi T, et al. Enteral nutrient supply for preterm infants: commentary from the European Society of Paediatric Gastroenterology, Hepatology and Nutrition Committee on Nutrition. J Pediatr Gastroenterol Nutr. Jan;50(1):85-91. PubMed PMID: 19881390.

3.Lapillonne A, Groh-Wargo S, Gonzalez CH, Uauy R. Lipid needs of preterm infants: updated recommendations. J Pediatr. 2013 Mar;162(3 Suppl):S37-47. PubMed PMID: 23445847. Epub 2013/03/06. eng.

4.Carlson SE, Cooke RJ, Werkman SH, Tolley EA. First year growth of preterm infants fed standard compared to marine oil n-3 supplemented formula. Lipids. 1992 Nov;27(11):901-7. PubMed PMID: 1491608. Epub 1992/11/01. eng.

5.Carlson SE, Werkman SH, Tolley EA. Effect of long-chain n-3 fatty acid supplementation on visual acuity and growth of preterm infants with and without bronchopulmonary dysplasia. Am J Clin Nutr. 1996 May;63(5):687-97. PubMed PMID: 8615350. Epub 1996/05/01. eng.

6.Fewtrell MS, Morley R, Abbott RA, Singhal A, Isaacs EB, Stephenson T, et al. Double-blind, randomized trial of long-chain polyunsaturated fatty acid supplementation in formula fed to preterm infants. Pediatrics. 2002 Jul;110(1 Pt 1):73-82. PubMed PMID: 12093949. Epub 2002/07/03. eng.

7.O'Connor DL, Hall R, Adamkin D, Auestad N, Castillo M, Connor WE, et al. Growth and development in preterm infants fed long-chain polyunsaturated fatty acids: a prospective, randomized controlled trial. Pediatrics. 2001 Aug;108(2):359-71. PubMed PMID: 11483801. Epub 2001/08/03. eng.

8.Ryan AS, Montalto MB, Groh-Wargo S, Mimouni F, Sentipal-Walerius J, Doyle J, et al. Effect of DHA-containing formula on growth of preterm infants to 59 weeks postmenstrual age. American journal of human biology : the official journal of the Human Biology Council. 1999;11(4):457-67. PubMed PMID: 11533965. Epub 2001/09/05. eng.

9.Vanderhoof J, Gross S, Hegyi T. A multicenter long-term safety and efficacy trial of preterm formula supplemented with long-chain polyunsaturated fatty acids. J Pediatr Gastroenterol Nutr. 2000 Aug;31(2):121-7. PubMed PMID: 10941962. Epub 2000/08/15. eng.

10.D'Ascenzo R, D'Egidio S, Angelini L, Bellagamba MP, Manna M, Pompilio A, et al. Parenteral nutrition of preterm infants with a lipid emulsion containing 10% fish oil: effect on plasma lipids and long-chain polyunsaturated fatty acids. J Pediatr. 2011 Jul;159(1):33-8 e1. PubMed PMID: 21362575. Epub 2011/03/03. eng.

11.Siscovick DS, Barringer TA, Fretts AM, Wu JH, Lichtenstein AH, Costello RB, et al. Omega-3 Polyunsaturated Fatty Acid (Fish Oil) Supplementation and the Prevention of Clinical Cardiovascular Disease: A Science Advisory From the American Heart Association. Circulation. 2017 Apr 11;135(15):e867-e84. PubMed PMID: 28289069.

12.Biagetti C, Vedovelli L, Savini S, Simonato M, D'Ascenzo R, Pompilio A, et al. Double blind exploratory study on de novo lipogenesis in preterm infants on parenteral nutrition with a lipid emulsion containing 10% fish oil. Clin Nutr. 2016 Apr;35(2):337-43. PubMed PMID: 25912232. Epub 2015/04/29. eng.

13.Collins CT, Makrides M, McPhee AJ, Sullivan TR, Davis PG, Thio M, et al. Docosahexaenoic Acid and Bronchopulmonary Dysplasia in Preterm Infants. The New England journal of medicine. 2017 Mar 30;376(13):1245-55. PubMed PMID: 28355511. Epub 2017/03/30. eng.

14.Kapoor V, Glover R, Malviya MN. Alternative lipid emulsions versus pure soy oil based lipid emulsions for parenterally fed preterm infants. Cochrane Database Syst Rev. 2015 Dec 02(12):CD009172. PubMed PMID: 26630252. Epub 2015/12/03. eng.

15.D'Ascenzo R, Savini S, Biagetti C, Bellagamba MP, Marchionni P, Pompilio A, et al. Higher docosahexaenoic acid, lower arachidonic acid and reduced lipid tolerance with high doses of a lipid emulsion containing 15% fish oil: a randomized clinical trial. Clin Nutr. 2014 Dec;33(6):1002-9. PubMed PMID: 24525115. Epub 2014/02/15. eng.

16.Savini S, D'Ascenzo R, Biagetti C, Serpentini G, Pompilio A, Bartoli A, et al. The effect of 5 intravenous lipid emulsions on plasma phytosterols in preterm infants receiving parenteral nutrition: a randomized clinical trial. Am J Clin Nutr. 2013 Aug;98(2):312-8. PubMed PMID: 23761482. Epub 2013/06/14. eng.

17.Walsh MC, Wilson-Costello D, Zadell A, Newman N, Fanaroff A. Safety, reliability, and validity of a physiologic definition of bronchopulmonary dysplasia. J Perinatol. 2003 Sep;23(6):451-6. PubMed PMID: 13679930.

18.Rayyan M, Devlieger H, Jochum F, Allegaert K. Short-term use of parenteral nutrition with a lipid emulsion containing a mixture of soybean oil, olive oil, medium-chain triglycerides, and fish oil: a randomized double-blind study in preterm infants. JPEN J Parenter Enteral Nutr. 2012 Jan;36(1 Suppl):81S-94S. PubMed PMID: 22237883. Pubmed Central PMCID: 3332303. Epub 2012/01/18. eng.

19.Skouroliakou M, Konstantinou D, Koutri K, Kakavelaki C, Stathopoulou M, Antoniadi M, et al. A double-blind, randomized clinical trial of the effect of omega-3 fatty acids on the oxidative stress of preterm neonates fed through parenteral nutrition. Eur J Clin Nutr. 2010 Sep;64(9):940-7. PubMed PMID: 20551967. Epub 2010/06/17. eng.

20.Tomsits E, Pataki M, Tolgyesi A, Fekete G, Rischak K, Szollar L. Safety and efficacy of a lipid emulsion containing a mixture of soybean oil, medium-chain triglycerides, olive oil, and fish oil: a randomised, double-blind clinical trial in premature infants requiring parenteral nutrition. J Pediatr Gastroenterol Nutr. 2010 Oct;51(4):514-21. PubMed PMID: 20531018. Epub 2010/06/10. eng.

21.Vlaardingerbroek H, Vermeulen MJ, Carnielli VP, Vaz FM, van den Akker CH, van Goudoever JB. Growth and fatty acid profiles of VLBW infants receiving a multicomponent lipid emulsion from birth. J Pediatr Gastroenterol Nutr. 2014 Apr;58(4):417-27. PubMed PMID: 24667866. Epub 2014/03/29. eng.

22.Najm S, Lofqvist C, Hellgren G, Engstrom E, Lundgren P, Hard AL, et al. Effects of a lipid emulsion containing fish oil on polyunsaturated fatty acid profiles, growth and morbidities in extremely premature infants: A randomized controlled trial. Clinical nutrition ESPEN. 2017 Aug;20:17-23. PubMed PMID: 29072164. Pubmed Central PMCID: 5784264.

23.Groh-Wargo S, Jacobs J, Auestad N, O'Connor DL, Moore JJ, Lerner E. Body composition in preterm infants who are fed long-chain polyunsaturated fatty acids: a prospective, randomized, controlled trial. Pediatr Res. 2005 May;57(5 Pt 1):712-8. PubMed PMID: 15718356. Epub 2005/02/19. eng.

Chapter 9

Directions for Future Research

We are interested in studying the effect of FO on phytosterol metabolism in infants receiving new generation lipid emulsions. We are measuring plasma phytosterols in patients who received Omegaven® due to parenteral nutrition associated cholestasis (conjugated bilirubin concentration> 2.0 mg/dl). Our preliminary data support the view that intravenous FO have a positive effect on liver metabolism in infants with severe cholestasis. The biochemical process involves in this preliminary clinical finding is also the subject of our ongoing study.

Phytosterol accumulation in the neonatal tissues is an important point of future research projects. Previous studies found a phytosterol accumulation in tissues of infants receiving vegetable oil-infant formulas. We would measure the phytosterol content in tissues of infant on parenteral nutrition with vegetable oils. At present, no data is available on the effect of phytosterol accumulation in brain and lung. An excessive accumulation of phytosterols during the neonatal period may potentially expose this group of patients to adverse outcomes in adulthood.

According to our data (*Chapter 8*), intravenous FO did not have a relevant role in improving growth in preterm infants on routine parenteral nutrition. However, we are interested in evaluating the effect of FO on the growth of selected organs, such as lung. By using a sensible non-invasive parameter, SFR (saturation to FiO_2 ratio) as indicator of lung growth and function, we are comparing infants who received or not FO during the hospital stay.

Understanding parenteral lipid management to avoid metabolic complications, such as hyperglycaemia, elevated urea, etc, potentially associated with worst growth and developmental outcomes is also a topic of interest. We are working on retrospective nutritional data to set-up better feeding protocols to reduce metabolic complications and other adverse outcomes. Preliminary data-analyses suggest that metabolic complications are strongly associated to neonatal diseases and slightly to characteristics at birth (i.e. birth weight, gestational age, etc) as generally considered.

Chapter 10

Summary

This thesis describes a number of investigations on lipid metabolism in enterally and parenterally fed infants. Both our experimental and clinical studies increased the knowledge of lipid handling in these critically ill patients. Phytosterols, fish oil and handling of metabolic complications on parenteral nutrition are topics of interest in Neonatology.

Chapter 1 introduces the importance of lipids in neonatal nutrition. Enteral and parenteral sources of lipids were extensively discussed. A special focus was placed on what is known about plant-derived sterols: phytosterols.

The investigations in this thesis were performed to:

1. study the phytosterol metabolism in preterm infants receiving vegetable oil-based intravenous lipid emulsions and infant formulas (*Chapters 2 - 6*).
2. evaluate the factors associated to hypertriglyceridemia and its impact on the daily macronutrient (lipids, amino acids and carbohydrates) intakes in preterm infants on routine parenteral nutrition (*Chapter 7*).
3. study the growth of preterm infants on parenteral nutrition with FO containing lipid emulsions (*Chapter 8*).

Chapter 2 characterizes the maternal-fetal gradient of free and esterified phytosterols. Fatty acids, cholesterol, cholesterol metabolites and phytosterols were measured in both maternal and cord blood at the time of delivery. Our data suggest that the human placenta tends to limit the phytosterol availability to the foetus, free phytosterols cross the placenta more easily than phytosterol esters.

Chapter 3 describes the phytosterol and cholesterol esterification in preterm and term infants and in human adults on parenteral nutrition. We demonstrated that plasma free phytosterols were esterified to a lesser extent than cholesterol in preterm and term infants and in human adults, and this phenomenon was independent from the intravenous lipid intake.

Chapter 4 reports the plasma phytosterol half-life in very-low-birth-weight preterm infants receiving parenteral with vegetable oil-based lipid emulsions. We demonstrated that all phytosterols were eliminated very slowly after the stop of intravenous lipid emulsions

Chapter 5 describes the plasma phytosterol half-life in preterm infants on routine parenteral nutrition who developed cholestasis. Preterm infants with cholestasis had higher plasma phytosterol half-lifes than patients without cholestasis.

Chapter 6 reports preliminary data on plasma and dietary phytosterols in preterm and term infants fed with human milk and infant milk formulas.

Chapter 7 describes the modifications of intravenous lipids, amino acids, carbohydrates and non-protein energy intakes in infants with a birth weight less than 1250 g on routine parenteral who developed hypertriglyceridemia. In a case-control study, we did not find any association between hypertriglyceridemia and reduced growth and poorer neurodevelopment in the study infants.

Chapter 8 describes the effect of fish oil containing intravenous lipid emulsions vs standard intravenous lipid emulsions on growth in a large cohort of preterm infants who received routine parenteral nutrition. We found that the use of intravenous fish oil did not negatively affect weight gain in a cohort of preterm infants.

Future directions of research are proposed in *Chapter 9.*

FULL-TEXT JOURNAL ARTICLES

1: Nobile S, Marchionni P, Gidiucci C, Correani A, Palazzi ML, Spagnoli C, Rondina C; Marche Neonatal Network, Carnielli VP. Oxygen saturation/FIO2 ratio at 36 weeks' PMA in 1005 preterm infants: Effect of gestational age and early respiratory disease patterns. Pediatr Pulmonol. 2019 Jan 27. doi: 10.1002/ppul.24265. [Epub ahead of print] PubMed PMID: 30688034.

2: Biagetti C, Correani A, D'Ascenzo R, Bellagamba MP, Marchionni P, Antognoli L, Pompilio A, Cogo P, Carnielli VP. Does intravenous fish oil affect the growth of extremely low birth weight preterm infants on parenteral nutrition? Clin Nutr.2018 Oct 16. pii: S0261-5614(18)32480-4. doi: 10.1016/j.clnu.2018.10.009. [Epub ahead of print] PubMed PMID: 30392754.

3: Verlato G, Simonato M, Giambelluca S, Fantinato M, Correani A, Cavicchiolo ME, Priante E, Carnielli V, Cogo P. Surfactant Components and Tracheal Aspirate Inflammatory Markers in Preterm Infants with Respiratory Distress Syndrome. J Pediatr. 2018 Dec;203:442-446. doi: 10.1016/j.jpeds.2018.08.019. Epub 2018 Sep 27. PubMed PMID: 30270169.

4: Correani A, Pignotti A, Marinelli L, Biagetti C, D'Ascenzo R, Vedovelli L, Verlato G, Cogo P, Rocchi MBL, Carnielli VP. Plasma Phytosterol Half-Life and Levels Are Increased in Very Low Birth Weight Preterm Infants with Parenteral Nutrition-Associated Cholestasis. Lipids. 2018 Jul;53(7):717-725. doi: 10.1002/lipd.12072. Epub 2018 Sep 6. PubMed PMID: 30187918.

5: Giambelluca S, Ricci F, Simonato M, Correani A, Casiraghi C, Storti M, Cogo P, Salomone F, Carnielli VP. Estimating the contribution of surfactant replacement therapy to the alveolar pool: An in vivo study based on (13) C natural abundance in rabbits. J Mass Spectrom. 2018 Jul;53(7):560-564. doi: 10.1002/jms.4088. PubMed PMID: 29633450.

6: Correani A, Visentin S, Cosmi E, Ponchia E, D'Aronco S, Simonato M, Vedovelli L, Cogo P, Carnielli VP. The maternal-fetal gradient of free and esterified phytosterols at the time of delivery in humans. Clin Nutr. 2018 Dec;37(6 Pt A):2107-2112. doi: 10.1016/j.clnu.2017.10.004. Epub 2017 Oct 13. PubMed PMID: 29102321.

7: Pupillo D, Correani A, Biagetti C, D'Ascenzo R, Simonato M, Verlato G, Cogo P, Rocchi MBL, Carnielli VP. Half-life of plasma phytosterols in very low birth weight preterm infants on routine parenteral nutrition with vegetable oil-based lipid emulsions. Clin Nutr. 2018 Feb;37(1):262-269. doi:10.1016/j.clnu.2016.12.022. Epub 2016 Dec 30. PubMed PMID: 28063717.

8: Savini S, Correani A, Pupillo D, D'Ascenzo R, Biagetti C, Pompilio A, Simonato M, Verlato G, Cogo P, Taus M, Nicolai A, Carnielli VP. Phytosterol Esterification is Markedly Decreased in Preterm Infants Receiving Routine Parenteral Nutrition. Lipids. 2016 Dec;51(12):1353-1361. Epub 2016 Sep 24. PubMed PMID: 27663254.

9: Lange-Consiglio A, Romaldini A, Correani A, Corradetti B, Esposti P, Cannatà MF, Perrini C, Marini MG, Bizzaro D, Cremonesi F. Does the Bovine Pre-Ovulatory Follicle Harbor Progenitor Stem Cells? Cell Reprogram. 2016 Apr;18(2):116-26. doi: 10.1089/cell.2015.0062. Epub 2016 Mar 16. PubMed PMID: 26982278. (**)

10: Corradetti B, Correani A, Romaldini A, Marini MG, Bizzaro D, Perrini C, Cremonesi F, Lange-Consiglio A. Amniotic membrane-derived mesenchymal cells and their conditioned media: potential candidates for uterine regenerative therapy in the horse. PLoS One. 2014 Oct 31;9(10):e111324. doi: 10.1371/journal.pone.0111324. eCollection 2014. PubMed PMID: 25360561; PubMed Central PMCID: PMC4216086. (**)

ABSTRACTS

A. Simonato M., Verlato G., Bassi E., Fantinato M., Correani A., Giambelluca S., Baraldi E., Carnielli V., Cogo P. Alveolar surfactant composition in preterm infants with respiratory distress syndrome before exogenous surfactant administration. 2018. PAS meeting conference.

B. A. Correani; S. Visentin; E. Cosmi; E. Ponchia; S. D'Aronco; M. Simonato; L. Vedovelli; P. Cogo; V.P. Carnielli. The maternal-fetal gradient of free and esterified phytosterols at the time of delivery in humans. 2017. 2nd Jens Congress of joint European Neonatal societies.

C. G. Verlato; M. Simonato; E. Bassi; M. Fantinato; A. Correani; I. Giretti; E. Baraldi; P. Cogo; V.P. Carnielli. Alveolar surfactant composition in preterm infants with respiratory distress syndrome before exogenous surfactant administration: effect of gestational age and inflammation. 2017. 2nd jENS Congress of joint European Neonatal societies.

D. Correani A, Pignotti A, Biagetti C, D'Ascenzo R, Simonato M, Verlato G, Cogo P, Rocchi M, Carnielli VP. Cholestasis affects plasma phytosterol half-life in very low birth weight preterm infants receiving parenteral nutrition. 2017. PAS meeting conference.

E. Correani A, Savini S, Pupillo D, D'Ascenzo R, Biagetti C, Pompilio A, Verlato G, Cogo P, Taus M, Nicolai A, Virgilio VP. Phytosterol Esterification Is Markedly Reduced In Preterm Infants Receiving Routine Parenteral Nutrition. 2016. PAS meeting conference.

F. Lange-Consiglio A., Correani A., Romaldini A., Corradetti B., Cremonesi F., Bizzaro D. In Vitro Characterization Of Granulosa Progenitor Cells Isolated From Bovine Ovarian Follicles, 2014. 18th Annual Conference of the European Society for Domestic Animal Reproduction (ESDAR) and the Annual Meeting of EU-AI-Vets. (**)

G. Barbosa L.R.S., Correani A., Romaldini A., Spinozzi F., Mariani P., Itri R. The Uni- to Multilamellar Transition of Mixed Anionic and Zwitterionic Vesicles Induced by Cytochrome-C: A Small Angle X-Ray Scattering Study, 2012. Biophysical Journal, 102: 497a. (**)

OTHER

- Mariani P., Ortore M.G., Spinozzi F., Funari S.S., Correani A. and Romaldini A. Analysis of Guanosine Quadruplex Formation and Elongation in Dilute Aqueous Solutions: Salt Effects, 2010. Photon Desy Science. Annual Report. (**)

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