Complications in home parenteral nutrition patients:
from lock solutions to lipids

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General introduction

PARENTERAL NUTRITION PATIENT

Total parenteral nutrition (TPN) as a therapeutic strategy to treat severe intestinal failure became available in the early 1960s when a soybean oil-based lipid emulsion named Intralipid was developed by Wretlind and colleagues in Sweden that lacked the severe side effects of cotton-oil derived lipids and which was safe enough to be used in daily practice. TPN is a nutritional formulation that is administered intravenously and which contains all necessary micro- (electrolytes, trace elements, vitamins) and macronutrients (amino acids, carbohydrates, lipids). TPN is a truly life-saving therapy for patients who suffer from severe intestinal failure. The latter implies a gastrointestinal dysfunction that results in a deficient absorption of nutrients and, if untreated, leads to malnutrition. Gastrointestinal dysfunction can be due to a lack of remaining intestine following resection (short bowel syndrome), mostly as a result of aggressive inflammatory bowel disease or mesenteric infarction, or as a result of a motility disorders. Depending on the length and anatomy of the remaining functional intestine patients may be fully or partly dependent on TPN or fluids and electrolytes. The latter scenario is often seen in patients with high output stomas, especially in patients with a jejunostoma. TPN treatment can be given continuously or cycled (mostly overnight), depending on the patient's preference and/or condition.

Short-term (days to weeks) TPN is mostly given immediately after (abdominal) surgical procedures or on the Intensive Care Unit (ICU). Indications for long-term use of parenteral nutrition (mostly 3 months or more) in the Netherlands may include temporary indications, such as recovery from complex post-surgical complications with or without enterocutaneous fistulae prior to surgical repair, or permanent administration of TPN at home (home PN, HPN) because of permanent intestinal failure. The latter is mostly due to short bowel syndrome or severe motility disorders. It is important to realize that gut function after large resection may improve by the administration of enteral feeding due to a process that is called intestinal adaptation. Adaptation may continue over a period of up to two years. Hence, oral feeding is usually recommended, to stimulate normal hormonal and gut secretory (bile) functions, even if nutrient absorption can be expected to be minimal. A substantial proportion of patients suffering from severe intestinal failure can be weaned off TPN due to this process of adaptation of the remaining gut over time.[1]

For long-term parenteral nutrition therapy, a subcutaneously tunneled central venous catheter is inserted into one of the large-bore central veins - typically the jugular or subclavian vein. In case the patient has no remaining patent jugular or subclavian vein left, femoral veins or the inferior caval vein may be used to obtain venous access. The choice of venous access depends on the estimated duration of HPN dependency, the condition of the veins and the personal preference of the patient, with respect to aesthetics (visibility of access device) and need for (self-)puncturing. In the Netherlands, and specifically in Nijmegen, the type of venous access for administration of TPN can be categorized into one of three major groups (Figure 1). First, a subcutaneously

tunneled central venous catheter, mostly the Hickman (Figure 1A) or Broviac type, is used. This catheter has a tunneled portion exiting the skin and a dacron cuff that is positioned in the catheter tunnel near the exit site. The cuff occludes the subcutaneous tunnel, preventing the migration of pathogenic microorganisms into the bloodstream. The second type of venous access is a totally implantable device, called a Port-a-cath (PAC, Figure 1B), which is a subcutaneous port that is accessible through a self-sealing septum. PACs are positioned in a subcutaneous pocket and can be accessed by (self-)puncturing by means of a hooked needle through the intact skin. The third type of venous access is an arteriovenous fistula (AVF, Figure 1C), which is a surgically created connection between a vein and an artery, mostly positioned in the forearm. The AVF can be punctured for TPN administration, similar to the use of these devices for renal replacement therapy in hemodialysis. To become familiar with the use of their venous access, patients and (if possible) their spouses are admitted to the hospital for a brief training course in aseptic catheter care and handling of the venous access. This training comprises preparation and administration of the TPN formulation using an

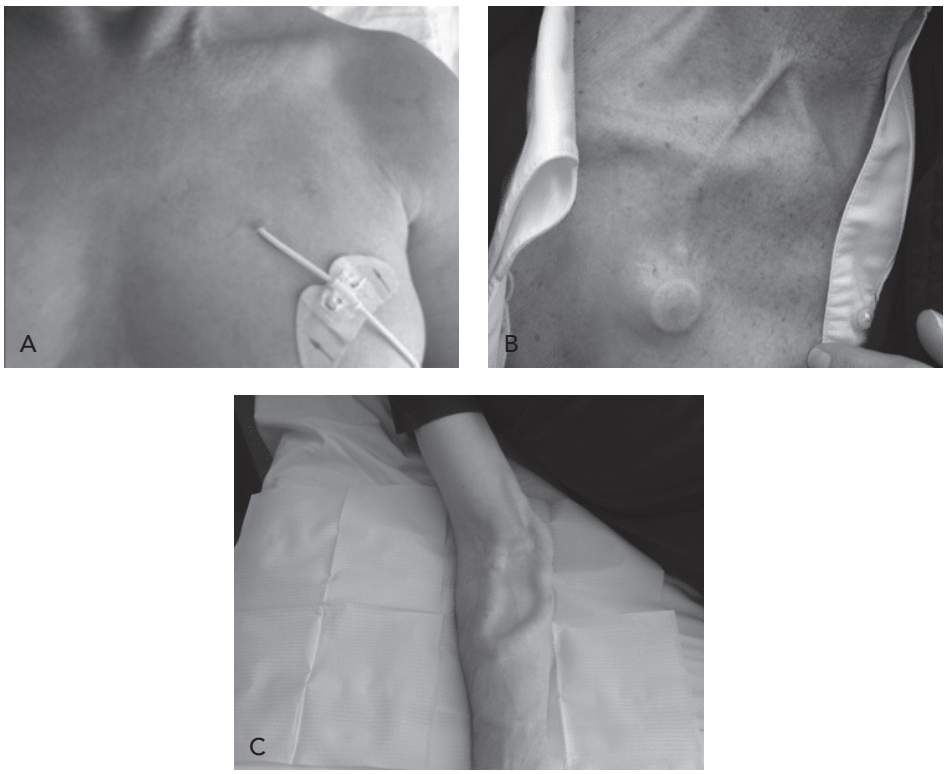

Figure 1. Types of venous access for administration of TPN[1]: central venous catheter (A), Port-a-cath (B), arteriovenous fistula (C).

infusion pump. These techniques return autonomy to these patients and enable them to perform this treatment in a home setting.[1, 2]

Although HPN undoubtedly is a life-saving treatment, this treatment comes with drawbacks. For instance, liver enzyme elevations are frequently seen and may result in end-stage liver disease. In addition, altered bone metabolism with a disturbed mineralization is commonly seen in HPN patients and frequently results in osteoporosis. The Achilles'heel of HPN treatment are complications associated with the presence of venous access, with venous occlusions and (more frequently) infections. Since a repeated loss of catheters compromises the options to keep adequate venous access, and in view of the fact that most patients will remain dependent on PN administration, catheter related complications should be prevented whenever possible.[1]

Catheter related bloodstream infection

Catheter related infection is defined as the presence of bacteraemia originating from an i.v. catheter. The spectrum of infections encompass bloodstream infections (80%), or may involve the catheter exit site of the catheter (17%) or the subcutaneous tunnel tract (2%).[1] Catheter-related bloodstream infections (CRBSI) remain the most threatening complication of HPN therapy. In Europe, the reported incidence of CRBSI in experienced centres ranges from 0.03 to 1.79 per catheter year.[3]

A definitive diagnosis of CRBSI requires that the same organism grow from at least one percutaneous blood culture and from a culture of the catheter tip, or that two blood samples be drawn (one from a catheter hub and the other from a peripheral vein) that, when cultured, meet CRBSI criteria for quantitative blood cultures or differential time to positivity (DTP). For quantitative blood cultures, a colony count of microbes grown from blood obtained through the catheter hub that is at least three-fold greater than the colony count from blood obtained from a peripheral vein best defines CRBSI. For DTP, growth of microbes from a blood sample drawn from a catheter hub at least two hours before microbial growth is detected in a blood sample obtained from a peripheral vein best defines CRBSI.[2, 4] However, in clinical practice, CRBSI is diagnosed on the basis of presence of symptoms (fever, chills) in association with positive blood cultures (blood drawn from peripheral vein and/or from venous access device) in the absence of other evident infectious foci that likely could explain such an infection.[3] The most common microbial species that cause CRBSI are skin-derived Gram-positive bacteria (~70%), followed by Gram-negative bacteria (~20%) and fungi (~10%).[5]

Several risk factors for the development of CRBSIs have been identified.[6] Some of these are disease-related, whereas other risk factors seem more treatment-related, including (possibly) the composition of the lipid component of TPN and the use of immunosuppressive medication. The presence of a venous access device on its own establishes a certain risk factor because of the direct connection of the internal (bloodstream) and external (skin) environment. The magnitude of this risk depends on the frequency and duration of the use of the device, the site of catheter insertion, the

coating of the catheter, type of solution to lock the catheter, and composition of the administered PN formulation (especially fluids vs TPN).

Whenever a CRBSI is suspected, patients are treated with antimicrobial agents, initially with antibiotics, if possible after blood withdrawal to obtain cultures. Given that most of these patients will depend on HPN support for the rest of their lives and since repeated catheter loss compromises the options to obtain venous access due to damaged and obstructed (thrombosis) vessels, catheter removal is only considered as a last resort in septic hemodynamically unstable patients. The considerations for catheter removal in these patients therefore differ considerably from those in other patient categories, for instance in the ICU. While treatment of CRBSI is performed whenever possible, the possible drawback of this strategy is that patients may develop severe infection-related complications, such as septic thrombosis or metastatic infections resulting in endocarditis or osteomyelitis. Depending on culture results the antimicrobial regimen often is tailored later on. [1, 2, 4]

Catheter related occlusion

Catheter occlusion is the most common non-infectious complication of HPN treatment. In the Netherlands, the reported incidence of catheter occlusions in the setting of HPN support ranges from 0.26 to 0.60 per catheter year.[3] Occlusion can be located at the level of the vein or at the venous access device (catheter or port) itself. Arteriovenous fistulae may also become obstructed due to thrombosis: most frequently this occurs based on an anastomotic stenosis at the arteriovenous junction. Catheter occlusions, either partial or complete, result in a luminal obstruction that limits or prevents the ability to withdraw blood, flush the catheter, and/or administer parenteral nutrition or medication. When a catheter occlusion develops in a gradual manner and is partial this is indicated by repeating alarming of the infusion pump and confirmed by a decreased inflow of fluids (with a droplet count of less than 100/minute) when a free-hanging bag (i.e. without pump) containing saline is administered. The occlusion and its nature can be diagnosed by fluoroscopy, and, in case of an arteriovenous fistula, by ultrasound (duplex).[1] Differences in catheter coating, TPN composition, the frequency of catheter manipulation and catheter flushing, position of the catheter tip, reflux of blood into catheter, and patient-related factors such as underlying disease and anticoagulant treatment can influence the risk for developing catheter occlusion.[6]

The origin of the occlusion can be thrombotic or non-thrombotic in nature. Non-thrombotic occlusions mostly result from mechanical problems like catheter tip migration or damage, or from precipitations related to lipid depositions or incompatibilities of medication with TPN. These non-thrombotic occlusions develop gradually and can be treated by careful (repeated) flushing with sodium hydroxide. Catheter occlusion by a thrombus, called catheter thrombosis, usually present in a more acute manner may be treated by means of thrombolytic agents such as urokinase. When left untreated, catheter occlusions almost invariably result in catheter removal.[1]

Catheter lock solution

After the administration of the HPN formulation central venous catheters, such as Hickman catheters and PACs, are rinsed with saline to remove residual HPN from the catheter. Subsequently a lock solution is instilled, which remains in the catheter until the next TPN is administrated. These catheter locks should be safe for the patient, since some of the contents spills over to the circulation and because the lock solution is usually flushed into the bloodstream prior subsequent TPN treatment. The latter is done because withdrawal of a lock from the catheter is not possible due to vascular collapse because of underpressure. In addition lock withdrawal implies that blood in the line will lead to the formation of a fibrin sheath that ultimately may enhance the formation of a biofilm as a precursor of a CRBSI.[7]

Several lock solutions have been used, some of which have been advocated to prevent occlusions, while others serve to prevent CRBSI. Anticoagulant and fibrinolytic lock solutions, like heparin, ethylene diamine triacetic acid (EDTA) and citrate, are especially important in hemodialysis practice, since in these patients blood needs to be aspirated via the catheter and anticoagulants are crucial to prevent catheter clogging. In HPN patients the choice of lock focuses more on the prevention of CRBSI and anticoagulants are not invariably deemed necessary. As mentioned, central venous catheters may be rapidly (within days) colonized with microorganism resulting in the formation of a biofilm, and these biofilms may cause CRBSIs that can hardly be treated due to the fact that most antimicrobial agents do not penetrate into this biofilm. In line with this notion, antibiotics and antiseptics, like ethanol, have been introduced as lock solutions to prevent or decrease biofilm formation and CRBSIs, but their beneficial effects in most cases remain to be proven in well-designed randomized controlled trials. An important issue in this respect is the concern for the development of microbial resistance.

Taurolidine

A novel catheter locking solution containing the potent antiseptic agent taurolidine, as a 2% (w/v) solution, has recently been shown to be effective in the prevention of CRBSI in the setting of HPN at our own center in Nijmegen.[8] Several structurally different taurolidine-based solutions are commercially available, containing different taurolidine concentrations, and some of which contain citrate and/or heparin. Taurolidine has been shown to reduce the risk for CRBSIs compared to traditional catheter locks like heparin in several patient groups who depend on a reliable central venous access device; the patient populations range from paediatric patients to adults, and from cancer patients to patients on haemodialysis or on HPN support. [8-15] A recent meta-analysis confirms these beneficial effects but did not identify which taurolidine formulation is optimal in which setting, or whether taurolidine is superior to other lock solutions than heparin, such as saline.[16]

Taurolidine is non-toxic for humans and is rapidly metabolized into the amino acid taurine, water and carbon dioxide. It has a broad spectrum of antimicrobial activity.

Fungi are however less sensitive to taurolidine than Gram positive and Gram negative bacteria, with a higher minimum inhibitory concentration 50 (MIC_{50}) of taurolidine, i.e. the required taurolidine concentration to inhibit the growth of 50% of a microorganism.[17, 18] The exact mechanism of action of taurolidine has not been completely elucidated for all microorganisms, but it seems to be a biocidal agent rather than antibiotic by nature, based on its mechanism of action in Gram negative bacteria. Antibiotics act specifically via structures or metabolic processes of the microorganism, while biocides inactivate the microorganism through other nonspecific or several different mechanisms. The mechanism of action of taurolidine against Gram negative bacteria involves a chemical reaction with the microbial cell wall, endotoxins and exotoxins, thus inhibiting both pathogenicity and microbial adhesion to inert and living surfaces. [19] Furthermore, taurolidine inhibited the adherence of bacteria and fungi to epithelial cells *in vitro*. [20, 21] The emergence of microbial adaptation or resistance to taurolidine has not been reported so far.

Immune function and lipid structure

The increased risk for infectious complications seen in patients treated by means of TPN has been related to lipid-induced disturbances of immune functions.[22] Lipids in TPN provide all essential fatty acid s(FA) and are an energy-dense source of calories that covers non-protein energy requirements. The lipids are administered as an emulsion consisting of minuscule triglyceride droplets (200 – 500 nm in diameter) that are surrounded by an envelope of phospholipids that acts as emulsifier. Lipids in TPN are triacylglycerols consisting of a glycerol backbone with 3 FAs attached to it. The chain length of the FAs in TPN can vary from 6 to 30 carbons.

Possible mechanisms that underlie the effects of lipids on immune function are altered physicochemical properties of immune cell membranes due to changes in the composition of cell membrane phospholipids and circumscript domains that contain the membrane-bound receptors, called lipid rafts. These lipid effects are considered to have distinct effects on crucial functions such as cell signaling and gene expression that depend on structural characteristics of these lipids. The structure of the lipids seems mainly important as far as the number and position of double bonds, fatty acid (FA) carbon chain length and the position of the carboxyl group is concerned, since it is these characteristics that determine the type of bioactive substances, such as 20-carbon based eicosanoids, that are formed when these lipids are metabolized.[23]

FA can be classified depending on the length of the carbon chain as short chain (SCT; $\leq$ 8 carbons), medium chain (MCT; 8-12 carbons) or long chain (LCT $\geq$ 14 carbons) FA. In the hydrocarbon chain, double bonds can be inserted. FAs can therefore be classified based on the number and position of these double bonds too. Saturated FAs have no double bonds, whereas monounsaturated FAs (MUFAs) have one double bond and polyunsaturated FAs (PUFAs) have two or more double bonds. PUFA's can be further divided into four major families according to the position of the first double

bond from the terminal methyl end of the hydrocarbon chain: these are the n-3, n-6, n-7 and n-9 families.

Essential fatty acids

Linoleic acid (n-6) and α-linolenic acid (n-3) cannot be produced *de novo* in humans and are therefore called essential fatty acids (EFAs). These EFAs have to be provided in the diet but can be metabolized through elongation and desaturation by mammalian enzymes, mainly in the liver. In line with this notion, TPN formulations will have to contain EFAs in order to be "total". The amount and type of lipids that should be provided to HPN patients depends on the remaining oral intake but has remained a matter of debate, also because of concerns with regard to the supposedly pro-inflammatory characteristics of some of these components (and especially linoleic acid). A low intake of EFAs increases the risk for the development of EFA deficiency, which can lead to increased water permeability of the skin, increased susceptibility to infections, lower resistance to irradiation injury and impaired wound healing, hematologic disturbances, fat infiltration of the liver, impaired chylomicron synthesis, and aggravated fat malabsorption.

End products of the metabolic breakdown of EFAs are arachidonic acid (AA) for linolenic acid and docosahexaenoic acid (DHA) and eicosapentaenoic (EPA) for α-linolenic acid (See Figure 2). Most of the EPA and DHA in humans however comes from the diet because the enzymatic turnover from ALA to EPA and DHA is slow. EPA, DHA and AA can be incorporated into the phospholipids of cell membranes and these components can influence the function of the immune cell by altering the function of membrane-related receptors, transporters and enzymes. EPA and AA can be released from the phospholipids of cell membranes by the action of phospholipase A2 and act as substrates for the synthesis of a group of bioactive 20-carbon mediators called eicosanoids. The pro-inflammatory eicosanoids prostaglandin E2 (PGE2), leukotriene B4 (LTB4) and platelet aggregation function (PAF) are derived from AA. Eicosanoids originated from EPA are prostaglandin E3 (PGE3), leukotriene B5 (LTB5) and tromboxane 3 (TX3) and these are considered to be less pro-inflammatory than AA-derived metabolites. Importantly, the n-6 (AA) and n-3 (EPA, DHA) substrates are in direct competition for their metabolic breakdown by using the same enzymatic machinery. This means that a diet that is richer in one component over the other will lead to a relative over production of its metabolites: for instance EPA can act as a competitive inhibitor of the conversion of AA to PGE2 and LTB4.

Lipid emulsions

Since the 1960's several different lipid emulsions have been developed (See Figure 3). The most frequently used lipid emulsions in HPN patients are based on soybean oil (SO, such as Intralipid® and Lipovenos®) and contain a high amount of n-6 PUFAs, thus promoting the production of pro-inflammatory eicosanoids. To reduce the amount of

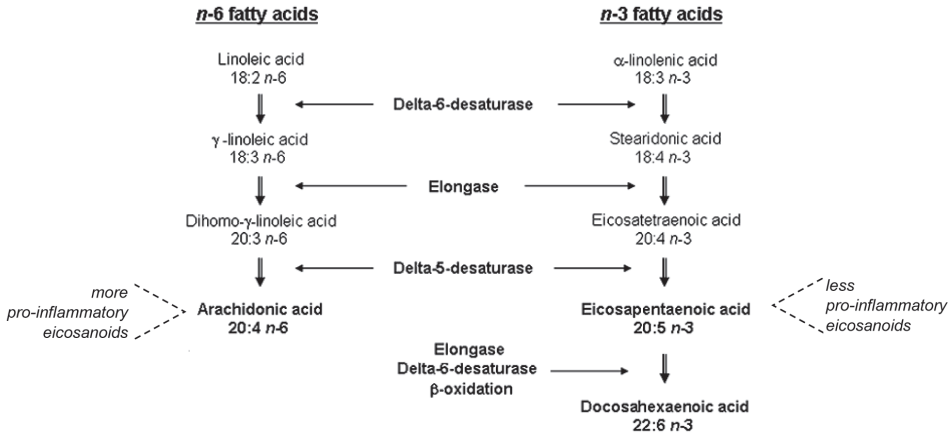

Figure 2. Synthesis of n-3 and n-6 fatty acids and the more and less pro-inflammatory eicosanoids.

linoleic acid and its pro-inflammatory properties, a physical lipid mixture (Lipofundin®) of 50% (v/v) SO and 50% coconut oil (CO), the latter containing mainly medium chain triglycerides (MCT), was developed in the mid 1980's. These MCTs were chosen because of their rapid metabolic breakdown and resistance to peroxidation (due to the absence of double bonds) and were expected to decrease the pro-inflammatory characteristics of the emulsion. Nowadays, structurally different lipids have become available to reduce the amounts of n-6 PUFAs. Lipid emulsions currently on the market for use as part of TPN formulations mostly are based on lipids that have more immune neutral or anti-inflammatory properties, such as olive oil (OO) or fish oil (FO) or synthetically structured lipids (SL). Structolipid® is a mixture of synthetically designed triglycerides in which both LCTs and MCTs are randomly attached to the 1st, 2nd, or 3th position of the same glycerol backbone. Clinoleic® contains 20% SO and 80% OO and was introduced for clinical use in the mid 1990's. Three FO containing lipid emulsions are currently commercially available. Omegaven® is a pure 10% (w/v) FO emulsion consisting of very long chain triglycerides (VLCT), while SMOF-lipid® consists of a mixture of 30% LCT, 30% MCT, 25% OO and 15% FO, and Lipoplus® consists of a mixture of 40% LCT, 50% MCT and 10% FO. So far only a limited number of head-to-head comparisons investigating the clinical effects of these structurally different lipid emulsions on the immune system is available.

LCT/MCT

Some *in vitro* studies demonstrated dramatic effects of LCT/MCT on the immune function. For instance LCT/MCT induced neutrophil activation by modulating the leukocyte phenotype in a number of studies. The expression of surface membrane activation markers that are involved in the immune activation cascade from rolling of

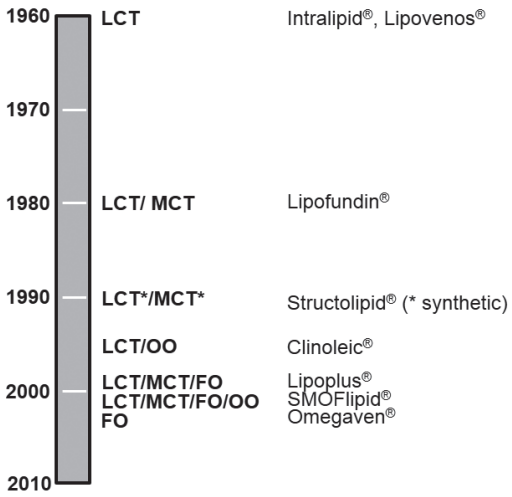

Figure 3. Timeline development commercial available lipid emulsions.

leukocytes (CD62L), to firm adhesion (CD11b), and eventually degranulation (CD63 and CD66b) to eliminate a pathogen, was clearly changed in a manner that is compatible with cell activation.[23] This was confirmed by a decrease in migration and an altered reactive oxygen species (ROS) production by neutrophils.[24, 25] These changes in immune function by LCT/MCT resulted in a decreased ability of neutrophils to kill *Candida albicans* and to eliminate *Streptococcus pneumoniae*, probably due to a rapid exhaustion of the neutrophil cellular metabolism in the presence of LCT/MCT and MCT emulsions.[26, 27]

To explain the in vitro LCT/MCT effects on the immune system, we have shown previously that lipids distinctively influence leukocyte signaling and stimulation through effects on intracellular calcium mobilization and protein kinase C (PKC) activation. More specifically, MCTs, but not LCTs sensitize neutrophils for activation by yeast particles in a PKC-dependent manner.[28] In addition we found that parenteral lipids can evoke a prompt and significant attenuation of hormone (fMLP)-induced neutrophil stimulation.[29] We could rule out a potential mechanism for LCT/MCT-induced immune activation. Although medium chain fatty acids have been described as ligands for a pertussis toxin (PT) -sensitive G-protein-coupled receptor 84 (GPR84), *in vitro* studies showed that the LCT/MCT-induced neutrophil activation does not involve the action of this receptor.[30] The exact mechanism by which LCT/MCT exerts these effects on the immune system therefore remains to be elucidated.

Toll like receptor 4

Toll like receptors (TLRs), which are present in the cell membranes, are signaling receptors that distinguish different types of pathogens by recognizing microbial

molecular patterns. These receptors help to direct immune responses and their actions are therefore crucial to establish an adequately functioning host defense system. TLR-4 is located on the outer cellular surface and recognizes parts of lipopolysaccharides (LPS), like LPS lipid A, from Gram-negative bacteria.[31] Ligand binding to TLR-4 leads to formation of a homodimer, activation of the transcription factor NFκB and its translocation into the nucleus to induce the expression of pro-inflammatory genes, like the pro-inflammatory cytokine TNFα.

TLR-4 is an interesting receptor in the search to elucidate the mechanism behind MCT-induced immune activation, because saturated FAs (SFAs) have functional groups that are similar in structure to lipoteichoic acid and LPS lipid A. Ligand binding, receptor dimerization, translocation of the receptor into lipid rafts, and recruitment of the immediate downstream signaling molecules are the potential steps through which SFAs can modulate activation of these receptors.[32] Lauric acid, a medium chain SFA, might activate NFκB and induce TNFα production, through TLR-4.[33]

This NFκB activation is of interest, because we have shown that MCTs sensitize neutrophils for activation by yeast particles in a PKC-dependent manner.[28] PKC can in turn activate MAPKs, such as JNK and p38. This leads to generation of transcription factors in the AP-2 and C/EBP families, respectively. These transcription factors can increase the transcriptional effectiveness of NF-kB by forming enhanced-activity complexes between the transcription factors, and/or by binding to multiple binding sites in the promoter segments of pro-inflammatory genes such as interleukin 6 (IL-6) and interleukin (IL-8), greatly increasing their transcription.

Olive oil based parenteral nutrition

Several *in vitro* studies showed that olive oil, compared to other lipid emulsion, have less immune modulating effects on proliferation, activation, migration and cytokine production of immune cells, concluding that olive oil has more immune neutral properties.[23, 29, 34-36] Studies in rats confirm that olive oil based TPN an immunologically neutral alternative to soybean oil-based lipid emulsions is.[37, 38] However, data on *in vivo* effects of olive oil based lipid emulsions on the immune system are limited. Clinical trials are more focused on safety and efficacy, rather than immune modulating properties of olive oil based lipid emulsions, meaning only plasma inflammatory markers (c-reactive protein and cytokine levels) and plasma oxidative stress markers were assessed.[39-43] With respect to the latter, long-term HPN use does not result in oxidative damage or an altered oxidant-antioxidant balance in HPN patients.[44]

Outline of this thesis

This thesis focuses on the complications experienced by patients on long-term (total) parenteral nutrition that is administered in at home. Catheter-related complications, mainly infections and occlusions, are the most frequent problems and have a profound impact on patients' quality of life as well as on hospital resources. It remains unclear

whether parenteral nutrition components, and especially the type of lipids, apart from the presence of a venous access device, affect the immune system and contribute to the increased infection risk seen in these patients. Another issue here is that although several types of catheter lock solutions have been suggested to lock the CVC to prevent occlusions and/or CRBSI, the scientific evidence to support the use of any of these locks is lacking.

Since it is unclear at this point whether catheter locks prevent CRBSIs or whether immune modulation by certain lipids contributes to the increased risk for CRBSIs in HPN patients, the investigations in this thesis aimed to A) evaluate the effects of various available catheter lock solutions as well as B) explore the immune modulating properties of structurally different lipids.

LOCK SOLUTIONS

In the first part of this thesis we address the effect of catheter lock solutions by answering the following research questions:

- *Is taurolidine lock superior to low-dose heparin lock in the prevention of catheter related bloodstream infections and occlusions? (**Chapter 2**)*
- *Is microbial adaptation to taurolidine seen in patients on home parenteral nutrition who develop catheter related bloodstream infections and use taurolidine locks? (**Chapter 3**)*
- *Do microbiocidal effects of various taurolidine-containing catheter lock solutions differ? (**Chapter 4**)*

Is taurolidine lock superior to low-dose heparin lock in the prevention of catheter related bloodstream infections and occlusions?

A randomized trial in our own tertiary HPN referral center comparing the catheter lock strategy using 2% taurolidine versus low dose (150 U/mL) heparin on the recurrence of CRBSIs was prematurely terminated due to the dramatic decrease in CRBSIs observed in patients using taurolidine locked catheters that became apparent because of the open label character of this study.[8] Although the sample size of this formal randomized trial was low (heparin: n=14), taurolidine (n=16), the results were considered evident enough to switch from low dose heparin to 2% taurolidine catheter locking in all of our HPN patients in the fall of 2008. To provide further and more robust evidence for the beneficial effects of taurolidine over heparin locks we performed a retrospective analysis of our HPN population several years after the switch in catheter lock type in order to study the effects of taurolidine compared to heparin locking in a large cohort of a single center during several years, during which no other procedural changes than the catheter lock strategy were implemented. Although we fully recognize the drawbacks of retrospective studies, in our opinion this design here provides additional proof for the efficacy of taurolidine over heparin. Thus, in **Chapter 2**, we compared

heparin-locked catheters and taurolidine locked catheters in a retrospective study design for their efficacy to prevent catheter-related complications, i.e. infections and occlusions, and evaluated the effect of this strategy on hospital admissions, covering the period from 2000 to late 2008 (using low-dose heparin) and the period from 2008 to 2011 (using taurolidine). The study comprises 212 patients, 745 central venous catheters and more than 200,000 catheter days, and provides by far the most robust dataset in this field so far. We initiated the investigation with the hypothesis that the switch from low dose heparin to 2% taurolidine, would result in a decrease of catheter-related complications and –hence- hospital admissions.

Is microbial adaptation to taurolidine seen in patients on home parenteral nutrition who develop catheter related bloodstream infections and use taurolidine locks?

We noticed that some HPN patients still developed catheter related bloodstream infections, despite the use of taurolidine and wondered whether the microorganisms responsible for these catheter related bloodstream infections might have adapted to taurolidine after long-term use of this locking agent. Our aim was therefore to assess the susceptibility to taurolidine of these micro-organisms. In order to stay as close as possible to clinical practice, we investigated this issue in an *ex vivo* setting in microorganisms that were obtained from cultures of HPN patients who developed CRBSIs during the use of taurolidine as a catheter lock. The mininimum inhibitory concentration is an often used quantitative presentation for the effect of a substance on the inhibition of microbial growth in microbiology. In **Chapter 3** we therefore determined the *in vitro* taurolidine minimum inhibitory concentration of CRBSI-causing microorganisms in HPN patients with CRBSIs during taurolidine lock therapy and compared these concentrations to values of sensitivity to taurolidine found in the literature. During the period 2009 - 2011, 9 patients of our HPN population developed 14 luminal CRBSIs in total, resulting in 27 clinical isolates of which the taurolidine minimum inhibitory concentration was determined for the most important microbial pathogens. Since the mechanism of taurolidine is a chemical reaction and adaptation of microorganisms to taurolidine has not been reported before, our hypothesis was that we would not find such adaptation to taurolidine, i.e. we did not expect to find a shift towards higher minimum inhibitory concentrations for the pathogens under investigation.

Do microbiocidal effects of various taurolidine-containing catheter lock solutions differ?

Besides the taurolidine used in our tertiary referral centre, other taurolidine-containing lock solutions are commercially available that contain citrate and/or heparin. Taurolidine locks with citrate and/or heparin as anticoagulants are mainly used in hemodialysis patients, because in this situation blood is aspirated via the catheter and anticoagulants

are considered necessary to prevent catheter clogging. In our HPN population only in the case of an infection, blood is withdrawn from the catheter, or when several attempts at venapuncture fail. Other than that all fluids, including catheter lock solutions, are flushed into the bloodstream, with no risk of blood getting into the catheter. Addition of anticoagulants is therefore not considered to be necessary in our HPN population in general. These agents may even cause harm since heparin and citrate promote biofilm formation, and because citrate is a substrate for *Escherichia coli* and *Klebsiella pneumoniae*, i.e. microbial strains that are known to cause CRBSI. Furthermore, higher doses of citrate can cause neurologic side effects including paresthesias and tetany, due to the calcium-binding properties of this agent. Our aim was to evaluate whether different types of catheter locks have different antimicrobial activities. To this end we performed a head-to-head comparison between various locks in an *in vitro* study design, given that a clinical study to address this question and compare various locks would never obtain sufficient power in light of the limited number of patients that is eligible for inclusion in such an investigation. In **Chapter 4**, we therefore investigated the *in vitro* effect of different dilutions of catheter lock solutions (taurolidine with or without anticoagulants, heparin and citrate) on the growth and biofilm formation of microorganisms. To cover all relevant pathogenic microorganisms, the effects on growth and biofilm formation of the most frequent CRBSI-causing Gram negative (*Escherichias coli*), Gram positive (*Staphylococcus aureus*) and fungal pathogens (*Candida glabrata*) were investigated. Since some studies suggest that heparin and citrate negatively influence the antimicrobial activity, we hypothesized that when used in a setting where anticoagulants are not deemed necessary, catheter lock solutions containing taurolidine without additives are more bactericidal compared to formulations containing citrate and/or heparin.

LIPIDS

In the second part of this thesis we address the effect of lipid structure on immune function by answering the following research questions:

- *Do MCT-containing lipid emulsions that contain fish- and/or olive oil activate the immune system similar to MCT and is this MCT effect mediated by TLR-4? (**Chapter 5**)*
- *Do HPN patients who fully depend on parenteral lipids for their EFA intake have an adequate EFA status and immune function compared to healthy controls? (**Chapter 6**)*
- *Is the function of the innate immune system of long-term HPN patients different from that of healthy controls? (**Chapter 7**)*

Do MCT-containing lipid emulsions that contain fish oil activate the immune system similar to MCT and is this MCT effect mediated by TLR-4?

Since the introduction of HPN, several lipid emulsions based on different oil sources have been developed that show distinct effects on immune function. Medium chain

triglycerides, present in LCT/MCT lipid emulsions, have been shown to activate the immune system in the *in vitro* setting. The mechanism behind this MCT-induced immune activation is not known, but saturated fatty acids have been shown to activate toll-like receptor 4, this seems a possible mechanism to consider. Modern parenteral emulsions still contain LCTs and MCTs, but also fish oil, which have more anti-inflammatory properties. Since we do not understand the immune activating effect of MCT at this point, our aim was to investigate whether these new MCT-containing lipid emulsions which contain fish oil still activate the immune system in the manner known by MCT and whether the MCT-induced effects are mediated by TLR-4. We choose to explore these effects in an *in vitro* setting because, immune parameters vary even within one person, and at different time points. The *in vitro* setting enables comparisons between several structurally different lipids in a highly controlled environment. In order to stay as close as possible to clinical practice, we did not use (immortalized) cell-lines, but chose to spike freshly drawn blood of healthy volunteers with the different emulsions for one hour, i.e. a period of lipid exposure that has previously shown to result in significant effects on leukocyte function and phenotype. In **Chapter 5** we assessed whether leukocyte activation by MCT involves signaling pathways that these saturated lipids share with highly unsaturated n-3 PUFA in fish oil. Therefore, we investigated the effect of novel mixed lipid emulsions containing MCTs and fish oil on leukocyte function. In this chapter we also explored whether TLR-4 is involved in the activation of immune cells by MCT, based on the hypothesis that *in vitro* immune activation by MCTs in HPN, might be mediated by TLR-4 and abolished by the addition of anti-inflammatory lipid emulsions, like FO.

Do HPN patients who fully depend on parenteral lipids for their EFA intake have an adequate EFA status and immune function compared to healthy controls?

Essential fatty acids *de facto* cannot be synthesized by humans and need to be provided in the diet. The first lipid emulsions available on the market were prepared from pure soybean oil, which especially contains high amounts of the EFA linoleic acid (C18:2). The most frequently used lipid emulsion in our patient population contains 80% olive oil and 20% soybean oil, resulting in a significantly (three times) reduced amount of linoleic acid compared to pure soybean oil. Our aim was to study whether our long-term HPN patients who fully depend on parenteral lipid emulsions for their EFA intake that is based on this 80% olive oil and 20% soybean oil emulsion still have an adequate EFA and immune status compared to healthy controls. In **Chapter 6** we determined the fatty acid composition of lipid membranes of PBMCs and plasma, and the immune function of HPN patients who used PN more than 5 times per week and compared these data with age- and sex-matched healthy controls. We hypothesized that although the olive-oil based lipid emulsion contains a lower amount of EFA, HPN

patients who are (almost) fully dependent on HPN still have an adequate EFA and immune status in the long run.

Is the function of the innate immune system of long-term HPN patients different from that of healthy controls?

The HPN population establishes a heterogeneous group with different underlying diseases at different stages and with different co-morbidities. Since most of our patients depend on parenteral nutrition permanently, it is highly undesirable that the nutrition formulation impairs crucial body functions such as host defense. Most of our patients are on parenteral nutrition based on olive-oil. Although olive oil has been characterized as an immune-neutral agent, the question remains whether this characteristic holds true for the lipid emulsion that we use for our HPN patients and which includes other components including soybean oil, phospholipids derived from egg-yolk that function as emulsifier, and various lipid-soluble vitamins. Our aim was to investigate whether long-term use of this olive oil-based lipid emulsion does affect the function of innate immune system. We chose to study the long-term effects of this lipid emulsion in an *ex vivo* setting, investigating blood of patients who had used HPN at least three times per week for at least six months, ensuring sufficient lipid incorporation and -dependency. To eliminate possible confounding effects of immune-mediated diseases and use of immune suppressive medication, these two characteristics were exclusion criteria in our study. Thus, in **Chapter 7** we compared the immune function of 21 healthy controls and 20 HPN patients by determining several aspects of the innate immune function; the capacity of leukocytes to eliminate *Streptococcus pneumoniae*, the expression of activation and degranulation markers, and the stimulus-induced oxygen radical production of leukocytes and (anti-)oxidant balances. Our hypothesis was that the function of the innate immune system, to which phagocytes such as neutrophils belong, is not affected in patients on olive oil-based HPN compared to healthy controls.

Finally, a General Discussion and Future Perspectives is provided in **Chapter 8** that integrates and discusses the main findings of this thesis. **Chapter 9** presents a summary of this thesis in English, and **Chapter 10** in Dutch.

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A

Lock solutions

2

Taurolidine lock is superior to heparin lock in the prevention of catheter related bloodstream infections and occlusions

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ABSTRACT

Background

Patients on home parenteral nutrition (HPN) are at risk for catheter-related complications; mainly infections and occlusions. We have previously shown in HPN patients presenting with catheter sepsis that catheter locking with taurolidine dramatically reduced re-infections when compared with heparin. Our HPN population therefore switched from heparin to taurolidine in 2008. The aim of the present study was to compare long-term effects of this catheter lock strategy on the occurrence of catheter-related bloodstream infections and occlusions in HPN patients.

Methods

Data of catheter-related complications were retrospectively collected from 212 patients who received HPN between January 2000 and November 2011, comprising 545 and 200 catheters during catheter lock therapy with heparin and taurolidine, respectively. We evaluated catheter-related bloodstream infection and occlusion incidence rates using Poisson-normal regression analysis. Incidence rate ratios were calculated by dividing incidence rates of heparin by those of taurolidine, adjusting for underlying disease, use of anticoagulants or immune suppressives, frequency of HPN/fluid administration, composition of infusion fluids, and duration of HPN/fluid use before catheter creation.

Results

Bloodstream infection incidence rates were 1.1/year for heparin and 0.2/year for taurolidine locked catheters. Occlusion incidence rates were 0.2/year for heparin and 0.1/year for taurolidine locked catheters. Adjusted incidence ratios of heparin compared to taurolidine were 5.9 (95% confidence interval, 3.9 – 8.7) for bloodstream infections and 1.9 (95% confidence interval, 1.1 – 3.1) for occlusions.

Conclusions

Given that no other procedural changes than the catheter lock strategy were implemented during the observation period, these data strongly suggest that taurolidine decreases catheter-related bloodstream infections and occlusions in HPN patients compared with heparin.

INTRODUCTION

Catheter-related bloodstream infections (CRBSIs) remain the major, potentially life-threatening, complication of home parenteral nutrition (HPN) therapy. As such, CRBSIs pose a massive burden on the patients' quality of life and hospital resources due to the frequent need for hospital admission, surgical and medical interventions and, eventually, the need for intestinal transplantation when venous access becomes irreversibly compromised.[1]

Patient-, therapy- and device-related risk factors for CRBSIs have been characterized previously in detail.[2] The nature of the underlying disease leading to intestinal failure may increase the risk of CRBSI.[3] Also factors that are related to the composition of the parenteral nutrition formulation, such as caloric content and the presence of a lipid emulsion play a role[4], as well as the frequency and duration of the use of the venous access device.[5] The presence of a venous access device that bypasses the natural host barriers by directly connecting the external environment to the patients' central bloodstream, has been identified as an independent risk factor for the occurrence of CRBSIs.[6] The magnitude of the risk also depends on catheter material[5], site of catheter insertion[7], and catheter coating.[8] Finally, the agent that is used to lock the central venous catheter (CVC) after infusion of the parenteral nutrition is increasingly being recognized as pivotal in the prevention of CRBSIs.[9]

Several lock solutions, some of which include (combinations of) anticoagulants, fibrinolytic agents, antiseptics and antibiotics, have been introduced, but failed because of side effects, microbial resistance issues or lack in effectiveness.[10] Taurolidine, a microbiocidal agent, has a broad spectrum activity against bacteria and fungi.[11] The suggested microbiocidal activity of taurolidine involves a chemical interaction with the microbial cell wall resulting in irreparable injury.[12] Taurolidine has shown to reduce the risk for CRBSIs in several patient groups who depend on a reliable central venous access device.[9, 13-19] A recent meta-analysis confirms these beneficial effects, but also emphasizes low power and methodological flaws of the currently available studies.[20]

An randomized trial in our own tertiary HPN referral center comparing the catheter lock strategy using 2% taurolidine (Taurosept) and low dose (150 U/mL) heparin on the recurrence of CRBSIs was preliminary terminated due to the dramatic decrease in CRBSIs in taurolidine locked catheters that became apparent because of the open label character of this study.[9] Although the sample size of this formal randomized trial was very low (heparin: n=14), taurolidine (n=16), we considered the results evident enough to switch from low dose heparin to 2% taurolidine catheter locking in all of our HPN patients in the fall of 2008. In the present study, we provide further evidence that taurolidine may be more effective in preventing catheter-related complications in HPN patients compared to heparin, using a comprehensive dataset of long-term catheter locking, covering the period from 2000 to late 2008 (using low-dose heparin) and the period from 2008 to 2011 (using taurolidine). The study comprises 212 patients, 745 central venous catheters and more than 200,000 catheter days, and provides by

far the most robust dataset in this field so far. Importantly, none of our HPN practice procedures or materials, other than the catheter lock strategy, changed during the complete observation period.

PATIENTS AND METHODS

Ethics

The research ethics committee of the Radboud University Nijmegen Medical Centre (CMO Regio Arnhem-Nijmegen) approved this retrospective study under the protocol number (CMO: 2014/167). This ethics committee confirmed that individual written informed consent was not needed. Data was collected, anonymized and de-identified by the treating physician (GW). Subsequently data were entered in a database and statistical analysis was performed.

Patients

We enrolled all consecutive patients on long-term (>3 months and >1/ week used for HPN/fluid administration) HPN or fluids using a central venous catheter (Hickman or Port-A-Cath (PAC, implantable port)) at the Radboud University Medical Centre, Nijmegen, the Netherlands, between January 1st, 2000 and November 1st, 2011 (n=212). Medical records of all patients were reviewed. Patients were categorized into one of five underlying disease groups, based on the indication for fluid/HPN treatment: motility disorder, short bowel with stoma, short bowel without stoma, impaired intestinal absorption (mainly due to radiation enteritis), or other. Data on time and site of catheter insertion were checked in operation reports.

Cleaning protocol of catheter

Only single lumen catheters were placed. During the observation period of this study, none of our cleaning protocol procedures changed. In general, patients are trained in procedures including catheter handling and HPN/fluid administration during an inpatient training period of 1 to 2 weeks in our hospital.

Independent of the access type, the patient's or care-givers's hands are washed and subsequently disinfected with chlorhexidine in ethanol prior to HPN/fluid administration or cleaning of the exit site or skin. Cleaning protocols for Hickman catheter and PAC in our hospital are described in more detail in the following text.

For Hickman catheters, the catheter exit site is covered direct after catheter insertion with a Tegaderm pad (3M Health Care, Neuss, Germany) which is replaced every 96 hours. The exit site is disinfected by circular movements from the exit site to the outer circumference. Every circle is disinfected with a new sterile swap. The first 10 centimeters of the catheter is disinfected with a sterile swap and chlorhexidine in ethanol. After drying, the exit site is covered with a new Tegaderm pad. After 3 weeks, the catheter cuff has grown in the surrounding tissues and sutures for line fixation are

removed. From then on, the exit site is no longer covered with a Tegaderm pad and is cleaned daily by washing with water and soap and drying with a clean towel.

For PACs, after placement the wound is covered with sterile compresses. Before HPN/fluid administration, the skin overlying the subcutaneous port is cleaned with chlorhexidine by the described circular movement method above. After HPN administration, the puncture-site is covered with a sterile compress for a few minutes, until (minor) bleeding ends.

Catheter lock solution

Central venous catheters were locked after every administration of HPN or fluids. After a successful open label randomized controlled trial[9], all HPN patients with a Hickman or PAC switched from low dose (150 U/mL) heparin to 2% taurolidine (TauroSept®) in 2008. Of every individual patient the date of switching from heparin to taurolidine was used for data analysis.

In case a catheter was locked with heparin and subsequently with taurolidine, only the period before starting taurolidine was analyzed, since catheter related complications after start of taurolidine could possibly be a consequence of a carry-over effect of locking with heparin, since a biofilm that originated from the heparin period might lead to a CRBSI in the taurolidine period. In that case, the total number of catheter days was calculated from insertion of the catheter to the starting date of the catheter lock solution taurolidine (Figure 1).

Catheter related bloodstream infections and occlusions

CRBSIs were defined by the presence of symptoms (fever, chills) associated with positive blood cultures (blood drawn from peripheral vein and/or from venous access device) in the absence of other evident infectious foci that likely could explain the bloodstream infection. Episodes with fever and/or chills without positive blood cultures were only considered a bloodstream infection in case blood samples were drawn under antibiotic treatment (possibly leading to negative blood cultures) and in case patients were showing signs of sepsis.[5] The date of the CRBSI was the date of the first positive blood culture. A CRBSI was considered polymicrobial when different pathogens were found in the peripheral and/or venous access blood culture. All pathogens, were classified as Gram positive or Gram negative bacteria or yeast.

Catheter occlusions were defined by an obstruction of the central venous catheter, described in the medical record, or a vascular occlusion of more than 50% of a vein near the insertion side of the central venous catheter, described in report of a duplex scan. The date of occlusion was the date of the first time that this occlusion was described.

Treatment

Data on the use of anticoagulants and immune suppressive medication, and the type of HPN/fluid formulation and the frequency of HPN/fluid administrations per week was

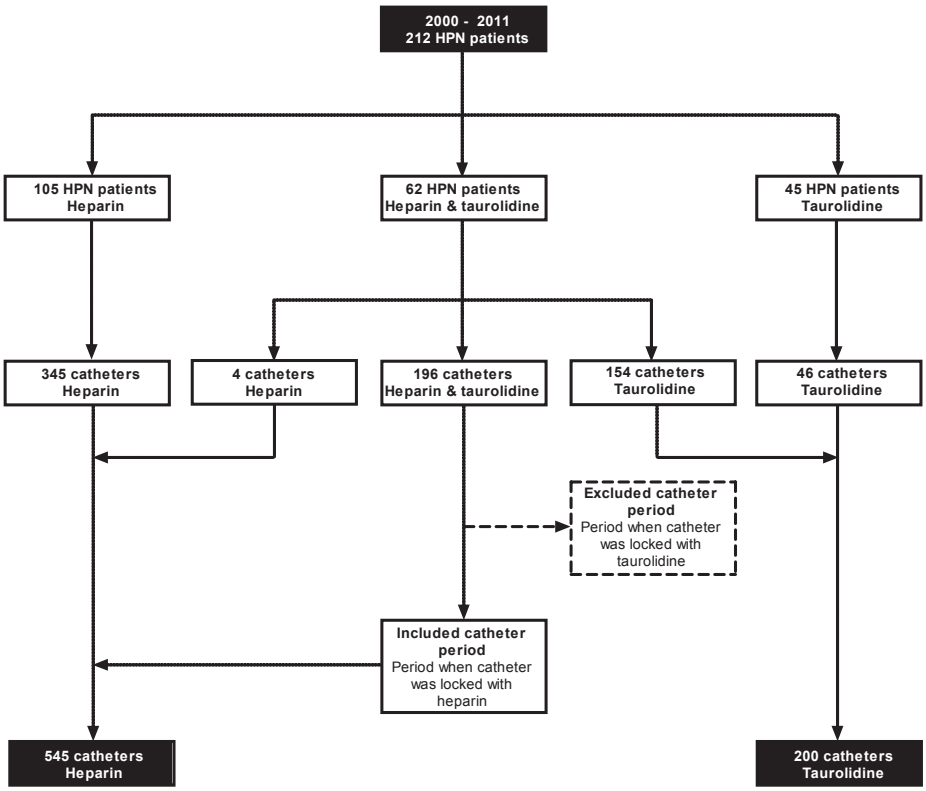

Figure 1. Flowchart of numbers of patients and catheters.

obtained from pharmacy prescriptions. Catheters were described as catheters which were exposed to only fluids, only HPN or a combination of both.

Hospital admission

We evaluated hospital admission data in the pre (2006-2007) and post (2009-2011) taurolidine eras. The transition year 2008 included hospital admissions with taurolidine and heparin locked catheters. No detailed hospital admission data were available of the period before 2006. All hospital admissions for in-hospital training of aseptic catheter techniques were excluded from analysis, since these admissions were not due to complications. Hospital admission were presented as the days that the patients were admitted to our ward in one year divided by the total number of catheter days in that year.

Adverse events

Adverse events resulting in discontinuation of the use of taurolidine were reported. If possible, patients switched first to another taurolidine-containing lock in the form of taurolidine-citrate (TauroLock®) and in case of repeated adverse effects switched to saline.

Statistical analysis

Characteristics of patients, vascular accesses and CRBSIs were presented as number (n) with percentage (%), or mean with 95% confidence interval (CI), or median with 25th and 75th percentile. Chi-square test for categorical data and Mann Whitney U test for continuous data were used to compare the heparin and taurolidine group in Table 1 and 2. Statistical significance was accepted if the probability of a type I error did not exceed 5%. We analyzed incidence rates and incidence rate ratios (i.e., the complication incidence rate that occurred with catheters locked with heparin divided by those locked with taurolidine) of CRBSIs and occlusions using random effects model with Poisson distributions for counts. Random effects for patients were incorporated in the modeling process to account for repeated vascular access periods within a patient. Possible confounders in the relation between catheter lock solution and complication rate were identified and reported, based on biological and clinical rationales[5], or a change of 10% or more on unadjusted complication rate ratios by a covariate. Adjusted complication incidence rate ratios for heparin over taurolidine were calculated with respect to these confounders. The NLMIXED procedure of SAS System for Windows version 9.2 was used (SAS Institute Inc, Cary, NC). Statistical significance was accepted if the probability of a type I error did not exceed 5%. To provide information about statistical significance, we reported 95% CI where appropriate. In case data were missing from medical records these were considered to be completely at random and were excluded from analyses.

RESULTS

Study population

212 HPN patients were included in the study: 62 patients had multiple catheters that were initially locked with heparin and later catheters that were locked with taurolidine, 105 patients had catheters that were exclusively locked with heparin, and 45 patients had their catheters exclusively locked with taurolidine (Figure 1). Most patients were male (n (%): 102 (61%) and 74 (69%) in heparin and taurolidine group, respectively). Patients started HPN at a mean (95% CI) age of 48 years (18 – 78 years) in the heparin group and 49 years (22 – 77 years) in the taurolidine group. The major indications for HPN (n (%)) use were motility disorder and short bowel syndrome with or without a stoma; 46 (28%), 32 (19%) and 69 (41%) patients in the heparin group, and 40 (37%), 28 (26%) and 34 (32%) patients in the taurolidine group, respectively. Less common indication of HPN was impaired intestinal absorption; 9 (5%) and 4 (4%) patients in the heparin and taurolidine group, respectively. All remaining patients were classified as other HPN indication (n (%)); 11 (7%) patients with heparin locked catheters and 1 (1%) patient with taurolidine locked catheters. The majority of patients (n (%)) was trained at our tertiary referral centre in aseptic catheter handling and parenteral nutrition administration; this concerned 143 (86%) patients for the heparin and 83 (78%) patients for the taurolidine group.

Table 1. Vascular access characteristics. Variables are shown per vascular access. Patients may have had multiple vascular accesses, total number of patients assessed is 212 (Figure 1). Characteristics were presented as number of events (n) with percentage (%) or median with 25th and 75th percentile. A p-value lower than 0.05 was considered statistically significant.

	Heparin (n=545)	Taurolidine (n=200)	p-value
Duration of HPN/fluid use before creation catheter			0.000*
(days: median (25th-75th percentile))	214 (34 – 765)	564 (103 – 1489)	
Unknown (n (%))	7 (1)	0 (0)	
Type of vascular access (n (%))			0.536
Hickman	368 (68)	140 (70)	
Port à cath	177 (32)	60 (30)	
Place of catheter insertion (n (%))			0.000*
Subclavian vein	337 (62)	57 (29)	
Jugular vein	63 (12)	105 (52)	
Femoral vein	33 (6)	19 (9)	
Inferior caval vein	13 (2)	6 (3)	
Unknown	99 (18)	13 (7)	
Catheter survival (days)			0.000*
Median (25th-75th percentile)	120 (43 – 310)	209 (65 – 611)	
Total number of catheter days of all catheters	147,842	71,112	
Number of catheters still in place (n (%))			0.000*
Yes	1 (0)	80 (40)	
No	544 (100)	120 (60)	
Composition of infusional fluid (n (%))			0.380
HPN alone	290 (53)	118 (59)	
Fluid alone	30 (6)	16 (8)	
HPN & fluid	178 (32)	61 (31)	
Unknown	47 (9)	5 (2)	
HPN/fluid administration frequency, per week (n (%))			0.529
1	3 (1)	1 (1)	
2	5 (1)	4 (2)	
3	32 (6)	14 (7)	
4	34 (6)	18 (9)	
5	50 (9)	21 (11)	
6	44 (8)	11 (5)	
7	319 (58)	111 (55)	
Unknown	58 (11)	20 (10)	
Immune suppressive use (n (%))			0.121
Yes	83 (15)	41 (21)	
No	437 (80)	154 (77)	
Unknown	25 (5)	5 (2)	

Table 1. Vascular access characteristics. Variables are shown per vascular access. Patients may have had multiple vascular accesses, total number of patients assessed is 212 (Figure 1). Characteristics were presented as number of events (n) with percentage (%) or median with 25th and 75th percentile. A p-value lower than 0.05 was considered statistically significant. (*Continued*)

	Heparin (n=545)	Taurolidine (n=200)	p-value
Anticoagulant use (n (%))			0.932
Yes	288 (53)	111 (55)	
No	220 (40)	83 (42)	
Unknown	37 (7)	6 (3)	

Vascular accesses

The characteristics of a total of 745 central venous catheters, of which 545 were locked with heparin and 200 with taurolidine were included (Figure 1) and analyzed (Table 1). In both groups Hickman catheters (about 70%) were used more frequently than PACs (about 30%). Around 75% of the catheters were inserted in the jugular or subclavian veins; in the heparin group the subclavian vein (62%) was the most common insertion place, while in the taurolidine group the jugular vein (52%) was mostly used. Catheter survival (days: median (25th – 75th percentile)) was longer in the taurolidine group (209 days (65 – 611 days)) compared to the heparin group (120 days (43 – 310 days)). The total number of catheter days was 147,842 for the 545 heparin locked catheters and 71,112 for the 200 taurolidine locked catheters. Patients with catheters in the taurolidine group were more experienced in the administration of HPN/ fluids (probably a consequence of having had heparin locked catheters before), since the median (25th – 75th percentile) duration from the start of HPN/fluid use to the creation of the venous access was longer; 214 days (34 – 765 days) for the heparin group and 564 days (103 – 1489 days) for the taurolidine group. Most catheters were used more than 5 days a week (75% and 71%, in the heparin and taurolidine group, respectively). HPN only was administered in 53% and 59%, in the heparin and taurolidine group, respectively, whilst a combination of HPN and fluids was administered in 32% and 31%, respectively. A minority, 6% of heparin and 8% of taurolidine catheters, was used for fluid administration only. Half of the catheters (53% and 55% in heparin and taurolidine group, respectively) were inserted in patients who used anticoagulants, and less than a quarter (15% and 21% in heparin and taurolidine group, respectively) of the catheters were inserted in patients who used immune suppressive medication.

Bloodstream infections and occlusion incidence rates

CRBSIs were 464 and 43 times detected in heparin and taurolidine locked catheters, respectively. Table 2 presents the characteristics of these CRBSIs. Forty-four percent of CRBSIs had both a positive blood culture of peripheral and venous access origin. The majority of CRBSIs (74 and 65% in heparin and taurolidine group, respectively)

Table 2. Characteristics of catheter related bloodstream infections. Characteristics were presented as number of events (n) with percentage (%). A p-value lower than 0.05 was considered statistically significant. *More pathogens than CRBSI, because of polymicrobial infections and differences in positive blood cultures between blood cultures of peripheral and venous access origin.

	Heparin	Taurolidine	p-value
Origin positive blood cultures (n (%))			0.000*
Peripheral	34 (7)	15 (35)	
Catheter	217 (47)	9 (21)	
Both	206 (44)	19 (44)	
Unknown	6 (1)	0 (0)	
Type of bloodstream infection (n (%))			0.147
Monomicrobial	345 (74)	28 (65)	
Polymicrobial	113 (24)	15 (35)	
Unknown	6 (2)	0 (0)	
Type of pathogens in peripheral blood culture (n (%))*			0.093
Gram positive bacteria	164 (57)	24 (55)	
Gram negative bacteria	94 (32)	11 (25)	
Yeast	26 (9)	9 (20)	
Unknown	6 (2)	0 (0)	
Type of pathogens in catheter blood culture (n (%))*			0.332
Gram positive bacteria	313 (55)	24 (57)	
Gram negative bacteria	209 (37)	12 (29)	
Yeast	43 (7)	6 (14)	
Unknown	6 (1)	0 (0)	

was based on a single type of pathogen. The most common microbial species that caused these CRBSIs were Gram-positive bacteria, followed by Gram-negative bacteria and fungi.

Table 3 shows the unadjusted incidence rates and the adjusted (for confounders) incidence rate ratios of heparin over taurolidine. Bloodstream infection incidence rates (per access year (95% CI)) were 1.1 per access year (0.9 – 1.3 per access year) in the heparin group and 0.2 per access year (0.1 – 0.2 per access year) in the taurolidine group. The bloodstream infection incidence ratio, of heparin compared to taurolidine incidence rates, adjusted for confounders (underlying disease, anticoagulant use, immune suppressive use, HPN/ fluid frequency per week, composition of infusional fluid, place of catheter insertion and HPN/fluid use before creation catheter), was 5.9 (95% CI, 3.9 – 8.7).

Catheter related vascular occlusions were 137 and 34 times detected in heparin and taurolidine locked central venous catheters, respectively. As presented in Table 3, occlusion rates (per access year (95% CI)) were slightly lower in the taurolidine group (0.1 per access year (0.1 – 0.2 per access year)) compared to the heparin group (0.2

Table 3. Catheter related bloodstream infection and occlusion incidence rates and incidence rate ratios in heparin and taurolidine locked catheters. Data were analyzed using random effects model with Poisson distributions for counts. *Adjusted values are corrected for: underlying disease, anticoagulant use, immune suppressive use, HPN/fluid frequency per week, composition of infusional fluid, and duration of HPN/fluid use before creation catheter. Random effecs for patients were incorporated to account for repeated vascular access periods within a patient.

	Incidence rate per access year (95% CI)	Adjusted incidence rate ratio (95% CI)*
Catheter related bloodstream infection		
Heparin	1.1 (0.9 – 1.3)	
Taurolidine	0.2 (0.1 – 0.2)	
Heparin/ taurolidine ratio		5.9 (3.9 – 8.7)
Catheter related occlusion		
Heparin	0.2 (0.2 – 0.3)	
Taurolidine	0.1 (0.1 – 0.2)	
Heparin/ taurolidine ratio		1.9 (1.1 – 3.1)

per access year (0.2 - 0.3 per access year)). The occlusion incidence ratio, of heparin compared to taurolidine incidence rates, adjusted for confounders (underlying disease, anticoagulant use, immune suppressive use, HPN/fluid frequency per week, composition of infusional fluid, place of catheter insertion and HPN/fluid use before creation catheter), was 1.9 (95% CI, 1.1 – 3.1).

Hospital admissions

From 2006 towards the end of 2011 the number of HPN-related admission days at our 15-bed clinical ward remained stable at a mean (± SD) of 1173 ± 159 days per year, while the HPN population increased from 61 to 133 patients, hence the number of catheter days increased from 21,619 to 45,960. Therefore, the ratio of hospital admission days per catheter day decreased by 60% from 0.055 in two pre-taurolidine years to 0.022 in 2011 (Figure 2).

Adverse events

Since the switch from heparin to taurolidine 8 of the 107 patients reported adverse events possibly related to use of taurolidine, and did not continue using pure taurolidine as a result. One patient experienced an anaphylactic-like reaction (vomiting, shortness of breath and urticaria) within minutes after the first administration and switched to saline for catheter locking. Five patients, who experienced a burning sensation, PAC occlusion, dizziness, nausea or pain and paresthesia, switched first to taurolidine-citrate, but experienced similar adverse reactions and switched thereafter successfully to saline. Two patients who experienced palpitations or discomfort over the chest switched successfully to taurolidine-citrate.

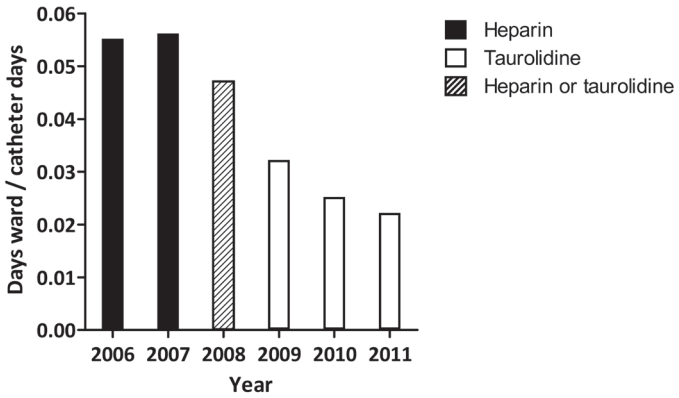

Figure 2. Hospital admissions in period 2006 until 2011. Catheters were locked with heparin (2006-2007) and taurolidine (2009-2011). In 2008 admissions of both lock strategies were included. Data are presented as the days that the patients were admitted to our ward divided by the total number of catheter days.

DISCUSSION

The availability and maintenance of an adequate and reliable venous access remains the foremost worry in long-term HPN care, with catheter related complications, mainly infections and occlusions, causing the majority of problems. This study presents by far the most robust data set so far to demonstrate that the use of taurolidine as catheter lock versus low-dose heparin decreases catheter related complications. During an observation period spanning over 200,000 catheter days, we found an impressive six times higher chance of developing CRBSI in heparin compared to taurolidine locked catheters. Also, a two times higher risk for developing catheter occlusions in heparin locked compared to taurolidine locked catheters was observed. Interestingly, we saw that this decrease in catheter complications was accompanied by a steep decrease in strain on healthcare resources in the form of diminished hospital admissions. Importantly, none of our other HPN-related procedures or techniques changed during this period suggesting that the type of catheter lock was instrumental in this observation.

The observed effectiveness of taurolidine to prevent CRBSI found in our study is in agreement with previous research in various patient populations.[9, 21-25] A meta-analysis including six studies covering 86,000 catheter days found a slightly lower than our study, but still an impressive three times (confidence interval: 1.8 – 4.8) higher risk for the development of CRBSI in CVCs locked with heparin compared to taurolidine. Due to small sample sizes as well as methodological deficiencies of the included studies in the meta-analysis these results should be interpreted with caution.[20] Also because these studies contain different heterogeneous patient populations with distinct risk profiles for the development of CRBSIs, and because the effects of diverse taurolidine

containing lock solutions, which differ in taurolidine concentration and the presence of other agents such as citrate and/or heparin, were pooled. Concerning the latter, minor differences between extremely diluted pure taurolidine and taurolidine-citrate(-heparin) solutions in the inhibition of growth of yeast, Gram negative and Gram positive bacteria *in vitro* have been found. The clinical relevance of these minor differences between different taurolidine solutions remains however unclear in the absence of clinical comparative studies.[26]

Although taurolidine has shown to be able to decrease thrombus weight, but is not as effective as heparin in this respect[27], we observed a lowered incidence of catheter related occlusions with its use. Previous studies described a relationship between the number of CRBSI and occlusions, possibly due to infection-induced activation of the coagulation system.[28] In the same vein, the decrease in catheter related occlusions in our study may be the result of diminished vascular damage because of the lower infection rate.[28] In contrast, the earlier mentioned meta-analysis (with its described limitations) found no significant difference between taurolidine and heparin-treated CVCs in incidence of catheter occlusions.[20]

The sharp decrease in catheter related complications that we observed after switching from catheter locking with heparin to taurolidine had a highly significant impact on the clinical burden that HPN care imposes on our clinical ward, as proven by the sharp decrease in number of days that HPN patients spent within the hospital. Keeping in mind that in Europe in general the cost of each case of catheter infection lies between 4,000 to 13,000 Euros[29], and the costs in the Netherlands for one year taurolidine locking (1,800 Euro per year) are about 300 Euros more than for heparin locking (1,500 Euro per year), taurolidine seems from a financial point of view promising. Still, a formal cost-effectiveness analysis is necessary to confirm that taurolidine is cost-effective.

In our study, approximately 7 percent of all patients who locked their catheter with taurolidine experienced (mostly mild) side effects, that urged us to stop the use of (any) taurolidine or switch to a different taurolidine formulation. This switch mostly resulted in similar side effects after which the patient used saline as a catheter lock. Only one probable anaphylactic reaction was observed and we did not dare to rechallenge this patient with the same or another taurolidine preparation. Although theoretically anaphylaxis seems unlikely due to the metabolization of taurolidine into taurine and carbon dioxide, other constituents such as polyvinylpyrrolidone (PVP-17), that is used as a stabilisator/ emulgator, might cause these problems, as described in a case study.[30] Side effects have been described before for taurolidine with citrate in a paediatric patient population with haematological malignancies. Twenty percent of the paediatric patients had side effects ranging from discomfort in chest and neck, perioral dysaesthesia, abnormal taste sensations to nausea, and half of these patients using the lock solution taurolidine with citrate was urged to stop with these lock solution because of the side effects they experienced.[22]

The statistical significant differences between the taurolidine and heparin locked catheters in HPN/fluid use before the creation of the catheter and the vascular acces *in situ* duration (Table 1) are a direct consequence of the fact that 62 of the 212 patients first locked their catheters with heparin and subsequently locked with taurolidine (Figure 1). The number of catheters that are still *in situ* are significantly higher in the taurolidine group (Table 1), primarily because the HPN population switched to taurolidine and none of the catheters were locked with heparin at the end of the study.

The place of catheter insertion is significantly different between the two groups. However, the place of insertion is dependent of different factors. The choice of venous access depends on the estimated length of parenteral nutrition dependency, the condition of the veins and the personal preference of the patient, with respect to esthetics (visibility of access device) and need for (self-)puncturing. Patients who have had more complications, may have less options left for venous access. A comparison between the place of insertion between catheters locked with taurolidine and heparin is therefore difficult. A recent systematic review did find similar risks for catheter-related complications in subclavian and internal jugular central venous catheters.[31]

A significant difference was found in the origin of positive blood cultures between heparin and taurolidine locked catheters (Table 2). We could not explain these differences. When we compare the type of pathogens that caused the CRBSI in the heparin and the taurolidine locked catheters (Table 2), it is interestingly to observe that in taurolidine locked catheters a higher percentage of the CRBSIs is caused by yeasts. This might theoretically be a consequence of the fact that yeast are less sensitive to taurolidine, since *in vitro* studies showed that higher taurolidine concentrations are necessary to eliminate yeasts compared to bacteria.[11]

The results of the statistical analysis in Table 1 and 2 should be interpreted with care, since the variables were not corrected for overrepresentation of patients who had multiple vascular accesses. To control for overrepresentation of certain patients, we used a specific statistical model that accounts for repeated measurements in the Poisson analysis, presented in Table 3. Variables of Table 1 and 2 were added as a covariate in the Poisson analysis, based on biological and clinical rationales, or based on the fact that the covariate changed the unadjusted complication rate ratios with 10% or more.

The retrospective nature of this study obviously has its pro's and con's. This study setting enabled us to collect and analyze a substantial number of patients (212), catheters (745) and catheter days (> 200,000) from a single center, which makes this by far the most extensive study in this field so far, with only a limited amount of missing data. The downside remains that this study setting hampers the drawing of any conclusions on causal relations.

A limitation of the study is that the diagnoses was based on the presence of symptoms (fever, chills) in association with positive blood cultures and in the absence of other evident infectious foci that likely could explain such an infection. Because of

the retrospective nature of the study, it was not possible to use the differential-time-to positivity criteria for diagnosis of CRBSI.

We chose to include all venous accesses of a single patient instead of only the first, since most long-term HPN patients are likely to have more than one catheter, making our approach more representative for clinical practice. Since none of our other HPN-related procedures or techniques changed during this period, except for the locking strategy, we don't think that bias was introduced by the fact that the cohorts of heparin and taurolidine catheters had not the same observation period.

An important remaining question is whether taurolidine should be used in all HPN patients or only in those who have a high risk for developing CRBSIs, also in light of the fact that it remains unclear from our study design whether the difference in infection rates between both strategies is caused by promotion of infections by heparin and/or prevention of this problem by taurolidine. We hope to shed light on this issue in a multicenter randomized controlled trial that is currently ongoing and which investigates the effectiveness of taurolidine versus saline in preventing CRBSI (ClinicalTrials.gov number, NCT01826526).

Despite the limitations of the study, we suggest that the long-term use of the lock solution taurolidine is more effective in preventing catheter related bloodstream infections and occlusions in HPN patients with CVCs than heparin.

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3

Absence of microbial adaptation to taurolidine in patients on home parenteral nutrition who develop catheter related bloodstream infections and use taurolidine locks

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ABSTRACT

Background

Some home parenteral nutrition (HPN) patients develop catheter related bloodstream infections (CRBSI) despite using an anti-microbial catheter lock solution taurolidine. The aim of this study was to assess whether long-term use of taurolidine leads to selective growth of microorganisms with increased taurolidine minimum inhibitory concentrations (MICs).

Methods

Bloodstream infections among 158 HPN patients with long-term taurolidine catheter locking were analyzed retrospectively. CRBSI-diagnosis was based on clinical symptoms, culture results, and absence of other sources of infections. CRBSIs were classified as definitive, probable or possible and exit site/ tunnel/ port or luminal infections. MICs were determined by broth microdilution.

Results

Between January 2009 and April 2011, 14 patients developed at least one luminal CRBSI episode during long-term taurolidine catheter locking (median (range)=451 (78-1394) days). Coagulase-negative *Staphylococcus species* or *Staphylococcus aureus* predominated among CRBSI-causing Gram-positive bacteria. Taurolidine MICs were 512 mg/l or less in 50% of these isolates (MIC_{50}). Taurolidine MIC_{50} for *Klebsiella pneumoniae* and *Escherichia coli*, the most common CRBSI-causing Gram-negative bacteria, were 256 and 512 mg/l, respectively. Taurolidine MIC_{50} among CRBSI-causing *Candida albicans* were 2048 mg/l.

Conclusion

Adaptation of microorganisms to taurolidine has not yet emerged as a factor in the pathogenesis of CRBSI in HPN patients with long-term taurolidine catheter locking.

INTRODUCTION

The main complication in home parenteral nutrition (HPN) patients is the development of repeated catheter-related bloodstream infections (CRBSI). Substantial morbidity and compromised options to obtain venous access are serious consequences of these CRBSI.[1]

In order to reduce the risk of developing CRBSI, several measures have been implemented in HPN care. As stated in the ESPEN guidelines, HPN patients and their caregivers should be trained in aseptic catheter handling, adopt an adequate policy of hand washing with disinfection of hubs and stopcocks and use 2% chlorhexidine as skin antiseptic. Furthermore, single lumen catheters should be used and subcutaneously cuffed to establish maximal barrier function.[2-4]

Prophylactic catheter locking with antiseptic agents is another strategy that might decrease the risk of developing CRBSI. Catheter locking with antibiotics does not seem to be effective in reducing the number of CRBSI and is not favorable because of the risk for the development of microbial resistance to these agents. Locking agents such as citrate and EDTA are not sufficiently promising for implementation in HPN care.[3] A recent meta-analysis promotes ethanol as an effective lock solution to prevent CRBSI in pediatric patients. Randomized controlled trials are however needed to confirm the efficacy of this locking solution.[5] A novel catheter locking solution with the potent antiseptic agent taurolidine, as a 2% (w/v) solution, dramatically decreased CRBSI in HPN patients.[6-8] In line with this finding, taurolidine has also shown efficacy in the prevention of a CRBSI in cancer and haemodialysis patients.[9-15]

Taurolidine is a bactericidal catheter lock solution that is rapidly converted into carbon dioxide and water. The mechanism of action of taurolidine has not been completely elucidated. Taurolidine reacts with the cell wall constituents of Gram-negative bacteria, and decreases microbial adhesion. These anti-microbial actions of taurolidine may be instrumental in prevention of CRBSI.[16, 17]

Because of the dramatic decrease in CRBSI in HPN patients using the taurolidine lock solution, our complete HPN population (currently 150 patients) switched from low-dose (150 u/mL) heparin to taurolidine catheter locking by the end of the year 2008. Although the emergence of microbial resistance to taurolidine has not been reported so far, the fact that some patients still develop CRBSI while using taurolidine might point towards selective growth of microorganisms with a phenotypic adaptation to taurolidine in the sense of increased minimum inhibitory concentrations (MICs) to this agent after its prolonged use. This notion urged us to assess taurolidine MICs of micro-organisms that caused CRBSI in HPN patients who used taurolidine as a catheter lock.

MATERIALS AND METHODS

Diagnosis of catheter-related bloodstream infection (CRBSI)

A diagnosis of CRBSI was made when the criteria described in Table 1 were met in patients who use taurolidine as a catheter lock, according to the protocol described

Table 1. Description of criteria for the likelihood and origin of catheter related bloodstream infection (CRBSI).

Likelihood of CRBSI	
Definitive [3]	Same microorganism detected in >1 percutaneous blood culture and in culture of catheter tip, or >2 hours before detection of microbial growth in blood sample from peripheral vein same microorganism detected in blood sample from catheter hub
Probable	positive blood culture, no proven criteria fulfilled and CRBSI highly likely based on clinical symptoms (fever > 38.5 °C, chills, hypotension, tachycardia, elevated white blood cell count and/or CRP rise) and no other apparent clinical and/or microbiological source of bloodstream infection.
Possible	positive blood culture, no proven criteria fulfilled, CRBSI possible based on clinical observations (fever > 38.5 °C, chills, hypotension, tachycardia, elevated white blood cell count and/or CRP rise)
Origin of CRBSI	
Luminal infection	Absence of clinical and microbiological proof of catheter tunnel or port infection.
Exit site/ tunnel/ port infection	Presence of clinical signs (pus seen on catheter removal, and/or redness, swelling or pain) and microbiological (same microorganism cultured from blood and catheter exit site or from pus at exit site) was seen as proof of a catheter tunnel or port infection. Absence of clinical signs (see above) of tunnel or port infection, but presence of a port culture with the same organism: origin of CRBSI left at discretion of attending physician.

by Bisseling *et al.*, from our tertiary referral centre for HPN care (Intestinal Failure Unit of the Department of Gastroenterology and Hepatology at the Radboud University Nijmegen Medical Centre) in Nijmegen, The Netherlands, from January 2009 until April 2011.[8] CRBSI episodes were categorized as being definitive, probable or possible (Table 1). An estimation was made as to whether the CRBSI originated from either the catheter lumen or from the exit site, catheter tunnel or -port (Table 1). Importantly, taurolidine is not used to treat CRBSI, but only in a prophylactic manner.

Minimum inhibitory concentration (MIC)

The determination of the taurolidine MIC by broth microdilution was performed according to the guidelines of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) [18, 19]. In short, taurolidine (kindly provided by Geistlich Pharma AG, Wolhusen, Switzerland) was diluted in cation-supplemented Mueller Hinton broth (CAMHB) at final concentrations of taurolidine: 0, 16, 32, 64, 128, 256, 512, 1024, 2048, 4092, 8192 mg/l CAMHB for bacteria or Roswell Park Memorial Institute (RPMI) 1640 with 2% dextrose for yeasts. To this end the stock solution of taurolidine (16384 mg/l) was prepared by mild heating and stirring of solid taurolidine into the CAMHB or RPMI 1640 with 2% glucose. The strains used in this study were isolates of microorganisms which caused the CRBSI in an HPN patient and reference strains. The strains were cultured overnight on blood agar plates for bacteria and Sabouraud agar plates for yeast. Inocula were diluted in 0.9% NaCl to a concentration of 0.5 McFarland.

Subsequently, the microorganisms were added to CAMHB or RPMI 1640 with 2% glucose in a final concentration of 5.0 * 10^5 CFU/ml. Plates were incubated at 35 ± 2°C in ambient air. The taurolidine MIC is defined as the lowest concentration of taurolidine without visible growth after 18 hours (bacteria) or 24 hours (*Candida species*) of culture. The taurolidine MIC of bacteria was manually assessed by two independent investigators. For yeasts, plates were read using an automated microplate reader spectrophotometer (Rosys Anthos HT3, Anthos Labtec Instruments GmbH, Salzburg, Austria).

Analysis

Total HPN use of the HPN patient was counted from the start of HPN use to the first positive blood culture from the luminal CRBSI. Total taurolidine use of the HPN patient was counted from the start of taurolidine use to the first positive blood culture from the luminal CRBSI. Catheter *in situ* duration of the catheter of the patient who developed a luminal CRBSI was counted from the day of insertion of the catheter to the first positive blood culture from this luminal CRBSI. Taurolidine use of the catheter of the patient who developed a luminal CRBSI was counted from the start of taurolidine use of this catheter to the first positive blood culture from this luminal CRBSI. MIC_{50} values were defined as the minimum inhibitory concentration required to inhibit the growth of 50% of these isolates. Values were expressed as median with range if applicable.

RESULTS

Diagnosis of luminal CRBSI in the HPN patients who use a taurolidine lock solution

During the study period, 17 out of 158 HPN patients developed 23 CRBSI episodes while using taurolidine as a catheter lock. In 9 out of these 23 CRBSI episodes, an exit site/tunnel infection could not be ruled out. The remaining 14 episodes were classified as either definitive, probable or possible luminal CRBSI (in 4, 4, and 6 episodes, respectively). Twenty-seven microorganisms caused eight monomicrobial and six polymicrobial cases of luminal CRBSI (Figure 1).

Description of HPN patients with the luminal CRBSI

As depicted in Table 2, a higher percentage of patients with a luminal CRBSI were female, and the median age of the patients with a luminal CRBSI was 49 years. Indications for HPN included motility disorder (n=5), high output stoma (n=2) and short bowel syndrome (n=2). The median duration of HPN use—counted from the start of HPN use to the first positive blood culture from the luminal CRBSI—was 569 days (range: 80-2786). The median duration of using taurolidine as a catheter lock—counted from the start of taurolidine catheter locking to the first positive blood culture from the luminal CRBSI—was 451 days (range: 78-1394).

Table 2. Characteristics of home parenteral nutrition (HPN) patients with luminal catheter related bloodstream infections. Where applicable, values are given as median (range) or number (n).

	Median (range)
Sex (n)	
Female	7
Men	2
Age, years	49 (17-66)
Indication for HPN (n)	
Motility disorder	5
Short bowel	2
High output stoma	2
Total HPN use, days	569 (80-2786)
Total taurolidine use, days	451 (78-1394)

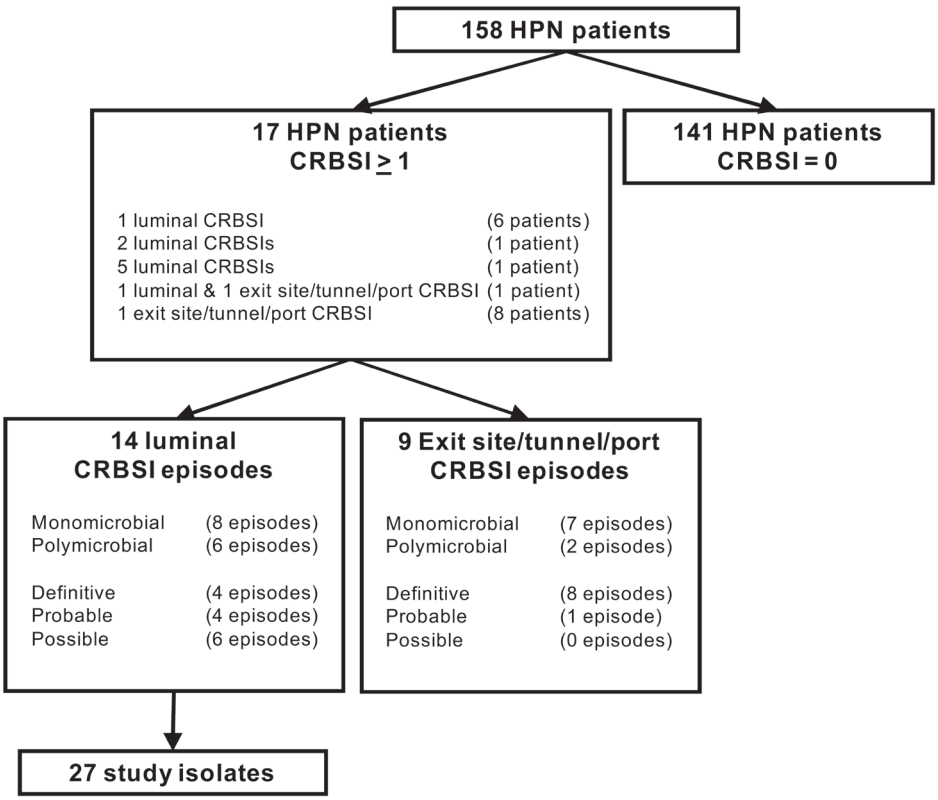

Figure 1. Flowchart showing home parenteral nutrition (HPN) patients with a catheter related bloodstream infection (CRBSI) and description of luminal and exit site/tunnel/port CRBSI resulting in 27 study isolates.

Description of the catheters of the HPN patients who developed a luminal CRBSI

Ten HPN patients with a Hickman catheter and 4 HPN patients with a port-a-cath (PAC) developed a luminal CRBSI (Table 3). These catheters had been in place for a median duration of 49 days (range: 3-994). Three of these 14 catheters could be salvaged despite the CRBSI, 11 catheters were removed. For these three catheters the minimal number of days *in situ* was taken – counted from the day of insertion of the catheter to the first positive blood culture of this catheter. Two catheters were initially locked with heparin after insertion and therefore not exposed to taurolidine for the whole placement duration: one catheter was exposed to taurolidine for 81 out of 442 days, the other for 702 out of 994 days, respectively. Ten out of 14 catheters were used daily for HPN administration. All catheters were locked with taurolidine after each round of HPN or fluid administration.

Minimum inhibitory concentration of luminal CRBSI causing microorganisms

The taurolidine MIC of reference strains of *Candida krusei* (ATCC 6258), *Candida parapsilosis* (ATCC 22019), *Escherichia coli* (ATCC 25922 and ATCC 35218), *Enterococcus faecalis* (ATCC 29212), *Staphylococcus aureus* (ATCC 29213) were 256, 64, 256, 256, 1024, 256, 512 mg/l, respectively. Among CRBSI causing isolates of HPN patients, Gram-positive bacteria, Gram-negative rods and Candida species accounted for 59, 22 and 19%, respectively. The CRBSI-causing Gram-positive bacteria were primarily coagulase-negative *Staphylococcus species* (n=10) or *Staphylococcus aureus* (n=8) with taurolidine MIC_{50} values of 512 mg/l. The taurolidine MIC_{50} for *Klebsiella pneumoniae* and *Escherichia coli*, the most common CRBSI-causing Gram-negative bacteria, were

Table 3. Characteristics of the catheters of the home parenteral nutrition (HPN) patients who developed a luminal catheter related bloodstream (CRBSI). Where applicable, values are given as median (range) or number (n). * The catheter was not removed during three episodes of luminal CRBSI. For these episodes, the minimal number of days was taken. ** Taurolidine was not started directly after insertion of two catheters.

	Median (range)
HPN/fluid use (n)	
7x HPN/week	10
6x HPN/week	2
3x HPN/week	1
3x Fluid/week	1
Type of catheter (n)	
Hickman	10
Port a cath	4
Catheter *in situ* duration (days)*	49 (3-994)
Taurolidine use (days)**	49 (3-702)

Table 4. Taurolidine minimum inhibitory concentrations (MICs) of luminal catheter related bloodstream infection causing microorganisms in home parenteral nutrition patients using taurolidine lock solution in this study and taurolidine MICs reported in the literature[21, 22]. Median (and range for this study) taurolidine MICs of this study are given for total number (n) of isolates of various microorganisms. * If the cumulative percentage of isolates of 50% are not exactly described in Nösner *et al.* [21], the values including the cumulative percentage of 50% are shown. N.D. = not determined. N.A. = not applicable.

	Taurolidine MIC (mg/l)				
	This Study			Nösner *et al.*	Torres-Viera *et al.*
	n	Median	Range	MIC^{50}*	MIC^{50}
Candida albicans	5	2048	2048-4096	500-1000	N.D.
Klebsiella pneumoniae	1	256	N.A.	125-250	250
Escherichia coli	2	512	512-512	250-500	500
Citrobacter freundii	2	256-2048	256-2048	125-250	500
Serratia marcescens	1	512	N.A.	125-250	500
Coagulase-negative staphylococci	9	512	256-512	125-250	500
Staphylococcus aureus	3	512	512-512	125-250	500
Enterococcus faecalis	2	512-1024	512-1024	250	500
Enterococcus faecium	1	512	N.A.	N.D.	500
Viridans Group streptococci	1	256	N.A.	60-125	250

256 (n=2) and 512 mg/l (n=2), respectively. CRBSI-causing *Candida albicans* isolates had higher taurolidine MIC_{50} (2048 mg/l) as compared to most bacteria (Table 4). The taurolidine MIC_{50}, defined as the minimum inhibitory concentration of taurolidine that is required to inhibit the growth of 50% of the isolates, was the same as the median taurolidine MIC concentration.

DISCUSSION

The main finding of the present study is that taurolidine MICs of CRBSI causing micro-organisms from HPN patients with a taurolidine lock are not different from previously reported taurolidine MICs. In other words, we found no evidence of the development of microbial adaptation to taurolidine during exposure to this catheter lock solution for up to 702 days. [20-22] In agreement with previous reports, taurolidine MICs for fungi were higher than for bacteria.[21]

For long-term HPN patients, it is crucial to have as few venous access complications as possible in order to maintain all options for adequate venous access. On the other hand, catheter lock therapy must be safe for these patients. Taurolidine is relatively non-toxic to humans, even at high concentrations.[20-24] This is exemplified by a case report of one HPN patient describing the administration of taurolidine as a 0.3% solution in the standard parenteral nutrition solution for five times a week during 12 months without side effects.[25] In line with these findings, our study shows for the first

time that the long-term use of taurolidine seems to be safe for up to 1394 days of taurolidine catheter locking.

Although taurolidine displays antimicrobial properties, it rather is a biocide and not an antibiotic.[26] Antibiotics act specifically via structures or metabolic processes of the microorganism, while biocides inactivate the microorganism through rather unspecific or several different mechanisms. Decreased activity of biocides does occur and is frequently a reversible phenotypic adaptation related to exposure to sublethal concentrations of the biocide. Nevertheless, some mechanisms of decreased activity of biocides may be stable genetic alterations, e.g. involving efflux pumps that might even negatively affect antibiotic sensitivity.[27]

Central venous catheters can be rapidly colonized with microorganism by formation of a biofilm, and these biofilms could potentially cause CRBSIs.[26] The type of lock solution can influence the formation of biofilms, and thereby the risk for developing a CRBSI. For example, 0.1 - 1,000 units/ml heparin and 0.2% citrate locks can stimulate biofilm formation, whereas 2 - 4% citrate and 0.2 – 50 mM EDTA prevent biofilm formation.[28] Ethanol seems to be effective in treatment and prevention of *C. albicans* and *S. aureus* monomicrobial and polymicrobial biofilms.[29] The effect of taurolidine on the development and progression of biofilm formation by various microbial species has not been characterized thoroughly. However, taurolidine showed anticolonizing activities, reduced initial counts of planktonic microbes and reduced the number of microorganisms from young biofilms, but not from older biofilms.[30, 31] Taurolidine is therefore suggested to be useful for the prevention of CRBSI.[26]

Whether the HPN patient is susceptible to developing a luminal CRBSI is likely determined by factors other than an increase in taurolidine MIC. In this respect, we recently found that immune functions are not compromised in HPN patients who do not have an active immune-mediated underlying disease.[32] The underlying disease and use of immunosuppressive medication can influence the immune function of patients and could result in increased susceptibility to developing a CRBSI. Other factors that may contribute to CRBSI in these patients, like patient compliance with taurolidine use, compliance with hygiene measures as well as taurolidine stability in relation to lock duration have not been assessed in this study.

Some limitations of the present study should be considered. Although most of the HPN patients in this study had used HPN for more than 1.5 years and used taurolidine as a catheter lock for more than 1 year, the catheters that became infected during taurolidine use had been in place for approximately 50 days. Therefore, the microorganisms that caused CRBSI in this study had been exposed to taurolidine for a relatively short periods, which makes predictions regarding taurolidine MICs after long-term exposure to taurolidine difficult.

Secondly, it was not always possible to determine retrospectively whether the patient had a luminal CRBSI or an exit site/tunnel/port infection. Because taurolidine is not expected to prevent exit site/tunnel/port infections, we excluded patients who had

a CRBSI with symptoms that could indicate an exit site/tunnel/port infection. Because of these strict exclusion criteria, the results were not biased by patients with such infections and we also eliminated the possibility of including false-positive exit site/tunnel/port CRBSI cases.

The best method to establish a diagnosis of CRBSI, if the catheter remains *in situ*, requires that the blood culture from the central venous catheter two hours earlier positive for the same organism as in the blood culture from the peripheral vein is, in short, that the differential time to positivity (DTP) is more than two hours. Unfortunately, in our study, the origin of the blood culture was not always registered.

The incidence of severe intestinal failure that necessitates HPN treatment is low. Combined with the decreased incidence of CRBSI when using taurolidine as the catheter lock solution, and our strict exclusion of potential exit site/tunnel/port CRBSI cases, this resulted in a rather small number of study isolates.[1] This limited power might rule out the detection of subtle changes in taurolidine MIC values. Future studies with more study isolates of microorganism causing CRBSI in home parenteral nutrition patients, maybe even in a multi-centre setting, are necessary to confirm that selective growth of microorganisms with a phenotypic adaptation to taurolidine in the sense of increased MICs is not a problem in using taurolidine as a catheter lock solution.

Furthermore, since the mechanism of action of taurolidine is only investigated in *E. coli*, it is important to investigate the mechanism of the antiseptic effect of taurolidine on gram-positive bacteria and fungi. More insight in the mechanism of action of taurolidine might elucidate why some patients who use taurolidine as a catheter lock solution still develop CRBSI.

In summary, although our results are based on a small number of samples, we found no evidence for adaptation of microorganisms to taurolidine in terms of altered taurolidine MICs, in HPN patients using this antiseptic catheter lock solution who still developed CRBSI.

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4

Microbiocidal effects of various taurolidine containing catheter lock solutions

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ABSTRACT

Background

We have recently shown that a catheter lock solution containing taurolidine dramatically decreases catheter related bloodstream infections (CRBSI) in patients on home parenteral nutrition (HPN) when compared to heparin. Since several taurolidine formulations are commercially available, some of which also contain citrate or heparin, we were interested in the effect of these different locks on growth and biofilm formation of fungal, Gram-negative and Gram-positive pathogens that are known to impede HPN treatment.

Methods

Clinical isolates obtained during CRBSI of HPN patients were grown in the presence of catheter locks (2% taurolidine, 1.34% taurolidine-citrate, 1.34% taurolidine-citrate-heparin, citrate and heparin) or phosphate buffered saline diluted in lysogeny broth medium for bacteria and sabouraud liquid medium for yeasts. Biofilm formation, assessed by crystal violet staining, and growth of clinical isolates were determined by optical density measurements.

Results

We found that 12.5x diluted solutions of all taurolidine containing formulations completely prevented growth of *Escherichia coli*, *Staphylococcus aureus* and *Candida glabrata*. Growth of these microbes was detected earlier in 1.34% taurolidine-citrate (-heparin) than in 2% taurolidine, while citrate and heparin did not inhibit growth of clinical isolates compared to PBS. No differences in biofilm formation were found between taurolidine containing solutions.

Conclusion

Taurolidine containing lock solutions prevent growth of fungal, Gram-negative and Gram-positive pathogens. While 2% taurolidine appears to be the most potent in this respect in this *in vitro* setting, the relevance of the small differences in growth inhibition between the commercially available taurolidine containing lock solutions for clinical practice remains to be established.

INTRODUCTION

Catheter-related bloodstream infections (CRBSI) are the foremost threat to continuation of treatment in patients who depend on central venous catheters (CVCs) for long-term intravenous (parenteral) nutrition due to severe intestinal failure.[1] CRBSIs are associated with a high risk for catheter loss, infection-associated morbidity and as such confer a considerable burden on healthcare resources. The most common microbial species that cause such infections are skin-derived Gram-positive bacteria, followed by Gram-negative bacteria and fungi. [2]

CRBSIs are usually preceded by catheter colonization, which implies deposition of microbes and biofilm formation on the extraluminal and intraluminal surfaces of these catheters, especially at the catheter hub.[3, 4] To prevent catheter contamination as a source of infection caregivers and patients are trained in meticulous aseptic catheter maintenance. Subcutaneously cuffed single lumen catheters are recommended for HPN care in order to establish maximal barrier function.[5]

Strategies to prevent intraluminal catheter colonization include use of anti-adhesive catheter biomaterials[6] and instillation of antimicrobial lock solutions to prevent bacterial attachment and minimize biofilm formation.[5] Taurolidine is such a potent broad spectrum antimicrobial agent that is used as part of catheter lock solutions. Taurolidine is non-toxic for humans and is rapidly metabolized into taurine, water and carbon dioxide.[7] The mechanism of action of taurolidine involves the chemical reaction with the microbial cell wall, endotoxins and exotoxins[8], thus inhibiting both pathogenicity and microbial adhesion[9] to inert and living surfaces. No evidence of the development of microbial adaptation to taurolidine after prolonged use of this catheter lock solution was found.[10]

Clinically, the use of taurolidine as catheter lock has been shown to decrease CRBSI rates in various patient groups compared to traditional catheter locks i.e. heparin.[11-15] Several structurally different taurolidine-based solutions are commercially available, some of which contain citrate and/or heparin because these locks are used in hemodialysis practice, implying that blood is aspirated via the catheter and anticoagulants are necessary to prevent catheter clogging.[14, 16-18] Although the relevance of citrate and heparin to prevent catheter-related thrombosis in this setting is undisputed because of their anticoagulant characteristics[16, 19], the use of these agents for prevention of CRBSI is under debate. Especially since heparin and citrate promote biofilm formation[20], and citrate is a substrate for the growth of *Escherichia coli*[21] and *Klebsiella pneumoniae*[22], i.e. microbial strains that are known to cause CRBSIs, and high-dose of 30% citrate can cause side effects, including tetany.[23]

We hypothesized that when used in a setting where anticoagulants are not deemed necessary, catheter lock solutions containing taurolidine without additives are more bactericidal compared to the same volume of taurolidine formulations containing citrate and/or heparin. To determine the antimicrobial effect of these formulations, the effects on growth and biofilm formation of fungi, Gram-positive and -negative bacteria were assessed.

MATERIALS AND METHODS

Growth of clinical isolates

Clinical isolates of *Escherichia coli* (*E. coli*), *Staphylococcus aureus* (*S. aureus*) and *Candida glabrata* (*C. glabrata*) were previously obtained during CRBSI episodes of HPN patients and were kindly provided by the Department of Medical Microbiology of the Radboud University Medical Center, Nijmegen, the Netherlands. Clinical isolates were collected during the hospital stay and diagnosis of the CRBSI. To determine the effect of lock solutions of growth inhibition, the clinical isolates were first cultured on agar plates at 37 °C, and eventually in Lysogeny Broth (LB) medium and Sabouraud Liquid Medium (SLM), for bacteria and yeasts, respectively, at 37 °C. Subsequently, the clinical isolates were incubated in 96 well plates (Greiner® flat bottom 96 well) at an initial optical density of 0.01 at 37 °C in the presence of 7x, 10x, 12.5x, 20x, 33x and 100x diluted lock solutions of catheter locks (Table 1) or phosphate buffered saline (PBS, control) in LB-medium and SLM medium for bacteria and yeasts, respectively. Growth of clinical isolates was evaluated by optical density measurement at 660 nm using a Fluorstar® Omega microplatereader continuously every 30 minutes for 60 hours (BMG Labtech, Germany) and data were analyzed using the MARS software package (BMG Labtech, Germany), and Microsoft Office Excel (Microsoft Corp, USA). [24]

Biofilm formation

After the evaluation of microbial growth of the clinical isolates, as described above, culture medium was removed, plates were washed four times in tap water and stained with 0.5% crystal violet solution for 10 minutes. Subsequently, the staining solution was aspirated, the plates were rinsed with tap water until all unbound crystal violet was removed. The plates were dried to air and 150 microliter of 95% ethanol with 2% acetic acid was added to dissolve the crystal violet bound to the biofilm.[25] Finally, absorption at 595 nm was measuredusing a Biorad iMark microplate reader (Bio-Rad Laboratories BV, Veenendaal, The Netherlands) to quantify the biofilm formation.

Table 1. Composition of evaluated catheter lock solutions according to the manufacturers.

	Manufacturer	Taurolidine (%)	Citrate (%)	Heparin (IU/mL)
Taurosept®	Geistlich	2	-	-
Taurolock®	TauroPharm	1.34	4	-
Taurolock-Hep®	TauroPharm	1.34	4	500
Heparin®	Pharmacy Radboud University Medical Center	-	-	500
Citrate	-	-	4	-

Statistical analyses

The effect of the catheter lock solutions on the growth of *E. coli*, *S. aureus* and *C. glabrata* was measured in duplicate, and each experiment was performed three times on separate days. A representative growth curve, presenting the optical density measurement at 660 nm every 30 minutes for 60 hours during incubation in media at 37 degrees, is presented in Figure 1 with the mean of two technical replicates. The growth time required for the culture to reach 50 percent of the maximum optical density of the PBS control (OD50) was determined in all individual growth curves. To calculate the effect of taurolidine on the growthrate, the growth time until OD50 in taurolidine containing lock solutions was divided by their respective PBS controls to correct for inter experimental differences. Next, we calculated the multiplicative inverse to obtain an growth value. This value was expressed as a growth percentage of the PBS control and presented in Figure 2. The experiment comparing the effect of 1.5 times diluted (to obtain similar taurolidine concentrations) pure 2% taurolidine to 1.34% taurolidine-citrate on growth of *E. coli*, *S. aureus* and *C. glabrata* was measured in quadruplicate and performed once. The mean of the in quadruplicate measured growth is presented in Figure 3. The effect of the catheter lock solutions on the biofilm formation of *E. coli*, *S. aureus* and *C. glabrata* was measured in duplicate, and each experiment was performed twice.

RESULTS

Taurolidine efficiently inhibits growth of *E. coli*, *S. aureus* and *C. glabrata*

The effect of different taurolidine-containing lock solutions (2% taurolidine, 1.34% taurolidine-citrate and 1.34% taurolidine-citrate-heparin), citrate, heparin and PBS on microbial growth was studied during 60 hours. Growth of *E. coli*, *S. aureus* and *C. glabrata* was inhibited in all taurolidine-containing catheter lock solutions which were less than about 12.5x diluted, growth of these microorganisms was still absent after 60 hours of culture. More than 100x diluted taurolidine containing solutions were not able to inhibit microbial growth, resulting in similar growth curves as during incubation with PBS. The growth of *E. coli*, *S. aureus* and *C. glabrata* in media with citrate or heparin was also similar to the growth in media with PBS.

Minor differences in microbial growth between extremely diluted taurolidine-containing lock solutions

Minor differences in efficiency of catheter lock solutions in inhibiting growth of *E. coli*, *S. aureus* and *C. glabrata* were observed (Figure 1 and 2). A solution containing 2% taurolidine was able to inhibit growth of *E. coli* about ten hours longer than 1.34% taurolidine-citrate-heparin and 1.34% taurolidine-citrate (Figure 1A). The growth of *S. aureus* was more efficiently inhibited using 2% taurolidine than 1.34% taurolidine-

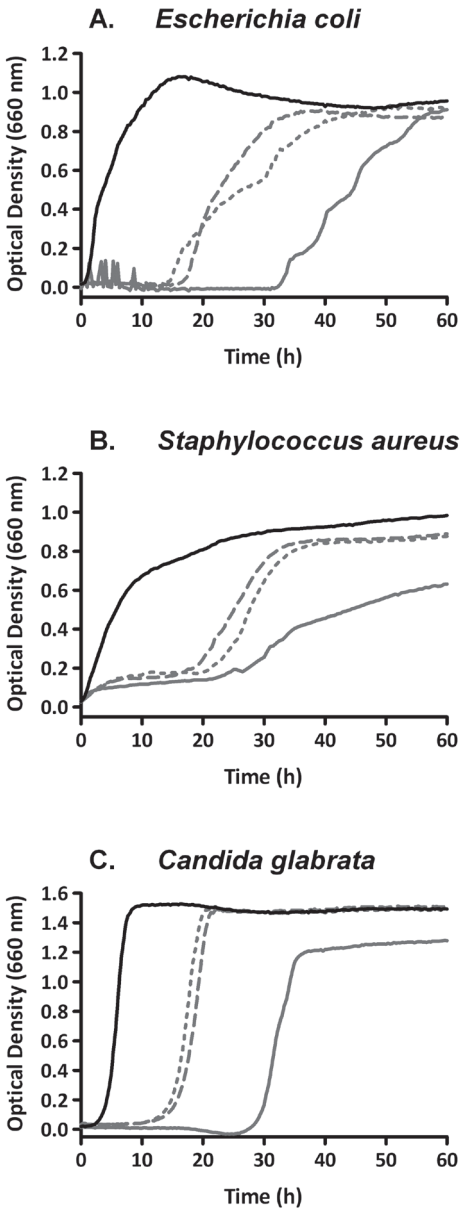

Figure 1. Differences in growth of *Escherichia coli* (A.), *Staphylococcus aureus* (B.) and *Candida glabrata* (C.) between different taurolidine containing catheter lock solutions and PBS as determined by optical density measurement at 660 nm every 30 minutes for 60 hours during incubation in media at 37 degrees. The presented lock solutions are stock solutions of 2% taurolidine (grey straight line), 1.34% taurolidine-citrate (grey dotted line), 1.34% taurolidine-citrate-heparin (grey striped line), and PBS (black straight line, control), which were before the start of the experiment 20x (A.), 50x (B.) and 33x (C.) diluted in LB-medium (A. + B.) or SLM medium (C.). Each figure is the result of one single experiment with mean values of two technical replicates. Each figure is representative for in triplicate performed experiments on separate days.

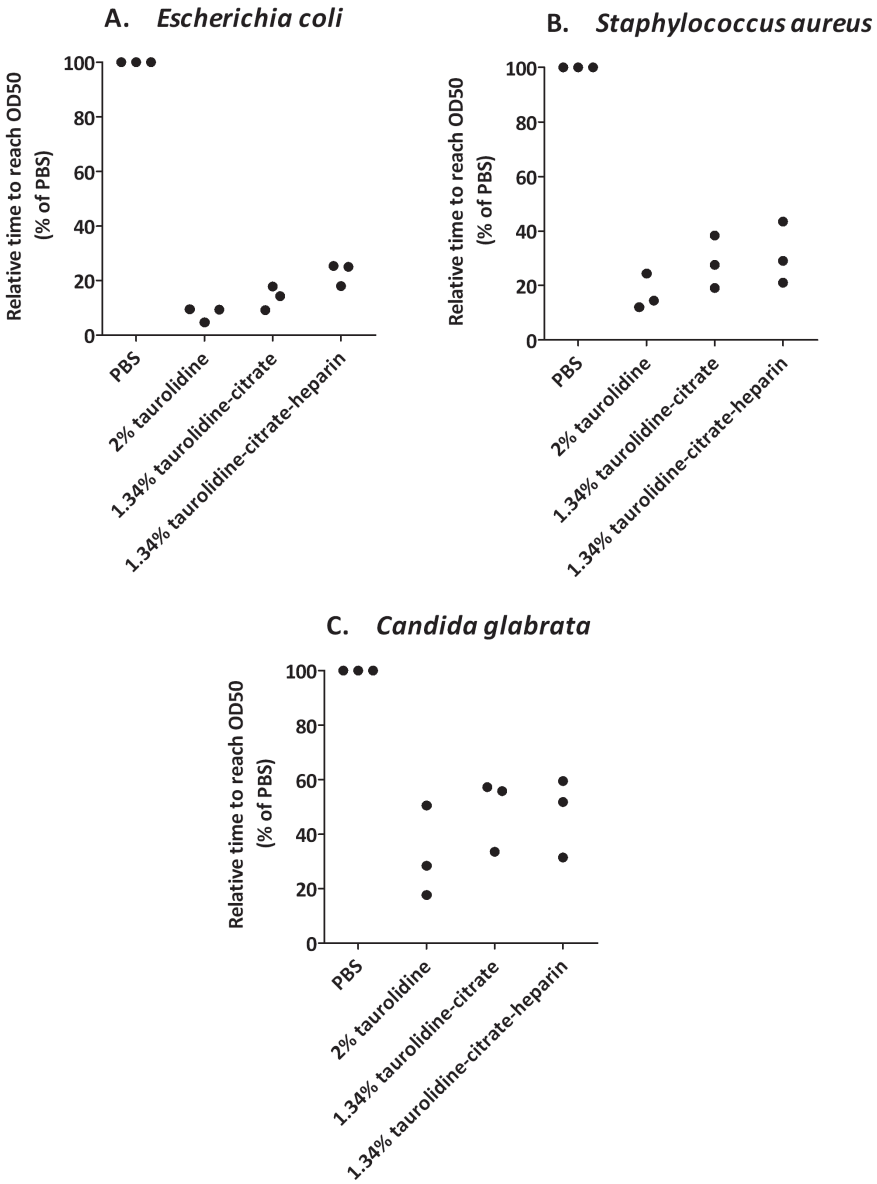

Figure 2. Differences in the time required to reach 50 percent of the maximum optical density (OD50) of *Escherichia coli* (A.), *Staphylococcus aureus* (B.) and *Candida glabrata* (C.) in different taurolidine containing catheter lock solutions: 2% taurolidine, 1.34% taurolidine-citrate and 1.34% taurolidine-citrate-heparin. Before the start of the experiment the lock solutions were 20 - 100 times diluted in LB-medium (*Escherichia coli* and *Staphylococcus aureus*) or SLM medium (*Candida glabrata*). The growth time required for the culture to reach 50 percent of the maximum optical density of the PBS control (OD50) was determined in all individual growth curves. The growth time until OD50 in taurolidine containing lock solutions was divided by their respective PBS controls. the multiplicative inverse was calculated was expressed as a growth percentage of the PBS control. The figure is the result of three independent experiments per clinical isolate with mean values of two technical replicates.

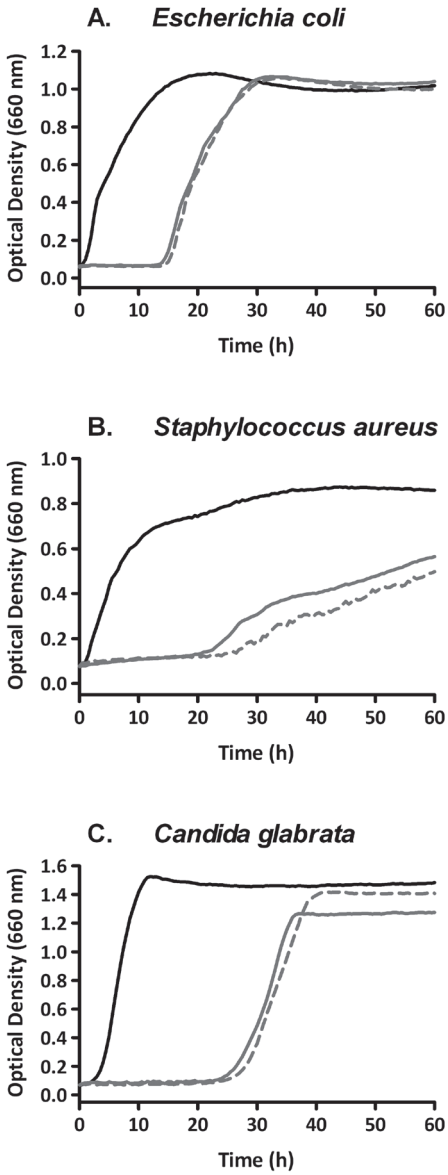

Figure 3. Differences in growth of *Escherichia coli* (A.), *Staphylococcus aureus* (B.) and *Candida glabrata* (C.) between two catheter lock solutions with similar taurolidine concentrations, and PBS, as determined by optical density measurement at 660 nm every 30 minutes for 60 hours during incubation in media at 37 degrees. The presented lock solutions are the 1.34% taurolidine-citrate solution (grey striped line) and the 1.5 times diluted 2% taurolidine solution (grey straight line), resulting in similar taurolidine concentration in both experimental conditions. PBS (black straight line) is used as a control. The lock solutions and PBS were diluted in LB-medium (bacteria) or SLM medium (yeast): A. + C.) 50x diluted 2% taurolidine, 33x diluted 1.34% taurolidine-citrate, 33x diluted PBS, B) 33x diluted 2% taurolidine, 22x diluted 1.34% taurolidine-citrate, 33x diluted PBS. Each figure is the result of one experiment with mean values of in quadruplicate determined optical densities.

citrate or 1.34% taurolidine-citrate-heparin (Figure 1B). Increased (by about 10 hours) growth inhibition of *C. glabrata* was found using 2% taurolidine compared to 1.34% taurolidine-citrate and 1.34% taurolidine-citrate-heparin (Figure 1C). No large differences in microbial growth were found between 1.34% taurolidine-citrate and 1.34% taurolidine-citrate-heparin. The time at which OD50 in taurolidine containing lock solutions was reached was lower than in PBS, and the OD50 of 2% taurolidine was slightly lower than 1.34% taurolidine-citrate and 1.34% taurolidine-citrate-heparin in all three clinical isolates: *E. coli* (Figure 2A), *S. aureus* (Figure 2B) and *C. glabrata* (Figure 2C).

Microbial growth is equally inhibited when 2% taurolidine is diluted to a taurolidine concentration of 1.34% and compared to 1.34% taurolidine-citrate

To investigate what the effect is of 1.5 times difference in taurolidine concentration and what the effect is of addition of citrate, the commercially available 2% taurolidine catheter lock solution was diluted to a taurolidine concentration of 1.34%, which was the taurolidine concentration present in the other two commercially available taurolidine containing lock solutions. Microbial growth is similarly inhibited when 2% taurolidine is diluted to a taurolidine concentration of 1.34% compared to 1.34% taurolidine-citrate (Figure 3).

No differences in biofilm formation between catheter lock solutions

To investigate the effect of different catheter lock solutions on biofilm formation, biofilm formation was assessed using crystal violet staining directly after ending the measurement of microbial growth (after 60 hours). In *E. coli*, *S. aureus* and *C. glabrata* an increase in the concentration of the lock solution resulted in a decrease in biofilm formation, which was directly correlated to the growth observed. No additional effect on biofilm formation was observed that could not be explained by the growth inhibition. No differences were found in biofilm formation between the different catheter lock solutions at the dilutions tested after 60 hours.

DISCUSSION

Our results confirm the very potent antimicrobial properties of taurolidine, present in concentrations ranging from 1.34% to 2% in currently available catheter lock solutions, against relevant microbial species. Our study showed that concentrations of 110 to 160mg/l of taurolidine, still successfully inhibited the growth of fungal, Gram negative and Gram positive pathogens. This is in agreement with earlier studies that showed minimal inhibitory concentrations of taurolidine ranging from 125 to 500 mg/l for bacteria, and 500 to 1000 mg/l for fungi[26-28], even after prolonged exposure to a taurolidine containing lock solution.[10]

The parallel evaluation of all catheter lock solutions enabled us to compare and detect even small differences in microbiocidal efficiency between these catheter lock

solutions. Over time some spilling of catheter lock solution into the bloodstream can be expected to occur in HPN patients, also depending on the type of catheter with or without side holes[29], making it useful to test different dilutions of the catheter lock solutions in our *in vitro* setting. Minor differences in growth inhibition were found between various commercially available taurolidine locks using similar volumes at high dilutions (Figure 1 and 2): here the strongly diluted pure 2% taurolidine formulations had the most potent antimicrobial effect compared to strongly diluted 1.34% taurolidine-citrate(-heparin) formulations. The growth of these pathogens in these strongly diluted taurolidine containing lock solutions started at least 13 hours later than in PBS. Since the growth is not completely inhibited, this suggests for clinical practice regular refreshing of the catheter lock solution in HPN patients. Nevertheless, at the concentration used normally in undiluted form in catheters of HPN patients, all tested lock solutions containing taurolidine completely inhibited bacterial growth. The implications of the minimal differences in microorganism growth at strong dilutes are not very likely to be relevant in the clinical setting. More clinical studies are needed to determine the clinical relevance.

The small differences in microbial growth between 2% taurolidine on the one hand and the catheter lock solutions containing the mixtures 1.34% taurolidine-citrate and 1.34% taurolidine-citrate-heparin on the other hand can be explained by a factor 1.5 difference in taurolidine concentration. Our study showed that dilution of the commercially available 2% taurolidine lock solution to a concentration of 1.34% taurolidine did not remain more efficient in inhibiting microbial growth (Figure 3), still keeping in mind that our tested lock solutions are used undiluted in catheters of HPN patients.

We did not find an additional effect of the taurolidine containing lock solutions on biofilm formation that could not be explained by an inhibition of growth. No differences in biofilm formation were found between the tested taurolidine containing lock solution dilutions. The absence of a difference in biofilm formation, where a small difference in growth was observed, can be explained since the biofilm formation was determined at only a single time point after 60 hours of growth, when growth, although delayed, had occurred.

We found that citrate and heparin alone did not inhibit the *in vitro* growth of *E. coli*, *S. aureus* and *C. glabrata*. Previous studies have shown that heparin and low concentrations of citrate may even stimulate biofilm formation of *S. aureus* [20] ,however, we did not observe this in our experimental conditions. Citrate has shown to possess antimicrobial properties, however, only at a concentration of 30%.[23, 30] A drawback of citrate is that *E. coli* and *Klebsiella pneumoniae*, which are known to cause CRBSI, metabolize citrate in fermentation products.[21, 22] This may imply that these microorganisms could benefit from the presence of citrate in catheter lock solutions. However, our study did not find any evidence for detrimental effects of citrate and/or heparin on microbiocidal growth or biofilm formation, since no major differences

were found in microbial growth at similar taurolidine concentrations with or without additional citrate and/or heparin.

Also, thrombosis and microbe-induced coagulopathy can dramatically increase the risk of CRBSI.[31] Taurolidine has shown to be able to decrease thrombus weight, but is not as effective as low-molecular weight heparin in this respect.[32] Patients at risk of thrombosis may therefore benefit from the addition of the anti-coagulants citrate and/or heparin to the taurolidine containing lock solutions. Evidently, such balancing of pro's and con's of the use of additives requires clinical studies for a final verdict.

In conclusion, we confirm that the taurolidine containing lock solutions have a potent microbiocidal effect on fungal, Gram-positive and Gram-negative pathogens. The commercially available lock solution with a higher concentration of taurolidine has a more potent effect on growth inhibition, but the relevance of this seemingly minor difference for clinical practice remains to be established. Furthermore, we have shown that the addition of citrate and/or heparin does not influence the microbiocidal effect of the taurolidine solution for the microbes under investigation in this *in vitro* setting.

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B

Lipids

5

Immune activation by medium-chain triglyceride-containing lipid emulsions is not modulated by n-3 lipids or toll-like receptor 4

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ABSTRACT

Background

Saturated medium-chain triglycerides (MCT) as part of the parenteral lipid regimen (50% MCT and 50% long chain triglycerides (LCT)) activate the immune system *in vitro*. Fish oil (FO)-derived n-3 fatty acids (FA) inhibit saturated FA-induced immune activation via a toll-like receptor (TLR)-4 mediated mechanism. We hypothesized that effects of parenteral MCTs on immune cells involve TLR-4 signaling and that these effects are modulated by n-3 FA that are present in FO.

Methods

To test this hypothesis we assessed effects of addition of various commercially available mixed parenteral lipid emulsions, n-3 FA and of TLR-4 inhibition on MCT-induced human immune cell activation by evaluation of the expression of leukocyte membrane activation markers and reactive oxygen species (ROS) production.

Results

All MCT-containing lipid emulsions activated leukocytes by inducing changes in expression of membrane markers and stimulus induced ROS production, whereas MCT-free lipid emulsions lacked this effect. Moreover, addition of n-3 FA to LCT/MCT did not prevent MCT-induced immune activation. TLR-4 inhibitors did not distinctly modulate MCT-induced changes in immune function.

Conclusion

Taken together, these findings suggest that leukocyte activation by parenteral MCTs does not involve TLR-4 signaling and is not modulated by n-3 FA in FO-, but is exerted via different signaling pathways.

INTRODUCTION

The first commercially available total parenteral nutrition (TPN) was based on 100% soybean oil, containing long chain triglycerides (LCTs). Later, part of the LCTs were replaced by medium chain triglycerides (MCTs), because of the suspected pro-inflammatory properties of LCTs, and this resulted in a 50/50% (v/v) mixed emulsion (LCT/MCT). More recently developed commercially available TPN also contain fish oil (FO), rich in the n-3 poly-unsaturated fatty acids (PUFAs) eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), and/or olive oil (OO), rich in the n-9 mono-unsaturated fatty acid (MUFA) oleic acid. Bioactive lipid mediators derived from EPA and DHA display anti-inflammatory properties and are involved in the resolving phase of inflammation, whereas oleic acid has been found to be more immune neutral in nature.[1]

The increased risk for infectious complications seen in patients on TPN has been related to lipid-induced disturbances of immune functions.[2] Changes in the composition of lipids in cell membrane phospholipids and lipid rafts influence physicochemical properties of immune cell membranes and may subsequently have distinct effects on cell signaling and gene expression.[2] *In vitro* studies suggest that especially the saturated MCTs present in the lipid emulsion LCT/MCT, can activate the immune activation, which seems to impair the functional capacity of leukocytes to migrate and to kill microbes *in vitro*.[3-15] To explain these *in vitro* MCT effects we showed earlier that nutritional lipids distinctively influence leukocyte signaling and stimulation through effects on intracellular calcium mobilization and protein kinase C (PKC) activation. More specifically, MCTs, but not LCTs sensitize neutrophils for activation by yeast particles in a PKC-dependent manner.[16] We also found that parenteral lipids can evoke a prompt and significant attenuation of hormone N-formyl-methionyl-leucyl-phenylalanine (fMLP)-induced neutrophil stimulation and that emulsions based on FO and MCTs are among the most potent ones in this respect.[11] The exact mechanism of MCT-induced immune activation remains however unknown.

A suggested mechanism for saturated fatty acid (SFA)-induced immune activation involves toll-like receptor 4 (TLR-4) signaling. TLR-4 is important during pathogenic infections since lipopolysaccharides (LPS) from Gram-negative bacteria are recognized by TLR-4.[24] SFAs, like the 12-carbon medium chain FA lauric acid, can directly induce TLR-4 dependent signaling leading to immune activation.[17-23] Unlike SFAs, n-3 PUFAs, such as DHA, can inhibit agonist-induced TLR activation.[18, 25, 26] *In vitro* addition of DHA inhibits SFA-induced activation of human monocytes and mouse dendritic cells.[17, 25, 26] The mechanism behind this effect is that DHA inhibits SFA-induced homodimerization of TLR-4 and recruitment of TLR-4 into lipid rafts, i.e. the initial step of TLR-4 signaling pathways.[17, 18, 26, 27]

Therefore, we hypothesized, that the previously observed *in vitro* immune cell activation by saturated MCTs in LCT/MCT, might be mediated by TLR-4 and might be abolished by the addition of anti-inflammatory n-3 PUFAs present in FO. To test this hypothesis we studied the effects of addition of various commercially available

MCT-containing and MCT-free parenteral lipid emulsions, n-3 FA and TLR-4 inhibition on MCT-induced immune cell activation by determination of expression of membrane activation markers and stimulus-induced ROS production.

METHODS

Materials

Ethyl (6R)-6- [N- (2-chloro-4-fluorophenyl) sulfamoyl] cyclohex-1-ene-1-carboxylate (TAK-242, Cayla Invivogen, Toulouse, France) selectively suppresses ligand-dependent and-independent TLR-4 signaling by binding to the amino-acid side chain at position Cys747 of the intracellular domain of TLR-4 and without antagonizing the binding of the TLR-4 agonist LPS.[28] A concentration of 5 µmol/L TAK-242 was used to inhibit TLR-4, based on manufacturer's instructions and Kawamoto *et al.*[28]

Bartonella quintana lipopolysaccharide (*B. quintana* LPS) was kindly provided by LA Joosten, Department of Internal Medicine, Radboud University Medical Center, Nijmegen, the Netherlands. *B. Quintana* LPS is a potent antagonist of TLR-4. A concentration of 0.1 ng/ mL was used, which can antagonize a strong immune activation due to a concentration of 0.01 ng/mL *Escherichias coli* LPS.[29]

Commercially available lipid emulsions included (Table 1): Lipofundin® (LCT/MCT), Lipoplus® (LCT/MCT/FO), SMOFlipid® (LCT/MCT/FO/OO), Structolipid® (SL), Intralipid® (LCT20%), Lipovenos® (LCT10%), Omegaven® (FO), Clinoleic® (LCT/OO).

Preparation of fatty acid micelles

Two mixtures of micelles of dipalmitoyl phosphatidylcholine (DPPC, Sigma Aldrich, USA) with EPA (Cayman chemicals, USA), and DPPC with DHA (Cayman chemicals, USA) were prepared as described previously.[8] Fatty acids (FA, 48 mmol/L) and DPPC (10.9 mmol/L) were dissolved in chloroform and 1:1 mixed in a 10 ml glass tube under nitrogen. After evaporation of the solvent under nitrogen, HBSS (Invitrogen/Life Technologies Corporation, USA) was added and the mixture was sonicated for 5 min at 48 kHz. The final concentrations of DPPC and FA in the micelles were 0.545 and 2.4 mmol/L, respectively.

Functional analysis of leukocytes

Blood samples drawn from human healthy volunteers were collected in 10 mL Monoject tubes with 170 IU of lithium heparin (Beliver Industrial Estate, Plymouth PL6 7BP, UK). Leukocyte functions were determined in whole blood by evaluating the expression of surface membrane activation markers and the production of reactive oxygen species.

Blood incubations

For the flow cytometric analyses and the determination of oxygen radical production whole blood was incubated with lipid emulsions, micelles or TLR-4-inhibitors. To assess the effect of commercial lipid emulsions on surface membrane markers and stimulus-induced ROS production, whole blood was incubated with lipid emulsions (LCT/MCT,

Table 1. Characteristics of parenteral lipid emulsions and fatty acid (FA) composition. Lipid emulsions contain long chain triglycerides (LCT), medium chain triglycerides (MCT), fish oil (FO), olive oil (OO) and/or structured lipids (SL). (3, 41, 42)

Characteristics	LCT/MCT	LCT/MCT/FO	LCT/MCT/FO/OO	SL	LCT20%	LCT10%	FO	LCT/OO
Brand-name	Lipofundin®	Lipoplus®	SMOFlipid®	Structolipid®	Intralipid®	Lipovenos®	Omegaven®	Clinoleic®
Manufacturer	B. Braun	B. Braun	Fresenius Kabi	Fresenius Kabi	Fresenius Kabi	Fresenius Kabi	Fresenius Kabi	Baxter
Lipid emulsion (%)	20	20	20	20	20	10	10	20
Mean molecular weight (g/mol)	634	636	732	683	865	865	882	873
Oil composition (% of total)								
LCT	50	40	30	36*	100	100	-	20
MCT	50	50	30	64*	-	-	-	-
OO	-	-	25	-	-	-	-	80
FO	-	10	15	-	-	-	100	-
Fatty acid composition (% of total)								
Saturated FA - MCT	50.0	51.9	28.7	36.3	-	-	-	-
Saturated FA - LCT	9.40	8.30	13.1	10.0	15.0	21.9	21.9	14.5
Mono- Unsaturated FA	11.0	11.9	30.0	14.0	24.0	25.9	25.9	63.7
Poly – Unsaturated FA	33.8	27.9	28.2	40.0	61.1	52.3	52.3	21.8

* Structolipid contains synthetic structured lipids, with medium- and long-chain fatty acids randomly distributed within a single triglyceride molecule.

LCT/MCT/FO, LCT/MCT/FO/OO, SL, LCT20%, LCT10%, FO or LCT/OO) at a clinically relevant concentration of 5 mmol/L by gentle head-over-head turning for 1 hour. The effect of two n-3 PUFAs, EPA and DHA was determined by the incubation of whole blood with LCT/MCT in the absence and presence of micelles with a concentration of 0.545 mmol/L EPA or DHA. Blood was pre-incubated for 1 hour with micelles of EPA or DHA, and subsequently incubated for 1 hour with these micelles and LCT/MCT. To investigate whether LCT/MCT induced immune activation is mediated by TLR-4, two specific TLR-4 inhibitors were used: 0.1 ng/ml *B. quintana* LPS or 5 μM TAK-242. Blood was pre-incubated for 1 hour with specific TLR-4 inhibitors, and subsequently incubated for 1 hour with these TLR-4 inhibitors and LCT/MCT. As a control, in all experiments whole blood without addition of lipid emulsions, micelles of EPA or DHA or TLR-4 inhibitors was also determined. All incubations were at 37 °C.

Surface activation markers

Immunofluorescent staining followed by flow-cytometric analysis was done to determine markers for activation. These activation markers are expressed on the membrane surface of granulocytes and monocytes, as described previously.[30] Characterization of surface markers was done using antibodies (purchased from Beckman Coulter, Miami, FL, USA). Monocytes and granulocytes were gated based on the expression of glycosyl-phosphatidylinositol-linked single-chain surface membrane glycoprotein (CD14-PE-Cy5) and tyrosine phosphatase (CD45-ECD), respectively. Antibodies directed against an adhesion molecule of the β2 integrin family (CD11b-PE), a degranulation marker of azurophilic (CD63-PE) or specific granulae (CD66b-FITC) and L-selectin (CD62L-FITC) were used to determine the surface membrane expression. Immune-fluorescent staining was performed according to the "lyse and wash" method (BD Biosciences, USA); 100 microliter of whole blood was incubated with an antibody mixture (CD14/CD45/63/CD62L or CD14/CD45/CD11b/CD66b) in tubes for 15 minutes at room temperature (RT), protected from light. Subsequently, 2 milliliter of BD Pharm Lyse™ lysing solution was added and incubated for 10-15 minutes at RT, protected from light. Tubes were centrifuged at 500*g* for 5 minutes at RT. Supernatant was removed, 2 ml of Phosphate Buffered Saline (PBS) was added and tubes were centrifuged at 500*g* for 5 minutes at RT. Supernatant was removed and 500 microliter of 1% paraformaldehyde was added. Flow cytometry analyses were performed on a Beckman Coulter Cytomics FC500 (Miami, FL, USA).

Oxygen radical production

Spontaneous and stimulus-induced oxygen radical production in whole blood was evaluated using Luminol-enhanced chemiluminescence and the total amount of ROS production was determined in an automated LB96V Microlumat Plus Luminometer (EG & G Berthold, Bald Wilberg, Germany), as described in detail previously.[30] Briefly, 1:100 in PBS diluted whole blood was added to a 96-well microplate, either without, or in the presence of 0.4 μg/mL receptor-independent (phorbol 12-myristate 13-acetate, PMA)

or 0.8 mg/mL receptor-dependent (serum-treated zymosan particles, STZ) stimulus. Luminol (Sigma Aldrich, USA) was added to each well to start the chemiluminescence reaction. Each measurement was carried out at least in triplo. Luminescence was expressed as relative light units per second (RLU/sec). Data were analyzed with Winglow software (EG & Berthold).

Overall antioxidant capacity

The balance between antioxidants and oxidants was assessed in the lipid emulsions by determining the overall antioxidant capacity of the emulsion. The antioxidant capacity was determined using the ferric reducing ability of plasma (FRAP) assay, using the method of Benzie and Strain[31], as previously described.[30, 32] Standards ($FeSO_4 \cdot 7H_2O$) and samples of the various lipid emulsions were added to the FRAP working solution (2.5 mL 10 mmol/L 2,4,6,-tripyridyl-s-triazine (Fluka Chemika) in 40 mmol/L HCl, 2.5 mL 20 mmol/L $FeCl_3 \cdot 6H_2O$ and 25 mL 300 mmol/L acetate buffer pH 3.6). In the presence of antioxidants, ferric ions are reduced to ferrous ions, which cause a colored ferrous-2,4,6-tripyridyl-s-triazine complex to be formed. Absorbance was measured at 593 nm on a Perkin-Elmer Spectrophotometer. Values are expressed in µmol Fe^{2+}/L. All solutions were measured in duplo.

Statistical analyses

Values are expressed as median with interquartile ranges and relative to control medium (meaning that control medium was set at 1). Stimulated ROS production was corrected for unstimulated ROS production. Differences between lipids and lipid-free medium were analyzed using the nonparametric Wilcoxon matched-pair signed-rank test. The additional effect of EPA, DHA, TAK-242 or *B. quintana* LPS to LCT/MCT induced immune-activation was analyzed using the Wilcoxon matched-pair signed-rank test. Differences were considered significant if a p-value of <0.05 was obtained. All statistical analyses were performed using SPSS software (version 20.0; SPSS, Inc., Chicago, IL, USA).

RESULTS

MCT-containing lipid emulsions activate leukocytes

To assess the effect of commercial lipid emulsions on surface membrane markers and stimulus-induced ROS production, blood was incubated with lipid emulsions at a clinically relevant concentration of 5 mmol/L by gentle head-over-head turning at 37 °C for 1 hour (Figure 1). Incubation with 5 mmol/L MCT-containing lipid emulsions (LCT/MCT, LCT/MCT/FO, LCT/MCT/FO/OO) resulted in activation of surface membrane markers compared to lipid-free incubations (Figure 1A & B): i.e. on granulocytes a significantly decreased expression of L-selectin (30, 36 and 24%, respectively) in combination with an increased expression of the adhesion marker (46, 48 and 37%, respectively) and the specific degranulation marker (71, 83 and 62%, respectively), and on monocytes

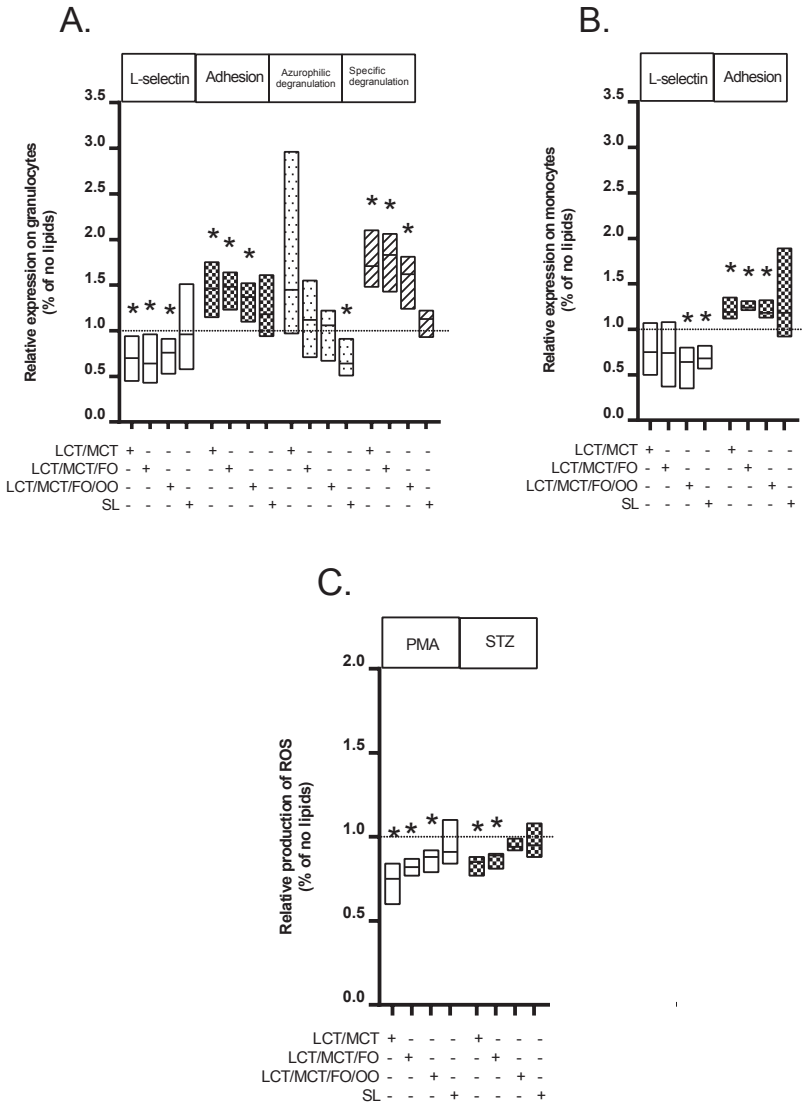

Figure 1. Effects of 5 mmol/L of MCT-containing parenteral lipid emulsions on the immune system. The expression of surface activation markers of L-selectin, adhesion, azurophilic and specific degranulation on A) granulocytes and B) monocytes, and the effect on C) phorbol 12-myristate 13-acetate (PMA) and serum treated zymosan (STZ) induced radical oxygen production (ROS) in whole blood were assessed. All samples were incubated for 1 hour at 37°C and compared to the control medium containing no lipids. The results are presented as median with interquartile range of six separate experiments. *A p-value of < 0.05 as tested by Wilcoxon's matched-pair signed-rank test was considered to be significantly different from lipid free medium.

an increased expression of the adhesion marker (19%, 24%, 18%, respectively) was found. The expression of L-selectin on monocytes was significantly decreased only after incubation in LCT/MCT/FO/OO (36%). The expression of the azurophilic degranulation marker was not different in the MCT-containing lipid emulsions. Incubation in SL, which contains synthetic structured lipids, resulted in a significantly decreased expression of azurophilic degranulation marker (36%) on granulocytes and L-selectin (32%) on monocytes, whereas other surface membrane markers were not affected. Incubation in MCT-containing lipids resulted in a decreased ROS production (Figure 1C). PMA- and STZ-induced ROS production was significantly decreased after incubation in LCT/MCT (25 and 15%, respectively), LCT/MCT/FO (18 and 11%, respectively) and LCT/MCT/FO/OO (PMA: 12%). ROS production was unaltered in the other conditions.

MCT-free lipid emulsions do not activate leukocytes

Immune cells exposed to 5 mmol/L MCT-free lipid emulsions (LCT20%, LCT10%, FO and LCT/OO) for 1 hour at 37 °C displayed similar outcomes when compared with cells not exposed to lipids. Only incubation in LCT10% and LCT/OO resulted in immune cell activation, as was seen by a significant decrease of expression of L-selectin on monocytes of 43% and 36% . The expression of other surface activation markers on granulocytes and monocytes were not statistically different from lipid-free medium, or were significantly decreased while an increase in expression would imply immune activation. PMA- and STZ-induced ROS production were not changed after incubation in MCT-free lipid emulsions, except for a significant (by 13%) decrease in PMA-induced ROS production after incubation in FO.

Differences in total antioxidant capacity between different lipid emulsions

The total antioxidant capacity of all commercial available lipid emulsion at a concentration of 5 mmol/L was measured in duplo. The order of highest to lowest total antioxidant capacity was: LCT20% (478 µmol Fe^{2+}/L), LCT/OO (386 µmol Fe^{2+}/L), FO (358 µmol Fe^{2+}/l), LCT/MCT/FO/OO (348 µmol Fe^{2+}/L), SL (301 µmol Fe^{2+}/L), LCT10% (266 µmol Fe^{2+}/L), LCT/MCT/FO (258 µmol Fe^{2+}/L) and LCT/MCT (196 µmol Fe^{2+}/L).

Leukocyte activation by LCT/MCT is not abolished by micelles of EPA or DHA

To assess the effect of two n-3 PUFAs, EPA and DHA, blood was incubated with LCT/MCT in the absence and presence of micelles of EPA or DHA (Table 2). Incubation with EPA and DHA alone did not significantly change the expression of surface membrane markers and stimulus induced ROS production. Incubation in LCT/MCT in the presence of micelles of EPA or DHA did not decrease the immune activation status of leukocytes compared to incubation with LCT/MCT alone. Incubation in LCT/MCT and EPA-containing micelles aggravated the MCT-induced immune cell activation: it significantly decreased L-selectin by 8% and, significantly increased specific degranulation marker

Table 2. Effects of 5 mmol/L long chain triglycerides (LCT)/medium chain triglycerides (MCT) in the presence or absence of 2.4 mmol/L micelles of eicosapentaenoic acid (EPA) or docosahexaenoic acid (DHA) on the immune system. The expression of surface activation markers of L-selectin, adhesion, specific and azurophilic degranulation on granulocytes and monocytes, and phorbol 12-myristate 13-acetate (PMA) and serum treated zymosan (STZ) induced radical oxygen production (ROS) in whole blood were assessed. All samples were incubated for 1 hour at 37°C. The effect of the lipids was presented relative to the control medium containing dipalmitoyl phosphatidylcholine. The results are presented as median with interquartile range of six separate experiments. *A p-value of < 0.05 as tested by Wilcoxon's matched-pair signed-rank test was considered to be significantly different from LCT/MCT.

	LCT/MCT		EPA + LCT/MCT		DHA + LCT/MCT	
Markers	Median	(25^{th} – 75^{th} percentile)	Median	(25^{th} – 75^{th} percentile)	Median	(25^{th} – 75^{th} percentile)
Membrane surface activation markers						
Granulocytes						
L-selectin	**0.45**	(0.36-0.48)	**0.33***	(0.27-0.39)	**0.43**	(0.35-1.62)
Adhesion	**2.26**	(2.08-2.73)	**2.32**	(2.15-2.77)	**2.27**	(2.00-2.59)
Specific degranulation	**2.39**	(1.93-2.88)	**2.54***	(2.12-3.36)	**2.37**	(1.87-2.79)
Azurophilic degranulation	**1.69**	(1.30-2.20)	**1.79**	(1.57-1.98)	**1.52**	(1.30-1.82)
Monocytes						
L-selectin	**0.37**	(0.23-0.53)	**0.27**	(0.22-0.38)	**0.33**	(0.25-0.47)
Adhesion	**1.84**	(1.72-2.23)	**1.93***	(1.83-2.30)	**1.80**	(1.69-2.17)
Stimulus induced ROS production						
Blood						
Phorbol 12-myristate 13-acetate	**0.85**	(0.82-0.90)	**0.85**	(0.78-0.95)	**0.83**	(0.75-0.91)
Serum treated zymosan	**0.98**	(0.95-1.01)	**0.94**	(0.89-1.03)	**0.93**	(0.85-1.01)

by 15% on granulocytes, and significantly increased adhesion marker on monocytes by 9%, compared to LCT/MCT alone. Other surface activation markers and PMA- and STZ-induced ROS production in whole blood were similar in leukocytes incubated in LCT/MCT containing micelles of EPA as compared to LCT/MCT alone. The addition of DHA micelles to LCT/MCT resulted in a similar expression of surface activation markers on granulocytes and monocytes and unaltered PMA- and STZ-induced ROS production in whole blood, compared to the effect of LCT/MCT alone.

LCT-MCT induced modulation of expression of surface membrane markers and ROS production is not abolished by TLR-4 inhibition

To investigate whether LCT/MCT induced immune activation is mediated by TLR-4, two specific TLR-4 inhibitors were used: 0.1 ng/ml *B. quintana* LPS (Table 3) or 5 μM TAK-242 (Table 4). Blood was pre-incubated for 1 hour with specific TLR-4 inhibitors, and subsequently incubated for 1 hour with these TLR-4 inhibitors and LCT/MCT. Expression of surface membrane markers and stimulus induced ROS production were determined to assess the effect of TLR-4 inhibition on LCT/MCT-induced immune

Table 3. Effects of 5 mmol/L long chain triglycerides (LCT)/medium chain triglycerides (MCT) in the absence and presence of 0.1 ng/mL of the TLR-4 inhibitor *Bartonella quintana* lipopolysaccharide (*B. quintana* LPS). The expression of surface activation markers of L-selectin, adhesion, specific and azurophilic degranulation on granulocytes and monocytes was assessed. All samples were incubated in the presence or absence of *B. quintana* LPS for 1 hour at 37°C, followed by incubation with or without LCT/MCT and/or *B. quintana* LPS, for 1 hour at 37°C. Incubation with *B. quintana* LPS alone did not alter the expression of surface membrane markers and stimulus-induced ROS production. All samples were compared to the lipid-free control. Results are presented as median value with interquartile range of four to six separate experiments. *A p-value of < 0.05 as tested by Wilcoxon's matched-pair signed-rank test was considered to be significantly different from LCT/MCT. No significant differences were found between LCT/MCT and LCT/MCT with *B. quintana* LPS.

	LCT/MCT		*B. quintana* LPS + LCT/MCT	
Markers	Median	(25th – 75th percentile)	Median	(25th – 75th percentile)
Membrane surface activation markers				
Granulocytes				
L-selectin	**0.62**	(0.52-0.74)	**0.73**	(0.54-0.86)
Adhesion	**1.34**	(1.32-1.37)	**1.23**	(1.13-1.39)
Specific degranulation	**1.39**	(1.30-1.69)	**1.27**	(1.16-1.55)
Azurophilic degranulation	**1.24**	(1.13-1.52)	**0.97**	(0.96-1.12)
Monocytes				
L-selectin	**0.64**	(0.52-0.76)	**0.57**	(0.54-0.95)
Adhesion	**1.24**	(1.18-1.29)	**1.12**	(1.09-1.37)
Stimulus induced ROS production				
Blood				
Phorbol 12-myristate 13-acetate	**1.08**	(1.05-1.27)	**1.08**	(0.96-1.25)
Serum treated zymosan	**1.11**	(1.03-1.27)	**1.13**	(0.99-1.18)

activation. Incubation with *B. quintana* LPS alone did not significantly change expression of surface membrane markers and stimulus-induced ROS production. The same was found for TAK-242, except that incubation with TAK-242 alone did significantly decrease the expression of azurophilic degranulation by 24%. The LCT/MCT-induced modulation of expression of surface membrane markers and of stimulus-induced ROS production was not abolished by *B. quintana* LPS (Table 3). TAK-242 did not alter the LCT/MCT-induced immune activation, except for the fact that TAK-242 significantly decreased the expression of specific and azurophilic degranulation markers on granulocytes with 11% and 36%, respectively (Table 4), compared to the effect of LCT/MCT alone.

DISCUSSION

The mechanism underlying the activating effects of MCTs on immune cells remains unclear, with several studies reporting detrimental effects whereas others report on beneficial effects during sepsis due to a positive effect on mitochondrial respiratory capacity.[3-11, 33] A relevant finding of this study is that, apart from the expected immune

Table 4. Effects of 5 mmol/L long chain triglycerides (LCT)/medium chain triglycerides (MCT) in the absence and presence of 0.5 μmol/L of the TLR-4 inhibitor TAK-242. The expression of surface activation markers of L-selectin, adhesion, specific and azurophilic degranulation on granulocytes and monocytes were assessed. All samples were incubated in the presence or absence of TAK-242 for 1 hour at 37°C, followed by incubation in the presence or absence of LCT/MCT and/or TAK-242 for 1 hour at 37°C. All results were compared to the lipid-free control medium. Values are presented as medians with interquartile range of three to five separate experiments. *A p-value of < 0.05 as tested by Wilcoxon's matched-pair signed-rank test was considered to be significantly different from LCT/MCT. Significant differences were found between LCT/MCT and LCT/MCT with TAK-242 in expression of degranulation markers. However, effects of TAK-242 alone were also found, as described in the text.

	LCT/MCT		TAK-242 + LCT/MCT	
Markers	Median	(25^{th} – 75^{th} percentile)	Median	(25^{th} – 75^{th} percentile)
Membrane surface activation markers				
Granulocytes				
L-selectin	**0.89**	(0.85-0.96)	**0.88**	(0.84-1.04)
Adhesion	**1.43**	(1.17-1.56)	**1.23**	(1.15-1.48)
Specific degranulation	**1.22**	(1.10-1.43)	**1.11***	(0.98-1.25)
Azurophilic degranulation	**1.17**	(1.02-1.24)	**0.81***	(0.66-0.97)
Monocytes				
L-selectin	**0.84**	(0.73-0.89)	**0.65**	(0.48-0.92)
Adhesion	**1.23**	(1.14-1.36)	**1.21**	(1.13-1.32)
Stimulus induced ROS production				
Blood				
Phorbol 12-myristate 13-acetate	**0.93**	(0.68-1.01)	**0.61**	(0.54-0.97)
Serum treated zymosan	**1.00**	(0.65-1.02)	**0.72**	(0.60-1.24)

activating effects of LCT/MCT we now have confirmed that similar effects can be expected in any MCT-containing emulsion irrespective of other n-3 anti-inflammatory FAs that are present, suggesting mechanisms of action for MCTs that are not modulated by these other FAs. i.e. involve different signaling pathways. The addition of DHA to LCT/MCT did not modulate the MCT-induced immune function, while the addition of EPA to LCT/MCT even aggravated the immune activation, shown by a further decrease of the expression of L-selectin, and a further increase of expression of specific degranulation and adhesion markers.

We also found that MCT-induced immune activation is not mediated by TLR-4, since inhibition of TLR-4 by TAK-242 or *B. quintana* LPS did not prevent cell activation by these lipids. We had some concerns with regard to the effect of TAK-242, because this TLR-4 inhibitor partially abolished the increase in the expression of activations markers by MCTs. However, since a similar decrease was found after incubation with 5 μM TAK-242 alone and we did not find more evidence for a role of TLR-4 in MCT-induced immune activation, we consider this effect of TAK-242 to be a confounding effect.

Contrary to our results, previous studies have suggested that TLR-4 signaling is involved in the effects of saturated fatty acids on immune cells. To determine whether recruitment into lipid rafts and receptor dimerization are part of the mechanism by which lauric acid induces immune activation via TLR-4, macrophages were transfected with tagged gene products of COX-2 and NF-κB expression, which are both molecules downstream of the TLR-4 pathway and TLR-4 itself. They confirm that lauric acid activates TLR-4 downstream pro-inflammatory genes by mechanisms that include TLR-4 recruitment into lipid rafts and TLR-4 receptor dimerization by means of isolation of lipid rafts and co-localization of TLR-4 molecules using immunoprecipitation.[17, 25, 27] This recently described reciprocal modulation of TLR-4 signaling pathways by saturated and polyunsaturated FA[25], which suggests that MCT-induced immune activation may be abolished by addition of FO, could also not be confirmed by our study, since addition of FO in parenteral mixed lipid emulsions or micelles of n-3 PUFAs did not affect MCT-induced immune activation.

To study MCT-induced immune activation in the context of TLR-4 signaling we used two mechanistically different TLR-4 inhibitors. TAK-242 does not inhibit the binding of ligands such as LPS to TLR-4, but binds to the amino-acid side chain at position Cys747 of the intracellular domain of TLR-4, which possibly changes the conformation and thus selectively blocks TLR-4 signaling.[34] On the other hand we used the TLR-4 inhibitor *B. quintana* LPS, a natural antagonist of TLR-4 that directly blocks the interaction of the agonist with TLR-4.[29] The fact that both TLR-4 inhibitors did not result in a clear reduction of several markers for immune activation, made us conclude that MCT-induced immune activation is not mediated by TLR-4.

Previous authors have linked effects of saturated fatty acids to TLR-4 signaling.[17-19, 21-23, 27] Most of these studies were based on saturated FA with a carbon length of 12 or more C atoms, whereas MCTs in parenteral nutrition formulations mainly are 8 to 10 C atoms in length. Since any effects of FA on immune function depend on carbon chain length, this might explain some of the discrepancies.[2, 3] Another explanation could be that we tested the effect of triglycerides, while other studies mostly focused on free FA.

The immune-activating effects of all MCT-containing emulsions were displayed by decreased expression of L-selectin, and increased expression of adhesion and specific degranulation markers. These effects do not seem to be strongly dose-dependent since LCT/MCT/FO/OO contains 20 percent less MCTs than LCT/MCT and LCT/MCT/FO, but displayed a more or less similar magnitude of immune activation. Interestingly, all MCT-containing mixed emulsions (LCT/MCT/(OO)/(FO), also decreased stimulus-induced ROS production suggesting a decreased capacity to eliminate pathogenic microbes, whereas this effect was absent in MCT-free emulsions.

Lipid emulsions differ with regard to their content of substances other than lipids. For instance, the amount of the anti-oxidant α-tocopherol differs among the different emulsions and is sensitive to storage conditions. The highest amount of α-tocopherol is found in FO, and α-tocopherol is added to LCT/MCT/FO/OO and LCT/MCT/FO.[35] To

determine whether differences in anti-oxidants in the emulsions explain differences in lipid effects, we assessed the total oxidant capacity of all tested lipids. Not unexpectedly, given the absence of double bonds in saturated FAs, MCT-containing emulsions showed a lower total anti-oxidant capacity than MCT-free lipid emulsions. However, the total antioxidant capacity of 5 mmol/L of LCT10% is 1.8 times lower than 5 mmol/L of LCT20%, and for 5 mmol/L of LCT/MCT this is also 1.8 times lower than for 5 mmol/L of LCT/MCT/FO/OO, and since no differences were detected in effects of these emulsions it seems that differences in total anti-oxidant balance do not explain our findings. This corroborates previous findings of a lack of effect of α-tocopherol on MCT-induced immune activation.[36] Also, it has been shown that the type of emulsifier in parenteral lipid formulations does not influence the function of neutrophils.[37]

Limitations of this study have to be taken into account, including the experimental setting, which precludes direct extrapolation of its finding into the clinical arena. For instance, since neutrophils in blood only remain vital for some hours, we only investigated lipid effects after a brief incubation period. On the other hand, some of these studies have shown that within one hour after infusion of LCT/MCT/FO, EPA, but not DHA was incorporated in leukocyte and platelet phospholipids, resulting in an increased n3:n6 ratio.[38-40] A second limitation is the variation in immune responses between the blood of different individuals, unfortunately resulting in sometimes different effects of LCT/MCT on stimulus induced ROS production in the experiments presented in the different tables. Still, in general, LCT/MCT activates the immune system, exemplified by increased expression of adhesion and degranulation markers and decreased expression of L-selectin.

In conclusion, our results suggest that leukocyte activation by MCTs is not mediated by TLR-4 and is not modulated by FO lipids, implying that MCTs exert their effects via different signaling pathways.

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No clinical or biochemical evidence for essential fatty acid deficiency in home patients who depend on long-term mixed olive- and soybean oil-based parenteral nutrition

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ABSTRACT

Background

Home parenteral nutrition (HPN) patients depend on lipid emulsions as part of their parenteral nutrition regimen in order to provide essential fatty acids (EFA). Mixed oil sources are used in modern lipid emulsions to decrease the amount of pro-inflammatory EFA, mainly linoleic acid, which is present in large amounts in soybean oil. It is unknown whether patients who fully depend on such mixed lipids have adequate EFA supply. We therefore evaluated whether HPN patients who depend on mixed olive- and soybean oil-based HPN show clinical or biochemical evidence of EFA deficiency.

Methods

Fatty acid status was assessed in plasma phosphatidylcholine (PC) and peripheral blood mononuclear cells from thirty patients on mixed olive- and soybean oil-based based HPN (>3 months, ≥5 times per week) and thirty healthy controls. Innate immune cell functions were evaluated by assessing expression of surface membrane molecules, and reactive oxygen species and cytokine production.

Results

None of the patients or controls showed clinical evidence (skin rash) or biochemical evidence (increased Holman index (>0.2)) for EFA deficiency. The Holman index in plasma PC (median (25^{th} – 75^{th} percentile)) was significantly higher in patients (0.019 (0.015 – 0.028)) compared with controls (0.015 (0.011 – 0.017)). No differences were found in innate immune cell functions between groups, except for a 3.6-fold higher TNF-alpha production in patients.

Conclusion

We found no clinical or biochemical evidence that HPN patients who fully and long-term depend on mixed olive- and soybean oil-based lipids have an increased risk for EFA deficiency.

CLINICAL RELEVANCY STATEMENT

Essential fatty acids (EFA) cannot be made endogenously, which makes intravenous lipid emulsions the only source of these fatty acids for patients who are fully dependent on parenteral nutrition. We studied whether patients reliant upon home parenteral nutrition (HPN) have clinical or biochemical signs of EFA deficiency when a mixture of soybean and olive oils is used as the intravenous lipid emulsion, and compared the fatty acid composition of these patients to healthy controls. The results of this study aid our understanding of the nutritional value of HPN for the most vulnerable patient group, those fully dependent on EFA intake from their parenteral nutrition.

INTRODUCTION

Lipid emulsions are essential components of total parenteral nutrition (TPN) formulations as a source of non-glucose calories and of fatty acids, including the essential fatty acids (EFA) alpha-linolenic acid (18:3n-3) and linoleic acid (LA; 18:2n-6). The first clinically available lipid emulsions were prepared from soybean oil (SO), which is rich in LA, an n-6 polyunsaturated fatty acid (PUFA). Due to the supposed adverse immune and inflammatory effects of mediators produced from the LA derivative arachidonic acid (20:4n-6), emulsions were developed where part of the SO was replaced by other lipids.[1] One such emulsion is based on 20% SO and 80% olive oil (OO). The latter oil is rich in the immune-neutral n-9 monounsaturated fatty acid oleic acid (18:1n-9). Compared with pure SO-based lipids, this mixed olive- and soybean oil-based contains three times less LA, which will comprise about 6.5 percent of energy intake when the emulsion is used as a component of TPN.[2] The minimum dietary requirements for adults to avoid EFA deficiency symptoms are estimated to be 0.5 percent energy from alpha-linolenic acid and 2.5 percent energy from LA.[3]

Low EFA intake eventually leads to EFA deficiency, in which case the synthesis of (mono-) unsaturated FA such as oleic acid and palmitoleic acid (16:1n-7) increases. This increased synthesis leads to the production of mead acid (20:3n-9), a n-9 PUFA derived from oleic acid. A mead acid/arachidonic acid ratio (the so-called Holman index) above 0.2 is most commonly used to diagnose EFA deficiency. [4,5] EFA deficiency has been associated with water losses from the skin due to increased permeability, susceptibility to infections, lowered resistance to irradiation injury and impaired wound healing, hematologic disturbances, fat infiltration of the liver, impaired chylomicron synthesis, and aggravated fat absorption. [1,6,7] In addition, changes in (essential) fatty acid status have shown an impact on various aspects of immune function. [8-10]

Evaluation of the EFA status of some HPN patients has revealed alterations in fatty acid profiles in line with a diagnosis of EFA deficiency.[7,11], but most patients on lipid containing parenteral nutrition do not have a Holman index above 0.2. [7,11-14] A previous double-blind randomized study compared mixed olive- and soybean oil-based PN with a pure SO-based lipid emulsion and did not find any evidence for EFA deficiencies after

short-term treatment (5 times/week, during 3 months) with either lipid emulsion. [14] We investigated whether patients who fully and long-term depend on mixed olive- and soybean oil-based HPN containing low LA concentrations also have adequate EFA intake. To this end, the plasma and cellular fatty acid profile and the presence of scaly skin lesions as a clinical symptom of EFA deficiency were evaluated. Besides the clinical effect, we were also interested in the effect of the EFA status at the cellular level. Accordingly, we compared the function and phenotype of cells of the innate immune system of HPN patients receiving mixed olive- and soybean oil-based lipid emulsion with healthy controls.

METHODS

Subjects

Thirty adult (> 18 years) HPN patients without active underlying immune-mediated disease, who had been using a parenteral nutrition formulation (Olimel® from Baxter, containing the 80% OO and 20% SO (v/v) lipid emulsion (Clinoleic®)) at least five times per week for at least 3 months and thirty sex- and age-matched healthy controls were included in the study. Subjects with metabolic disorders, active allergic, inflammatory or otherwise immune-mediated diseases, those who consumed more than two portions of fatty fish per week, smoked more than five cigarettes per day, or who used immune suppressive medication, vitamins or fish oil supplements, were excluded from enrollment. Four patients had been hospitalized for a few days due to infectious complications at the moment of inclusion: presuming that these events did not alter FA profile but did have an impact on immune function, immunologic assays were not performed in these patients. The Ethical Review Board of the Radboud University Medical Center approved the study. All procedures were performed after obtaining written informed consent from the patients and controls. The study was registered at ClinicalTrial.gov (NCT01986153).

Laboratory variables

Blood cell counts, including automated leukocyte differentiation, and C-reactive protein were determined on an automated analyzer (AdviaTM120; Siemens Medical Solutions, The Hague, The Netherlands).

Isolation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) were purified from venous whole blood in 10 ml Monoject tubes containing 170 IU of lithium heparin (Beliver Industrial Estate, Plymouth PL6 7BP, UK) as described previously.[15] Briefly, the blood, 1:1 diluted with phosphate buffered saline (PBS, B. Braun Melsungen AG, Melsungen, Germay), was layered on Ficoll-Paque™ Plus (GE Healthcare Life Sciences, Uppsala, SE) and centrifuged (700xg, 20 min, RT). The PBMC-containing interphase was collected, washed twice with PBS and suspended in RPMI medium to the desired final cell concentration.

Fatty acid composition assessment of PBMCs and plasma phosphatidylcholine

Total lipid was extracted from thawed plasma (0.5 mL, collected in EDTA and stored at -80 °C) and PBMCs (1 x 10^7 /mL RPMI medium supplemented with 500 units/ml penicillin/500 µg/ml streptomycin and stored at -80 °C) with 5 mL of chloroform:methanol (2:1) containing the antioxidant butylated hydroxytoluene (50 mg/ L). Solid phase extraction was used to isolate phosphatidylcholine (PC) from the total plasma lipid extract. Next, fatty acid methyl esters (FAMEs) from plasma PC and from PBMC total lipid were formed by incubation in methanolic H_2SO_4 for 2 h at 50 °C. FAMEs were separated using gas chromatography on a Hewlett Packard 6890 gas chromatograph fitted with a BPX70 fused silica capillary column (0.25 lm · 30 m · 0.22 mm); helium was used as the carrier gas. Following injection, the temperature was rapidly raised to 115 °C for 2 min. Then, the temperature was raised to 200 °C at the rate of 10 °C/ min, where it was held for 18 min. Finally, the temperature was raised to 245 °C at a rate of 60 °C/ min where it was held for 8 min. FAMEs were detected by flame ionization detection. FAMEs were identified by comparison with run times of authentic standards. CHEMSTATION software was used to calculate peak areas and the percentage contribution of each peak to the total.

Functional and phenotypic analysis of leukocytes

Leukocyte functions were determined by evaluating the expression of surface activation markers, the oxygen radical production and the cytokine production.

Cytokine production: TNF-alpha and IL-10

Isolated PBMCs (1x10^6 cells/mL) were cultured in RPMI. Cells were stimulated with phytohaemagglutinin (PHA; 10 µg/mL) at 37 °C and 5% CO_2. Aliquots of the culture supernatant were removed after 48 h of incubation and stored at -80 °C until use for determination of IL-10 and TNF-alpha concentrations. Cytokine concentrations were determined in the supernatants using a specific ELISA kit for human TNF-alpha and IL-10 (R&D Systems Europe, Abingdon, UK) according to instructions of the manufacturer.

Surface activation markers

Immunofluorescent staining followed by flow cytometric analysis was used to determine markers for activation, expressed on the membrane surface of neutrophils and monocytes, as described previously.[15] Monocytes and neutrophils were gated based on their CD14 and CD45 expression. Characterization of activation markers was performed using antibodies (purchased from Beckman Coulter (Miami, FL, USA)) directed against an adhesion molecule of the β2 integrin family (CD11b), a degranulation marker for specific granulae (CD66b) and L-selectin (CD62L). Immune-fluorescent staining was performed according to the "lyse and wash" method. Flow cytometry analyses were carried out on a Beckman Coulter Cytomics FC500 (Miami, FL, USA).

Oxygen radical production

Spontaneous and stimulus-induced oxygen radical production in whole blood was evaluated using Luminol-enhanced chemiluminescence and determined in an automated LB96V Microlumat Plus Luminometer (EG & G Berthold, Bald Wilberg, Germany), as described in detail previously.[15] Briefly, 200 microliters of 1:100 HBSS diluted blood were added to a 96 well microplate, either without stimulus, or in the presence of a receptor-independent (phorbol 12-myristate 13-acetate, PMA) or receptor-dependent (serum-treated zymosan particles, STZ) stimulus. Luminol was added to each well to start the chemiluminescence reaction. Each measurement was carried out in at least four replicates. Chemiluminescence was determined every 145 seconds at 37 °C for one hour. Luminescence was expressed as relative light units per second (RLU/sec). Data were analyzed with Winglow software (EG & Berthold). After subtraction of background signal, the signal intensity in whole blood samples was corrected for the neutrophil population count.

Statistical analyses

Values are expressed as median with interquartile range unless stated otherwise. Differences between numeric variables of patients and controls were analyzed using the nonparametric Mann-Whitney U test. To evaluate whether immune status was correlated with fatty acid profile of the PBMCs, all immune parameters were tested for the presence of a correlation with the value of the Holman index in PBMCs by using Pearson's correlation test. In order to correct for multiple testing, a p-value <0.01 was considered statistically significant. All statistical analyses were performed using SPSS software (version 20.0; IBM SPSS, Inc., Chicago, IL, USA).

RESULTS

Characteristics of HPN patients and healthy controls

The majority of patients (n=19/30) and controls (n=21/30) were female. The median age (25^{th} – 75^{th} percentile) of patients and controls was 57 (51 – 64) and 58 (46 – 61) years, respectively. The BMI (median (25^{th}- 75^{th} percentile) of patients (22.3 (19.4 – 23.8) kg/m^2) was significantly (p=0.009) lower than that of controls (23.8 (22.0 – 25.8) kg/m^2). The primary indication for intestinal failure was short bowel syndrome (SBS, in 15/30) while nine patients suffered from a gastrointestinal motility disorder and six patients had various problems, including systemic sclerosis, chronic intestinal pseudo obstruction, or Crohn's disease. Patients received parenteral nutrition five (n=8), six (n=8) or seven (n=14) times per week. The amount of fat (median (25^{th}- 75^{th} percentile) given to the HPN patient per kilogram bodyweight per day was 0.97 gram (0.79 – 1.23 gram). Most patients had been dependent on HPN for more than one year (median (25^{th}- 75^{th} percentile) 1151 (438 – 2241) days). None of the patients consumed relevant amounts of oral/enteral nutrition.

Increased n-9 and lower n-6 FA in plasma PC of HPN patients

Plasma PC FA profiles of patients and controls are presented in Table 1. Patients and controls differed in n-6 and n-9 FA: the relative amounts of LA and total n-6 FA were significantly lower in patients, while the relative amounts of oleic acid and total n-9 FA were significantly higher. Small but statistically significant differences in cis-vaccenic acid, total n-3 and total n-7 fatty acids were found between groups. Minor, but statistically significant, differences were found between patients and controls in myristic-, behenic-, α-linolenic-, eicosatetraenoic-, γ-linolenic-, eicosadienoic-, palmitoleic- and mead acid in plasma PC.

Increased Holman index in HPN patients

The Holman index (i.e. the mead acid/arachidonic acid ratio) in plasma PC that is regarded as a measure for EFA status and is suggestive for deficiency when increased above 0.2 is presented for all subjects in Figure 1. A Holman index above 0.2 was not found in plasma PC from any of the HPN patients or controls. The median (25^{th} – 75^{th} percentile) Holman index in plasma PC was significantly higher ($p < 0.01$) in patients (0.019 (0.015 – 0.028)) compared with controls (0.015 (0.011 – 0.017)).

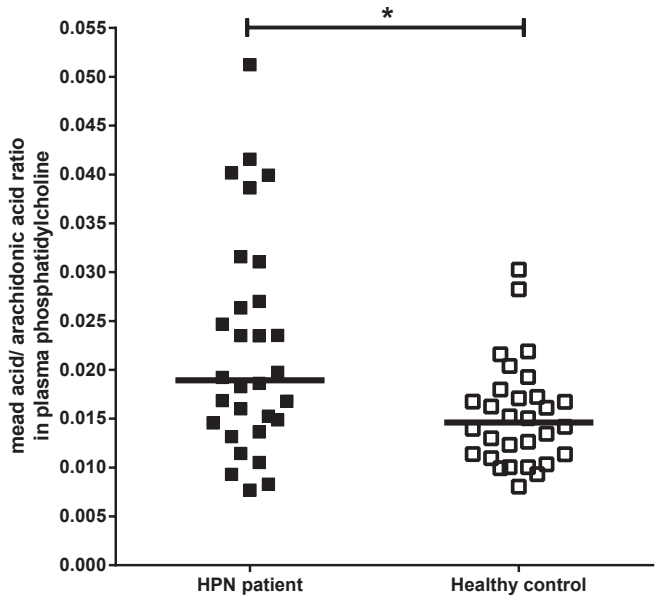

Figure 1. Mead acid/arachidonic acid ratio (Holman index) in plasma phosphatidylcholine of HPN patients and healthy controls. Essential fatty acid deficiency is characterized by a Holman index > 0.2. [4] All values are presented as medians. * A p-value of <0.01 was considered to be statistically significant.

Table 1. Fatty acid composition of plasma phosphatidylcholine (PC) and PBMCs as percentage by weight of total fatty acids. All data are presented as median with interquartile range. A p-value of <0.01 was considered statistically significant.

	PLASMA PC				PBMCs			
Fatty acids	Healthy controls (n=30)		HPN patients (n=30)		Healthy controls (n=30)		HPN patients (n=30)	
Saturated fatty acids	**43.1**	**(42.5 – 44.0)**	**42.7**	**(42.2 – 43.4)**	**45.7**	**(43.4 – 51.6)**	**46.4**	**(43.8 – 50.7)**
Myristic acid (14:0)	0.3	(0.3 – 0.4)	0.2	(0.2 – 0.3)[a]	1.0	(0.8 – 1.2)	1.2	(0.9 – 1.4)
Palmitic acid (16:0)	29.3	(28.3 – 30.0)	29.8	(29.1 – 30.5)	23.5	(21.3 – 26.7)	21.7	(20.4 – 24.0)[b]
Stearic acid (18:0)	13.5	(12.9 – 13.8)	12.5	(11.4 – 13.5)	20.2	(18.8 – 22.5)	22.3	(20.8 – 24.7)[b]
Arachidic acid (20:0)	0.1	(0.1 – 0.1)	0.1	(0.1 – 0.1)	0.7	(0.6 – 0.9)	0.8	(0.6 – 1.0)
Behenic acid (22:0)	0.2	(0.1 – 0.2)	0.1	(0.1 – 0.2)[b]	0.4	(0.3 – 0.5)	0.5	(0.4 – 0.8)[b]
Unsaturated fatty acids								
n-3 fatty acids	**5.2**	**(4.2 – 6.1)**	**4.5**	**(4.1 – 5.4)**	**3.5**	**(2.8 – 4.0)**	**4.5**	**(3.9 – 5.2)[a]**
α-linolenic acid (18:3n-3)	0.3	(0.2 – 0.3)	0.2	(0.2 – 0.2)[a]	0.5	(0.4 – 0.7)	1.0	(0.4 – 1.2)
Eicosatetraenoic acid (20:4n-3)	0.1	(0.1 – 0.2)	0.1	(0.1 – 0.1)[b]	0.1	(0.1 – 0.1)	0.2	(0.1 – 0.2)[a]
Eicosapentaenoic acid (20:5n-3)	0.9	(0.7 – 1.3)	0.8	(0.5 – 1.0)	0.2	(0.2 – 0.3)	0.4	(0.2 – 0.5)[b]
Docosapentaenoic acid (22:5n-3)	0.9	(0.8 – 1.1)	0.8	(0.6 – 1.0)	1.2	(0.8 – 1.4)	1.6	(1.2 – 1.9)[b]
Docosahexaenoic acid (22:6n-3)	2.9	(2.3 – 3.6)	2.8	(2.4 – 3.6)	1.4	(1.1 – 1.7)	1.6	(1.3 – 2.0)

Table 1. Fatty acid composition of plasma phosphatidylcholine (PC) and PBMCs as percentage by weight of total fatty acids. All data are presented as median with interquartile range. A p-value of <0.01 was considered statistically significant. (*Continued*)

	PLASMA PC				PBMCs			
Fatty acids	Healthy controls (n=30)		HPN patients (n=30)		Healthy controls (n=30)		HPN patients (n=30)	
n-6 fatty acids	**38.3**	**(37.3 – 40.1)**	**32.1**	**(30.3 – 33.2)[a]**	**22.6**	**(16.1 – 24.7)**	**26.8**	**(22.4 – 30.0)[b]**
Linoleic acid (18:2n-6)	24.7	(23.0 – 26.2)	17.2	(14.7 – 19.8)[a]	6.7	(5.7 – 8.1)	7.8	(6.5 – 9.3)
γ-linolenic acid (18:3n-6)	0.1	(0.1 – 0.2)	0.2	(0.1 – 0.2)[a]	0.1	(0.1 – 0.2)	0.3	(0.1 – 0.6)
Eicosadienoic acid (20:2n-6)	0.4	(0.3 – 0.4)	0.3	(0.2 – 0.4)[b]	0.7	(0.5 – 1.0)	1.3	(0.7 – 1.7)[a]
Dihomo-γ-linolenic acid (20:3n-6)	3.3	(2.9 – 3.8)	3.1	(2.4 – 3.7)	1.4	(1.1 – 1.8)	1.9	(1.4 – 2.2)
Arachidonic acid (20:4n-6)	9.1	(8.4 – 10.9)	10.8	(9.1 – 12.4)	13.4	(7.6 – 15.3)	14.4	(12.7 – 18.7)
Adrenic acid (22:4n-6)	0.01	(0.01 – 0.02)	0.02	(0.02 – 0.02)	0.02	(0.01 – 0.03)	0.02	(0.01 – 0.03)
n-7 fatty acids	**2.0**	**(1.8 – 2.2)**	**3.0**	**(2.7 – 3.2)[a]**	**3.0**	**(2.6 – 3.5)**	**2.6**	**(2.2 – 2.9)[b]**
Palmitoleic acid (16:1n-7)	0.6	(0.4 – 0.6)	0.7	(0.5 – 0.8)[b]	0.8	(0.7 – 1.1)	0.9	(0.6 – 1.3)
Cis-vaccenic acid (18:1n-7)	1.4	(1.4 – 1.6)	2.2	(1.9 – 2.5)[a]	2.1	(1.8 – 2.5)	1.7	(1.6 – 1.9)[a]
n-9 fatty acids	**10.8**	**(10.2 – 11.9)**	**17.5**	**(15.6 – 19.2)[a]**	**24.9**	**(22.5 – 27.1)**	**18.6**	**(17.6 – 20.3)[a]**
Oleic acid (18:1n-9)	10.5	(9.8 – 11.6)	17.1	(15.3 – 18.7)[a]	23.5	(21.5 – 25.6)	17.3	(16.0 – 19.2)[a]
Gondoic acid (20:1n-9)	0.2	(0.2 – 0.2)	0.2	(0.2 – 0.2)	0.9	(0.6 – 1.1)	0.8	(0.6 – 1.0)
Mead acid (20:3n-9)	0.1	(0.1 – 0.2)	0.2	(0.1 – 0.3)[a]	0.3	(0.2 – 0.3)	0.4	(0.2 – 0.9)

[a] HPN patients are statistically different from healthy controls with a p-value < 0.001.
[b] HPN patients are statistically different from healthy controls with a p-value <0.01.

Table 2. Innate immune function of HPN patients and age- and sex- matched healthy controls. Immune function was evaluated by determining C-reactive protein, the expression of membrane surface activation markers (L-selectin, adhesion, specific and azurophilic degranulation) on granulocytes and monocytes, the production of reactive oxygen species during stimulation with a receptor-independent (phorbol 12-myristate 13-acetate) and receptor-dependent (serum treated zymosan) stimulus and cytokine production (TNF-alpha and IL-10) in PBMCs. All data are presented as median with interquartile range. A p-value of <0.01 was considered to be statistically significant.

Characteristics	Healthy controls (n=30)		HPN patients (n=25)#	
C-reactive protein (mg/l)	5	(5 – 5)	5	(5 – 5)
Cytokine production (in PBMCs)				
TNF-alpha (pg/ml)	1020	(770 – 1610)	3640	(1170 – 4670)[a]
IL-10 (pg/ml)	410	(263 – 760)	533	(379 – 981)
Ratio IL-10/TNF-alpha	0.45	(0.23 – 0.76)	0.19	(0.10 – 0.60)
Membrane surface activation markers				
Granulocytes				
L-selectin (mean fluorescence of CD62L)	55	(44 – 71)	63	(43 – 89)
Adhesion (mean fluorescence of CD11b)	994	(697 – 1623)	935	(593 – 1750)
Specific degranulation (mean fluorescence of CD66b)	62	(51 – 81)	68	(51 – 102)
Monocytes				
L-selectin (mean fluorescence of CD62L)	59	(45 – 72)	69	(49 – 80)
Adhesion (mean fluorescence of CD11b)	943	(794 – 1310)	1180	(998 – 2090)
Stimulus induced ROS production (in whole blood)				
Phorbol 12-myristate 13-acetate (Relative light units * 10^4)	2.7	(2.0 – 3.2)	3.0	(2.5 – 5.1)
Serum treated zymosan (Relative light units * 10^4)	8.2	(6.2 – 9.6)	9.5	(6.3 – 10.3)

[#] Immune function was not assessed because they were hospitalized (n=4) or because of logistic problems (n=1).
[a] HPN patients are statistically different from healthy controls with a p-value < 0.001.

Lower n-9 and higher n-6 FA in PBMCs of HPN patients

The differences between groups in PBMC FA profiles were different from those of plasma PC (Table 1). PBMCs of patients and controls differed in n-6 and n-9 FA profiles. The relative amount of total n-6 FA was significantly higher in patients, whereas that of oleic acid and total n-9 FA was lower in patients. Minor, yet statistically significant, differences were found between patients and controls in palmitic, stearic, behenic, eicosatetraenoic, eicosapentaenoic, docosapentaenoic, eicosadienoic, cis-vaccenic, total n-3 and total n-7 FA in PBMCs. The median (25^{th} – 75^{th} percentile) mead acid/arachidonic acid ratio in PBMCs was not statistically different between patients (0.029 (0.011 – 0.076)) and controls (0.019 (0.013 – 0.040).

Increase in TNF-alpha production by PBMCs from HPN patients

Innate immune function was assessed by evaluating the expression of surface membrane activation markers, and the stimulus-induced production of ROS and cytokines by leukocytes (Table 2). A 3.6-fold increase in TNF-alpha production was found for PBMCs from patients compared to those from controls, while IL-10 production was not different between groups. A 2.4-fold decrease in IL-10/TNF-alpha ratio was found for PBMCs from patients compared to those from controls. No statistically significant differences between patients and controls were found in the expression of the activation marker L-selectin or of adhesion and degranulation markers of granulocytes and monocytes. Receptor-independent (PMA) and receptor-dependent (STZ) induced ROS production were not statistically different between groups.

No correlation between leukocyte functions and EFA status in PBMCs

To evaluate whether immune status was correlated with EFA status, cytokine production, ROS production and expression of surface activation markers were tested for the presence of a correlation with the mead acid/arachidonic acid ratio in PBMCs. None of the immune parameters was significantly correlated with the mead acid/arachidonic acid ratio in PBMCs.

DISCUSSION

In the present study we found no clinical or biochemical evidence for EFA deficiency in patients who long-term and fully depend on mixed olive- and soybean oil-based parenteral nutrition. None of the patients or controls had a Holman index above 0.2, meaning none of them met the criteria for the diagnosis of EFA deficiency. Scaly skin lesions, the most prominent feature of EFA deficiency, were not seen in any of the patients or controls. Accordingly, functional immunological parameters were not different between groups, with the exception of evidence for increased inflammatory potential (TNF-alpha production) in the patients.

We found significantly lower relative plasma PC concentrations of LA and alpha-linolenic acid in HPN patients than controls. About 90 percent of the total amount of EFA in plasma PC consisted of LA in both groups. We found average relative concentrations of LA in the plasma PC of 14.7 percent in the patients and 24.7 percent in the controls, which is in line with previous reports, ranging from 11 to 24 percent in patients and from 22 to 30 percent in controls.[7,10-14,16,17] The lower LA in plasma PC of HPN patients was compensated for by a higher average relative concentration of oleic acid (17.5 vs 10%). The different relative proportions of oleic acid and LA in plasma PC of patients and controls probably reflect the differences in relative supply of those two fatty acids in the TPN regimen (patients) compared with the diet (controls). Alpha-linolenic acid represents only a small proportion of the total amount of EFA present in plasma, as has previously been described for both HPN patients and healthy controls.[7,10-14,16,17]

EFA deficiency is traditionally defined as a mead acid/arachidonic acid ratio (Holman index) above 0.2 in plasma, since a low EFA intake will eventually lead to increased mead acid synthesis. Although a significant difference between patients and controls was found with a maximum Holman index in patients of 0.051 and in controls of 0.030, none of the patients or controls met the criterion for EFA deficiency. A Holman index above 0.2 is sporadically described [7,11], but most HPN patients like our patients have a Holman index lower than 0.2.[7,11-14]

To establish the clinical relevance of the fatty acid profile, we evaluated patients for the presence of the most prominent clinical sign of EFA deficiency, scaly skin lesions, on the day that blood was withdrawn for analysis. We did not find such evidence in any of the patients or controls. Interestingly, clinical signs of EFA deficiency have been described in HPN patients with a Holman index lower than 0.2. For instance, Jeppesen reported an median (25th -75th percentile) Holman index for HPN patients without skin problems of 0.10 (0.04 – 0.28), while for HPN patients with skin problems a Holman index of 0.05 (0.02 – 0.20) was found.[12] However, these skin problems were self-reported, and might not be related to the EFA status of the patients.

Besides the effects of EFA status on clinical symptoms, we were also interested in the effect of the EFA status at the cellular level, since it is known that EFA deficiency impairs cellular aspects of the immune response. [8] A previous study showed no major differences in FA profile between neutrophils and monocytes, so the PBMC lipids were considered to be representative for immune cells.[10] We therefore analyzed fatty acid profiles of PBMCs and performed functional tests to evaluate immune function. The FA profile of PBMCs was different from that in plasma PC: while the concentration of n-6 FA was lower and that of n-9 FA higher in plasma PC of patients compared to controls, the opposite was found in PBMCs. Differences between plasma and PBMC FA profiles have been described before, and may be explained by the possibility that cells exert a significant level of control over their plasma membrane composition. A second explanation may be that cells can metabolize PUFAs and thereby modify the FA composition of their plasma membrane. [10,15]

PBMC function was evaluated by measuring the stimulus (PHA)-induced production of pro- and anti-inflammatory cytokines, yielding a 3.6-fold increase in production of the pro-inflammatory cytokine TNF-alpha in patients compared to controls. Since the IL-10/TNF-alpha ratio was only 2.4 times lower in patients, the difference in TNF-alpha seems to be partly neutralized by the anti-inflammatory IL-10. The increased TNF-alpha production was not related to EFA status, since no correlation was found between TNF-alpha and the mead acid/arachidonic acid ratio in PBMCs. Other immune functions were not correlated to this ratio of fatty acids.

Differences in stimulus-induced cytokine production may have consequences in clinical situations where the immune system needs to be triggered, like during infection. However, we found no evidence of differences between patients and controls with regard to other markers of immune function (stimulus-induced ROS production,

expression of surface activation markers), a finding that is in line with previous work in this field. [18]

It was not unexpected that the healthy controls had no evidence of EFA deficiency, since EFA are abundantly present in the western diet. Although some HPN patients have been described with an increased Holman index, most patients with lipid containing parenteral nutrition did not meet the criterion of a Holman index above 0.2 before. [11,13] The LA caloric intake of about 6.5 percent present in OO-based HPN seems to be adequate for our patients to have a Holman index below 0.2.

Limitations of the present study should be taken into account. First, besides scaly skin lesions, no other, but also less prominent, clinical features of EFA deficiency, like infection susceptibility or impaired wound healing were evaluated in our study population. Secondly, only innate immune functions were evaluated, since these seem to be particularly affected in HPN patients, as is exemplified by the increased risk for pneumonia and wound infections in mildly malnourished surgical patients on PN.[19] Furthermore, the limited power because of small study groups precludes the detection of subtle changes in FA profile and immune function.

In conclusion, we found no clinical or biochemical evidence that HPN patients who fully and long-term depend on mixed olive- and soybean oil-based lipids have an increased risk for EFA deficiency.

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Long-term olive oil-based parenteral nutrition sustains innate immune function in home patients without active underlying disease

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ABSTRACT

Background

It remains unclear whether impaired host defenses contribute to the increased risk for infectious complications seen in patients on Home Parenteral Nutrition (HPN). The aim of this study was to compare the innate immune function of patients on olive oil-based HPN with that of healthy controls.

Methods

Innate immune functions and (anti-) oxidant balance were studied in 20 patients on olive oil-based HPN without an active underlying immune-mediated disease (Clinoleic®, ≥6 months; >3 times/ week), and 21 age and sex matched healthy controls.

Results

Neutrophils of patients and controls had a similar capacity to eliminate *Streptococcus pneumoniae*. Also, levels of activation markers (CD66b, CD11b, CD62L) in granulocytes and monocytes, phorbol ester- and zymosan- induced neutrophil oxygen radical production were not different between patients and controls. No differences in (anti-) oxidant status were found, except for higher concentrations of oxidized glutathione and lower plasma selenium and vitamin C in patients compared to controls.

Conclusion

Compromised innate immune function does not seem to explain the increased risk for infectious complications in HPN patients using olive oil-based lipid emulsions.

INTRODUCTION

The increased risk for infectious complications, mainly in the form of catheter-related sepsis, is still a reason for concern in patients treated with home parenteral nutrition (HPN). These infections establish a potentially life-threatening hazard that has a profound impact on the patients' quality of life as well as on hospital resources. In addition, repeated catheter loss eventually compromises the options to obtain adequate venous access.[1] Despite technical improvements, training of patients and medical staff in aseptic catheter handling, and the use of antiseptic catheter locks, which have decreased infection rates to some extent, the problem is still not solved.[1, 2] In addition, it remains unclear whether parenteral nutrition (PN) components, and especially lipids, apart from the presence of a venous access device contribute to the infection risk in HPN patients.

Since the introduction of PN, several lipid emulsions based on different oil sources have been developed that have shown distinct effects on immune function.[3] The first emulsion (Intralipid®) that became available in the early 1960s was based only on soybean oil (SO). This emulsion is still extensively used worldwide despite concerns with regard to its pro-inflammatory profile due to the high amount of n-6 fatty acids, specifically linoleic acid. To decrease the amount of n-6 fatty acids, several emulsions have been developed in which SO is partly replaced by one or more alternative oils. These mixtures of SO, fish oil (FO), olive oil (OO) and/or coconut oil (CO) seem to have less pro-inflammatory effects and to modulate immune function in a more beneficial way.[3, 4]

To rule out effects of underlying immunological diseases or immunosuppressive medications, the immune function of HPN patients without active underlying immune-mediated diseases or immunosuppressive medications were compared with the immune function of healthy controls. We focused on neutrophils, since these phagocytes seem to be particularly affected in patients on PN, as was exemplified by the increased risk for pneumonia and wound infections in mildly malnourished surgical patients.[5] To test the hypothesis that the first line of defense of the human immune system is not affected in patients on olive oil-based HPN, relevant aspects of the function of the innate immune system were determined. Therefore, the capacity of leukocytes to eliminate *Streptococcus pneumoniae (S. pneumoniae)*, the expression of activation and degranulation markers, and the stimulus-induced oxygen radical production of leukocytes and (anti-)oxidant balances were determined under the long-term influence of an OO-based parenteral nutrition formulation.

METHODS

Subjects

Twenty adult HPN patients without active underlying immune-mediated diseases, who had been using an OO-based PN formulation (Oliclinomel®, containing 80% OO and 20% SO) based lipid emulsion Clinoleic® for at least 6 months and at least three times per week and twenty-one adult sex- and age-matched healthy controls were

included in the study. Subjects with metabolic disorders, active allergic, inflammatory or otherwise immune-mediating diseases, who consumed more than two portions of fatty fish per week, smoked more than five cigarettes per day, or who used immune suppressive medication, vitamins or fish oil supplements, were excluded. The Ethical Review Board of the Radboud University Nijmegen Medical Center approved the study. All procedures were performed after obtaining written informed consent.

Laboratory variables

Blood cell counts, including automated leukocyte differentiation, hemoglobin, hematocrit, C- reactive protein, liver enzymes (alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), gamma-glutamyltransferase (gamma-GT)), total bilirubin, triglycerides, vitamin C, vitamin E and selenium were determined on an automated analyser (AdviaTM[120]; Siemens Medical Solutions, The Hague, The Netherlands).

Isolation of leukocytes

Granulocytes and peripheral blood mononuclear cells (PBMCs) were purified from venous whole blood in 10 ml Monoject tubes containing 170 IU of lithium heparin (Belliver Industrial Estate, Plymouth PL6 7BP, UK) as described previously.[6] Briefly, the blood, 1:1 diluted with phosphate buffered saline (PBS, B. Braun Melsungen AG, Melsungen, Germany), was placed on Percoll (ρ 1.076 g/ml, GE Healthcare Biosciences AB, Uppsala, Sweden) and centrifuged (700x*g*, 20 min, RT). The lymphocyte-containing interphase (PBMC) was washed twice with cold PBS and suspended in Hank's Buffered Salt Solution (HBSS) to the desired final concentration. The granulocyte-containing pellet was suspended in 50 ml ice-cold isotonic lysis solution (8.3 g/L NH_4Cl, 1.05 g/L $KHCO_3$, pH=7.4) for 10-15 min. After centrifugation (400x*g*, 5min) the remaining erythrocytes were lysed in fresh lysis solution for another 5 min. The granulocytes were washed twice with cold PBS and suspended in HBSS (with 0.5% bovine serum albumin (BSA)) to the desired final concentration.

Functional analysis of leukocytes

Leukocyte functions were determined by evaluating the capacity to eliminate bacteria, the expression of surface activation markers and the oxygen radical production.

Elimination of *S. pneumoniae*

With some modifications, the opsonophagocytic killing assay (OPKA) described by Nahm *et al.* was used to assess the capacity to eliminate *S. pneumoniae* by neutrophils.[7] We recently described this modified method in detail.[8] Briefly, *S. pneumoniae* (strain OREP-4, serotype 4) were opsonized with a dilution series of pooled and purified human IgG (Nanogam 50 mg/ml; Sanquin, Amsterdam, The Netherlands) in a 96-well microplate for 30 min at room temperature. Next, neutrophils purified from blood,

and rabbit complement (Pel-Freez Biologicals, Rogers, AR, USA) were added and the mixture was incubated for 45 min at room temperature. All of these incubations were performed in triplicate. Subsequently, all samples were spotted in duplicate on THYA medium plates. After drying, THYA overlay medium with 2,3,5-triphenyl tetrazolium chloride was added, to stain viable colony forming units (CFU) of *S. pneumoniae*. The plates were incubated overnight at 37 °C and 5% CO_2. The CFUs on the plates were determined by counting photographed plates using TotalLab (Nonlinear Dynamics, Newcastle, UK). The capacity to eliminate *S. pneumoniae* was expressed as the percentage elimination in samples incubated with neutrophils relative to the bacterial count in samples without neutrophils (i.e. with complement and IgG).

Surface activation markers

Immunofluorescent staining followed by flow-cytometric analysis was used to determine markers for activation, expressed on the membrane surface of neutrophils and monocytes, as described previously.[6] Monocytes and neutrophils were gated based on their CD14 and CD45 expression. Characterization of activation markers was performed using antibodies (purchased from Beckman Coulter (Miami, FL, USA)) directed against an adhesion molecule of the β2 integrin family (CD11b), a degranulation marker of azurophilic (CD63) or specific granulae (CD66b) and L-selectin (CD62L). Immune-fluorescent staining was performed according to the "lyse and wash" method. Flow cytometry analyses were carried out on a Epics XL (Beckman Coulter, Hialeah, FL).

Oxygen radical production

Spontaneous and stimulus-induced oxygen radical production in whole blood and of isolated neutrophils was evaluated using Luminol-enhanced chemiluminescence and determined in an automated LB96V Microlumat Plus Luminometer (EG & G Berthold, Bald Wilberg, Germany), as described in detail previously.[6] Briefly, 1:100 in PBS diluted blood or isolated neutrophils were added to a 96 well microplate, either without stimulus, or in the presence of a receptor-independent (phorbol 12-myristate 13-acetate, PMA) or receptor-dependent (serum-treated zymosan particles, STZ) stimulus. Luminol was added to each well to start the chemiluminescence reaction. Each measurement was carried out in at least four replicates. Chemiluminescence was determined every 145 seconds at 37 °C for one hour. Luminescence was expressed as relative light units per second (RLU/sec). Data were analyzed with Winglow software (EG & Berthold). After subtraction of background signal, the signal intensity in whole blood samples was corrected for the neutrophil population count.

(Anti-)oxidant balance measurements

The balance between antioxidants and oxidants was assessed in blood by determination of plasma concentrations of glutathione, lipid peroxidation- and protein oxidation products and total antioxidant capacity.

Glutathione

Total and oxidized glutathione were determined in whole blood and plasma by HPLC, as described previously.[6, 9] Whole blood was collected in EDTA tubes. For the GSH assay in whole blood, 500µl whole blood was added to 500µl ice-cold 12% perchloric acid (PCA), containing 2 mM bathophenan-throlinedisulfonic acid for the determination of total glutathione, and another 500µl whole blood was added to the same solution plus 40 mM N-ethylmaleimide for the determination of oxidized glutathione. Samples were thoroughly mixed and centrifuged at 16,000x*g* for 15 min at 4°C. Supernatants were stored at -80°C until analysis. The remainder of the blood was centrifuged at 3,000x*g* for 10 min. For the assay of oxidized glutathione, 35 µl KOH (2.0 M) was added to 50 µl of the appropriate supernatant, followed by 30 µl HCL (0.01 M in 3.0 M MOPS buffer) to remove the excess of *N*-ethylmaleimide. This mixture was centrifuged at 16,000x*g* for 3 min at 4°C. For total and oxidized glutathione in whole blood, and total glutathione in plasma the assay was performed using 50 µl sample runned in parallel with the glutathione standards. To the samples and standards, 5 µl Tris (carboxy-ethylene) phosphin (10% w/v) in 0.9% (w/v) NaCl/4 mM EDTA) was added, mixed and incubated for 30 min. After the reduction, 50 µl PCA/EDTA (0.6 M/1 mM) was added and mixed. The samples were left for 5 min to precipitate and then centrifuged for 5 min at 16,000x*g*. The supernatants (50 µl) were incubated for 60 min at 60°C with 120 µl reaction-mix containing 10 µl NaOH (1.55 M), 100 µl borate buffer (125 mM $K_2B_4O_7 \cdot 4H_2O$ and 4 mM EDTA) and 10 µl 7-fluorobenzoflurazane sulfonic acid (2 mg/ml in borate buffer; Fluka Chemika). After cooling, the derivatized samples were neutralized with 5 µl 6M HCl, and a 20 µl sample was injected in the HPLC system with fluorescent detection (Jasco Inc., Easton, MD, USA). Obtained data were analyzed using the CROMPASS Chromatography Data System (Jasco Inc.). Glutathione concentrations were calculated using a four-point calibration curve and expressed in micromolar.

Overall antioxidant capacity

The antioxidant capacity in blood plasma was determined using the ferric reducing ability of plasma (FRAP) assay, according to the method of Benzie and Strain[10], as previously described.[6, 9] Standards ($FeSO_4 \cdot 7H_2O$) and samples were added to the FRAP working solution (2.5 mL 10 mM 2,4,6,-tripyridyl-s-triazine (Fluka Chemika) in 40 mM HCl, 2.5 mL 20 mM $FeCl_3 \cdot 6H_2O$ and 25 mL 300 mM acetate buffer pH 3.6). In the presence of antioxidants, ferric ions are reduced to ferrous ions, which cause a colored ferrous-2,4,6-tripyridyl-s-triazine complex to be formed. Absorbance was measured at 593 nm on a Perkin-Elmer Spectrophotometer. Values are expressed in mmol Fe^{2+}/L.

Lipid peroxidation

The peroxidation of lipids was measured as the amount of thiobarbituric acid reactive substances (TBARS) in plasma (blood collected in EDTA tubes), as described previously.[6, 9] Briefly, malondialdehyde (MDA) generated during lipid peroxidation

reacts with thiobarbituric acid. After incubation, TBARS are extracted using butanol. Analysis of the supernatants was performed by fluorometry on an Infinite® 200 PRO series (Tecan Group Ltd., Männedorf, Switzerland), with an excitation wavelength of 520 nm and an emission wavelength of 550 nm. A standard curve was prepared using 1,1,3,3-tetramethoxy-propane (Fluka Chemika, Buchs SG, Switzerland). Concentration of TBARS was expressed as µmol MDA/L.

Protein oxidation

The amount of oxidative protein damage products was determined in an enzyme linked immunosorbent assay (ELISA) for estimation of protein carbonyls according to the method of Buss *et al.*[11], as described previously.[6, 9] Briefly, standards and plasma samples were diluted in PBS to a protein concentration of 4 mg/mL. Proteins were derivatized with 2,4-dinitrophenyl-hydrazine and coated on an ELISA plate (NUNC-Immuno plate maxisorp; Nunc, Roskilde, Denmark) before probing with biotinylated anti 2,4-dinitrophenyl-hydrazine antibody (Molecular Probes Inc, Eugene, OR, USA). Subsequently, the wells were incubated with streptavidin biotinylated horseradish peroxidase (Amsterdam Life Science Inc, Arlington Heights, IL, USA). Staining was carried out with *o*-phenylenediamine. Absorbance was read at 490 nm in a ThermoMax microplate reader (Molecular Devices Corp, Sunnyvale, CA, USA). Concentrations of carbonyls are expressed in nmol/mg protein.

Statistical analyses

Differences between numeric variables of patients and controls were analyzed using the nonparametric Mann-Whitney U test. Differences were considered significant if a p-value of <0.05 was obtained. Values are expressed as median with interquartile range unless stated otherwise. All statistical analyses were performed using SPSS software (version 15.0; SPSS, Inc., Chicago, IL, USA).

RESULTS

Characteristics of HPN patients and healthy controls

The cause of intestinal failure in 11 out of 20 HPN patients was a motility disorder of the gut, while 6 had a short bowel following extensive resection with a colon in continuity and 3 had a short bowel with a high output stoma (Table 1). The laboratory parameters of patients and controls are presented in Table 2. A significant increase in serum triglyceride concentrations was found in HPN patients compared to healthy controls. As expected, several (35%) HPN patients still had mildly increased triglyceride levels at the time of blood collection, which was performed between 4 and 7 hours after the termination of PN administration. Also as expected , mildly perturbed liver function tests were found in most HPN patients. This concerned both transaminases (ALT and AST were increased in 20% of patients compared to reference values) as

Table 1. Indications for long-term HPN of the 21 patients: divided in 3 categories; motility disorder, short bowel and high output stoma.

Indication (n)	
Motility disorder (11)	Chronic idiopathic intestinal pseudo-obstruction (3)
	Multiple adhesions (3)
	Idiopathic (5)
Short bowel (6)	Mesenteric thrombosis (4)
	Radiation enteritis (1)
	Resection; multiple adhesions (1)
High output stoma (3)	Resection; multiple adhesions (3)

Table 2. Laboratory parameters of HPN patients and healthy controls. Results are presented as median with interquartile range, unless stated otherwise in the table. * A p-value of < 0.05 was considered to be significant. NS = not significant.

	Reference value	Healthy controls		HPN patients		p-value
		Median	(25th – 75th)	Median	(25th – 75th)	
Gender (male/total (% of total))	-	6/21	(29%)	5/20	(25%)	
Age (years)	-	51	(40-58)	54	(47-62)	NS
Lipid status						
Triglycerides (mmol/ l)	0.80 – 2.00	0.9	(0.7-1.4)	1.8	(1.2-2.6)	0.001*
Hepatic function						
ALP (U/ l)	<120	59	(44-71)	121	(81-141)	<0.001
AST (U/ l)	<40	26	(23-30)	33.5	(28-43)	0.006*
ALT (U/ l)	<45	22	(17-27)	30	(23-45)	0.026*
Gamma-GT (U/ l)	<35	16	(11-23)	34	(19-149)	0.002*
Bilirubin total (µmol/ l)	<17	10	(8-12)	8	(6-11)	NS
Inflammatory status						
CRP (mg/ l)	<10	<5	(<5-<5)	<5	(<5-5.8)	NS
Blood cell count						
Leucocyte differentiation						
Trombocytes (*10^{E9}/ l)	-	240	(189-274)	201	(163-273)	NS
Leucocytes (*10^{E9}/ l)	-	5.2	(4.4-6.5)	5.9	(5.1-9.0)	NS
Neutrofilic granulocytes (10^{E9}/l)	-	3.5	(2.3-3.9)	3.6	(2.8-6.1)	0.041*
Lymfocytes (10^{E9}/l)	-	1.7	(1.4-2.2)	1.9	(1.4-2.2)	NS
Monocytes (10^{E9}/l)	-	0.33	(0.24-0.40)	0.34	(0.26-0.43)	NS
Eosinofilic granulocytes (10^{E9}/l)	-	0.12	(0.087-0.19)	0.29	(0.12-0.41)	0.011*
Basofilic granulocytes (10^{E9}/l)	-	0.00	(0.00-0.05)	0.00	(0.00-0.06)	NS
Hemoglobin (mmol/ l)	-	8.1	(7.7-8.6)	7.5	(0.7-7.9)	0.015*
Hematocrit (l/ l)	-	0.40	(0.37-0.40)	0.36	(0.34-0.38)	NS

well as cholestatic markers (50% percent of patients had increased levels of ALP and/or gamma-GT compared to reference values). Hemoglobin levels were significantly different between HPN patients and healthy controls, but all remained within normal ranges. A small but significant difference in the number of neutrophilic and eosinophilic granulocytes was found between healthy controls and HPN patients, whereas gender, age, total bilirubin, CRP levels and other subtypes of leukocytes than neutrophilic and eosinophilic granulocytes were not different between groups.

No difference in elimination of *S. pneumoniae*

The capacity of isolated neutrophilic granulocytes to eliminate *S. pneumoniae* was assessed by means of the OPKA and revealed no differences between patients and controls (Figure 1, p=0.513). The elimination of these pneumococci depends on the concentration of the used human IgG concentration. All bacteria were eliminated at an IgG dilution below 1:80, while at an IgG dilution of 1:40960 the maximum elimination of 49% and 55% of *S. pneumoniae* in healthy controls and HPN patients, respectively, was reached. Due to technical problems, the capacity to eliminate *S. pneumoniae* was not determined in 1 healthy control and 1 HPN patient.

Similar expression of membrane surface activation markers

The expression of activation markers on the membrane of different leukocyte subpopulations was analyzed after fluorescent staining using flowcytometry (Figure 2). No statistical significant differences in expression were found between patients and controls. More specifically, the expression of the adhesion marker CD11b was similar in controls and HPN patients in granulocytes (median (range)= 662 (381-947) and 516 (298-681), respectively, p=0.106) as well as monocytes (median (range)= 631 (530-1079)

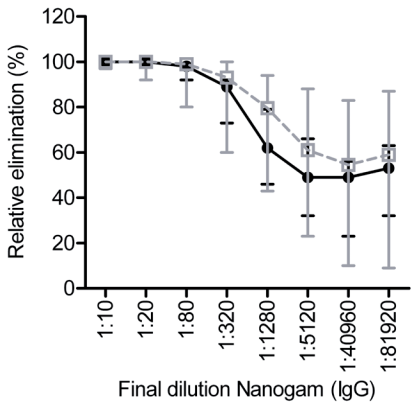

Figure 1. Relative percentage of eliminated *Streptococcus pneumoniae* at different dilutions of Nanogam (IgG) by isolated neutrophils of HPN patients (grey open, n=19) and healthy controls (black filled, n=20). Data are presented as median with interquartile range. A p-value of < 0.05 was considered to be significant. No significant differences between patients and controls were observed.

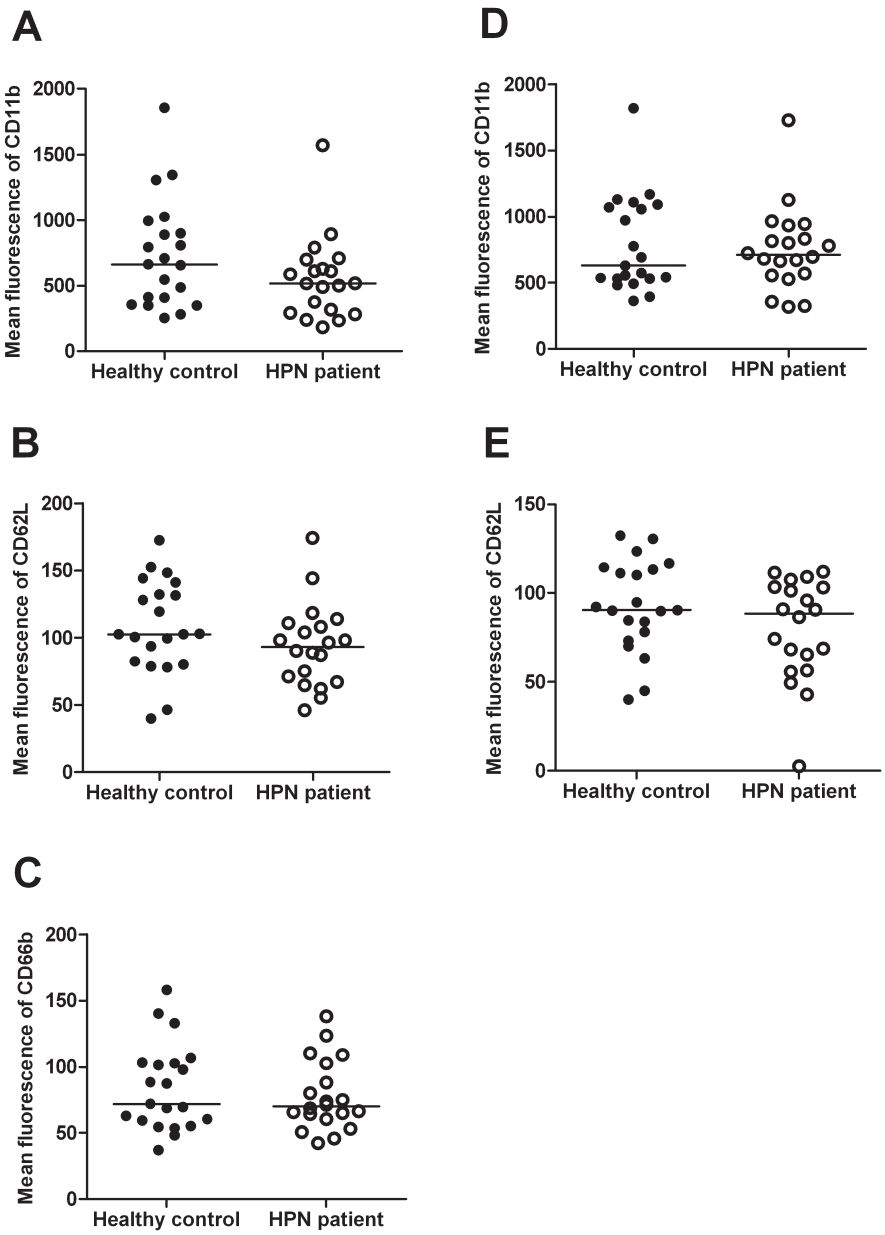

Figure 2. Expression of surface activation markers on neutrophils (A, B, C) and monocytes (D, E) of HPN patients (open, n=20) and healthy controls (filled, n=21): adhesion marker: CD11b (A, D), activation marker: CD62L (B, E), degranulation marker: CD66b (C). All data are presented as median. A p-value of < 0.05 was considered to be significant. No significant differences between patients and controls were observed.

and 710 (559-907) respectively, p=0.958). L-selectin (CD62L), which is downregulated upon activation, was also expressed at a similar level in controls and HPN patients in granulocytes (median (range)= 103 (81-137) and 93 (68-110), respectively, p=0.121) and monocytes (median (range)= 90 (76-114) and 88 (59-103), respectively, p=0.183). The expression of a marker for specific degranulation (CD66b) was also not different between controls and patients in granulocytes (median (range)= 72 (57-103) and 70 (61-99) respectively, p=0.752). The expression of the azurophilic degranulation marker (CD63) on granulocytes was below detection limits in all subjects and therefore this is not depicted in Figure 2.

No differences in oxygen radical production

Stimulus-induced oxygen radical production was evaluated by means of luminol-enhanced chemiluminescence. No significant differences in PMA and STZ stimulated ROS production were found in whole blood and isolated neutrophils between controls and HPN patients (Figure 3).

Some differences in oxidant- antioxidant status

Plasma concentrations of vitamins C and E, selenium, glutathione, total plasma antioxidant capacity (FRAP), plasma levels of protein carbonyls and level of lipid peroxidation, as well as the concentration of total and oxidized glutathione in whole blood were determined to evaluate the oxidant-antioxidant balance (Table 3). HPN patients had significantly lower plasma vitamin C and selenium levels compared to healthy controls (p= 0.029 and p<0.001, respectively). However, the plasma vitamin C

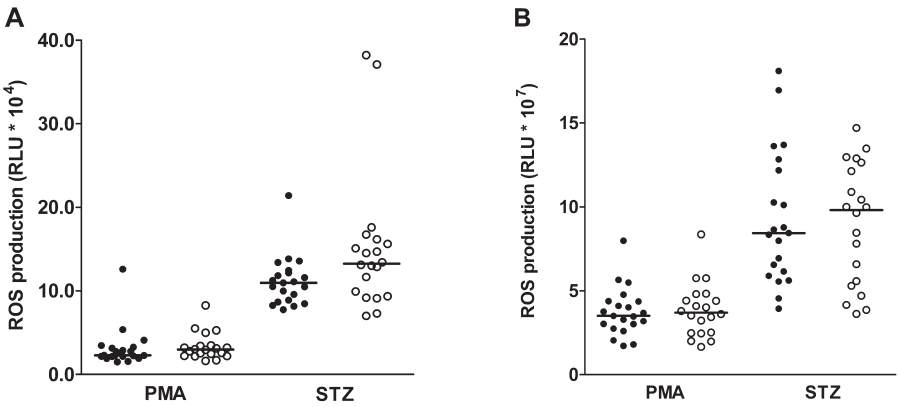

Figure 3. Phorbol 12-myristate 13-acetate (PMA) and serum-treated zymosan (STZ) induced radical oxygen production in (A) whole blood and (B) isolated neutrophils of healthy controls (filled, n=21) and HPN patients (open, n=20). All results are presented as median. A *p*-value of < 0.05 was considered to be significant. No significant differences between patients and controls were observed.

Table 3. Oxidant and antioxidant status of HPN patients and healthy controls. Results are presented as median with interquartile range. * A p-value of < 0.05 was considered to be significant. NS = not significant.

	Reference value	Healthy controls		HPN patients		P-value
		Median	(25th – 75th)	Median	(25th – 75th)	
Plasma						
Vitamin C (µmol/ l)	15 - 69	49	(46-59)	41	(31-54)	0.029*
Vitamin E (µmol/ l)	7 - 33	42	(30-49)	42	(31-52)	NS
Selenium (µmol/ l)	0.7 – 1.4	0.97	(0.93-1.11)	0.74	(0.64-0.91)	<0.001
Glutathione (µmol/ l)	-	7.4	(6.8-8.7)	5.8	(4.8-8.5)	NS
Lipid peroxidation (µmol/ l)	-	0.51	(0.47-0.68)	0.52	(0.40-0.64)	NS
Total antioxidant capacity (mmol Fe^{2+}/l)	-	0.82	(0.73-0.94)	0.84	(0.72-1.02)	NS
Whole blood						
Total free glutathione (µmol/ l)	-	1868	(1633-2089)	1899	(1628-2126)	NS
Oxidized glutathione (µmol/ l)	-	13	(9.5-26)	66	(49-92)	<0.001

and selenium concentrations were below the reference values in only 5% and 30% of the HPN patients, respectively. Total glutathione concentration between whole blood of patients and controls was not different, whereas a five times higher concentration of oxidized glutathione (p<0.001) was present in whole blood of HPN patients when compared to controls. In plasma, the amount of lipid peroxidation products, plasma concentrations of vitamin E and glutathione were not different between patients and controls (p=0.584, p=0.611 and p=0.060, respectively). The plasma levels of protein carbonyls were below detection limits in HPN patients and healthy controls, signifying the absence of evidence for protein damage in either group.

DISCUSSION

We found no evidence for the presence of compromised innate immune functions in patients with severe intestinal failure without immune-mediated underlying disease who were on long-term OO-based PN. Despite the known increased susceptibility to infections, neutrophils of these HPN patients had no impaired capacity to eliminate *S. pneumoniae* compared to neutrophils of healthy controls. Also, the expression of phenotypic markers of cell activation (CD11b, CD66b and CD62L) of cells of both the innate and adaptive immune system was not different between patients and healthy controls. Furthermore, the neutrophils of these HPN patients were not pre-activated ("primed") when compared with the neutrophils of healthy controls, since the neutrophils of both groups had a similar production of reactive oxygen species after *ex vivo* stimulation with STZ and PMA.

In line with the unaffected leukocyte activation, the balance between oxidants and anti-oxidants was comparable in both groups, except for decreased selenium and vitamin C levels, and a five-fold increase in oxidized glutathione concentrations in HPN patients compared to healthy controls. This finding might at least in part be explained by the fact that selenium can modulate inflammatory and immune responses through effects on redox functions, since glutathione peroxidase, a selenium-containing enzyme, is involved in oxidation reactions of glutathione.[12] It appeared that in our study HPN patients who did not receive daily PN including the multivitamin solution, may have more increased levels of oxidized glutathione. However, similar trends were not seen in selenium and vitamin C concentrations. Taken together, the differences in anti-oxidant balance observed in this study apparently did not affect the patients' innate immune function.

In vitro studies suggest that OO is more immune-neutral compared to other lipid emulsions. For instance, SO-induced inhibition of T cell proliferation, -activation and migration, was not observed after OO exposure, and OO was less potent in inhibiting inflammatory cytokine production by PBMCs and inducing leukocyte death.[13-15] Furthermore, emulsions based on OO have been shown to modify neutrophil responses *in vitro* to a lesser extent than emulsions based on SO or mixed SO and medium chain triglycerides (MCTs).[16] These immune-neutral effects of OO have been confirmed in animal studies.[17, 18] Data on immune modulation by OO-based emulsions *in vivo* are, however, sparse. Clinical trials included patients with several pathologies, and *in vivo* studies in healthy volunteers mostly concerned small short-term trials with low power. These studies mainly focused on safety and efficacy and did not provide any detailed insight into immune modulating properties of OO-based lipids.[19-26] Concerning the evaluation of immune modulation, only plasma inflammatory markers (C-reactive protein and cytokine-levels) and plasma oxidative stress markers were assessed. With respect to oxidative stress, our group has previously shown that long-term HPN use does not seem to result in significant oxidative damage or an altered oxidant-antioxidant balance in a group of 41 Dutch HPN patients, of whom 75% were using a OO-based emulsion.[9] In contrast to most clinical trials, in the present study the activation and function of leukocytes (mainly neutrophils) in HPN patients and healthy controls were assessed by multiple assays.

The innate host defense system of the HPN patients in this study appeared not to be affected by the administration of PN, in the absence of an active underlying disease and without the use of medication that might affect immune function. However, the presence of the venous access device, which per definition affects the host defense by providing a direct connection between the internal and external environment, has been identified as an independent risk factor for the occurrence of bloodstream infections.[27] To decrease this risk for microorganism invasion through the open connection, our HPN patients and medical staff are thoroughly trained in aseptic catheter management. In addition, based on a randomized clinical trial,

which presented a dramatic (>90%) reduction in the occurrence of catheter-related bloodstream infections (CRBSI), we recently (2008; i.e. >1 year before the start of the present study) switched from low-dose (150 U/ml) heparin to 2% taurolidine (TauroSept®) catheter lock solution.[2, 28]

It should be considered that the composition of the OO-based parenteral nutrition in this study differs from the normal diet of the healthy Dutch population. OO-based PN consists of 64% mono-unsaturated fatty acids (FAs), 22% polyunsaturated FAs and 14% saturated FA, while according to the Dutch National Food Consumption Survey the diet of the adult Dutch population consists of 53% unsaturated FA, 38% saturated FA, 3% trans FA and 6% other FA.[3, 29] It is known that saturated FAs and triglycerides can modulate immune function depending on their carbon chain-length *in vitro*.[30] However, it is unclear what the consequences of these different intakes of FAs are for cell membrane composition and the function of immune cells in patients on OO-based PN.

Another difference between the groups in this study is the hepatic function; HPN patients have significantly increased serum liver enzyme levels compared to healthy controls. Liver dysfunction occurs frequently in long-term HPN patients.[1] The difference in hemoglobin of 0.6 mmol/l between HPN patients and controls is not clinically relevant and is considered to be the effect of the underlying disease of HPN patients.

Limitations of the present study should be also considered. The incidence of severe intestinal failure that necessitates HPN treatment is low, only 4-6 million per year in patients with benign primary diseases in Europe. Since HPN patients with an active immune-mediated disease were excluded, this resulted in a small group of patients that could be included in this study.[1] This limited power might therefore preclude the detection of very subtle changes in innate immune functions. However, our detailed analysis clearly rules out significant differences between these HPN patients and healthy controls.

In conclusion, this study shows that innate immune function in patients with severe intestinal failure can be preserved during home treatment with OO-based parenteral nutrition. Therefore, a compromised innate immune function does not seem to be the explanation for the increased risk for infectious complications in these HPN patients.

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8

General discussion, implications and future perspectives

When a patient cannot meet his nutritional requirements because of an impaired uptake of nutrients via the gastrointestinal tract intravenous (parenteral) nutrition has to be initiated. In case of long-term and severe intestinal failure this usually means the start of a treatment with parenteral nutrition in the home setting (home parenteral nutrition, HPN). HPN is mostly administered via a catheter that is positioned in a large-bore central vein. Although a life-saving strategy, this therapy has several drawbacks too. These latter issues provided the research questions that sparked the research in the present thesis. This final chapter addresses possible implications, limitations and discusses future perspectives of these investigations.

RESEARCH QUESTIONS

As outlined in Chapter 1, this thesis focuses on catheter-related complications, like infections and occlusions, experienced by patients on long-term (total) parenteral nutrition. It remains unclear whether and, if so, which type of catheter lock solutions can prevent these complications. Furthermore, it is unclear whether lipid emulsions as part of parenteral nutrition formulations affect the function of the immune system and thus contribute to the increased infection risk seen in HPN patients. For this reason the effects of catheter locks on infection risk and the influence of lipids on immune functions are the two leading themes in this thesis. The first part of the thesis focuses on catheter lock solutions, and investigates the effects of a switch in catheter locking with heparin to taurolidine on catheter-related bloodstream infections (Chapter 2). We explored the development of microbial adaptation after long-term use of taurolidine as a catheter lock solution (Chapter 3), and we studied *in vitro* the microbiocidal differences between various structurally different taurolidine-containing catheter lock formulations that are available on the market today (Chapter 4). The second part of this thesis focuses on lipids, and here we studied the *in vitro* effects of lipid emulsions that are used as part of parenteral nutrition formulations on various aspects of immune function (Chapter 5). We also investigated the essential fatty acid status (Chapter 6) and several functional aspects of the immune system (Chapter 7) of patients who were long-term dependent on olive oil-based parenteral nutrition and compared these data with those obtained in healthy controls.

LOCK SOLUTIONS

In order to reduce the risk for developing CRBSI, several measures have been implemented in HPN care.[1,2] Prophylactic catheter locking to prevent CRBSI and/or occlusions is one of these strategies. Our interest was especially raised by taurolidine, a promising antimicrobial agent that has previously shown to prevent the development of intravenous catheter-related infections in several patient populations, ranging from patients on renal function replacement therapy (dialysis) to those suffering from cancer.[3-9] In the first part of this thesis we further explored the effects of this antimicrobial catheter lock solution. We addressed the following research questions:

Is taurolidine lock superior to low-dose heparin lock in the prevention of catheter related bloodstream infections and occlusions?

Since a randomized open-label trial in our own tertiary HPN referral center discovered a dramatic decrease in incidence of CRBSI with taurolidine compared to low-dose heparin catheter locking[10], our HPN center switched from using heparin to taurolidine to lock the catheters of all of our HPN patients. In **Chapter 2**, we provide further evidence that taurolidine is more effective in preventing catheter-related complications in HPN patients compared to heparin in a robust retrospective dynamic (members can leave or be added over time) cohort study as a follow-up to the prospective randomized open-label trial. During an observation period spanning over 200,000 catheter days, comprising 212 HPN patients and 745 CVCs, we found an impressive six times higher risk for developing CRBSI and a two times higher risk for developing catheter occlusions in heparin-locked compared to taurolidine-locked catheters. This decrease in catheter complications to 0.2 CRBSI and 0.1 occlusions per venous access year using taurolidine was accompanied by a substantially decreased strain on hospital resources, evidenced by a 60% decrease in ratio of hospital admission days per catheter day. This ratio decreased from 0.055 during heparin locking to 0.022 with the use of taurolidine as catheter lock.

Although several small and underpowered studies have evaluated the effect of taurolidine locking on CRBSI[11], this is the first robust study over a long observation period in a single-center patient population using the same catheter handling and training protocols. Nevertheless, studying catheter complications in this patient category remains challenging because of the clinical issues which may bias results; HPN patients may suffer from a range of underlying diseases and varying degrees of intestinal failure, which can influence for instance pharmacotherapy and HPN use. To account for repeated vascular access episodes within an individual patient, and to minimize bias introduced by confounders we analyzed our data using a random effects model with Poisson distributions for counts and corrected for confounders. Based on our results, the hypothesis that the switch from low-dose heparin to 2% taurolidine results in a decrease in catheter related complications and – subsequently- hospital admissions was accepted.

Is microbial adaptation to taurolidine seen in patients on home parenteral nutrition who develop catheter related bloodstream infections and use taurolidine locks?

Although the emergence of microbial resistance to taurolidine has not been reported so far, the fact that some patients still develop CRBSI while using taurolidine might point toward selective growth of microorganisms with a phenotypical adaptation to taurolidine. An increase in the concentration required to inhibit the growth of 50% of the microorganism (MIC_{50}) is most commonly used to provide evidence for the adaptation to an antimicrobial agent.[12,13] In **Chapter 3**, we found that the MIC_{50} of taurolidine of CRBSI-causing microorganisms in HPN patients using a taurolidine lock as

described in our study, are not different from previously reported MIC_{50} of taurolidine. Therefore, we found no evidence whatsoever for any adaptation of microorganisms to taurolidine in those HPN patients who still developed CRBSI when locking their catheters with this antiseptic agent.

It has to be mentioned here that the method used to determine MIC_{50} in its present form is not sensitive to very subtle changes. Minor adaptation to taurolidine could therefore not be ruled out in the results obtained in the experiments described in Chapter 3. Another limitation is that it is unknown how long CRBSI-causing pathogens had been exposed to taurolidine, and whether this exposure was long enough to develop any adaptation to taurolidine at all. With these limitations in mind, our hypothesis that adaptation to taurolidine did not occur in the clinical isolates from CRBSI of our patients, was accepted.

Do microbiocidal effects of various taurolidine-containing catheter lock solutions differ?

Taurolidine use appears to be safe, in the light of the absence of serious adverse events and in terms of the efficacy (Chapter 2) to prevent catheter infections. Still, the question remains which – if any- of the commercially available taurolidine-based solutions is superior over the others. In Chapter 4, we showed that in its undiluted form 2% taurolidine, 1.34% taurolidine-citrate and 1.34% taurolidine-citrate-heparin completely inhibit the growth of fungal, Gram positive and Gram negative pathogens. Only during extreme dilution (>20 times) of the lock solutions, 2% taurolidine has a more potent effect on microbial growth inhibition and biofilm formation than 1.34% taurolidine-citrate-(heparin). We showed that this difference in microbial growth inhibition was due to the 1.5 times difference in taurolidine concentration, and therefore, that the addition of citrate and/or heparin to the taurolidine containing lock solution seems to have no additional beneficial effects in terms of infection prevention. Moreover we observed a decrease in biofilm formation, which was directly correlated to the decreased microbial growth. Although these tests were performed in a limited number of pathogens (one Gram negative, one Gram positive and one fungus), the effects we saw were very similar in all micro-organisms, which convinced us to accept the hypothesis that catheter lock solutions containing taurolidine without additives are more bactericidal compared to the same volume of formulations with a lower concentration of taurolidine containing citrate and/or heparin, at least in the *in vitro* setting.

LIPIDS

Ever since the first lipid emulsion was developed and introduced in the clinical arena in the early 1960's, the discussion with regard to lipid effects on several issues in PN care, and mainly infections and liver function disturbances, has remained ongoing until this day. Pro-inflammatory effects have been linked to the use of n-6 PUFA in soybean

oil-derived emulsions, whereas anti-inflammatory effects have been associated with the use of fish oil formulations that are rich in n-3 PUFA, and immune neutral effects have been attributed to n-9 MUFA in olive oil.[14] In addition, the use of saturated MCTs that are present in coconut-oil has been advocated because the absence of double bonds in these compounds precludes the occurrence of detrimental effects as a result of lipid oxidation. In the second part of this thesis we sought to extend the discussion of the effects of parenteral lipids on immune cell functions. Especially because mixed lipid emulsions are increasingly being used as part of PN formulations, and because the mechanism behind the previously described immune cell-activating effects of MCT remain unclear we addressed the following research questions:

Do MCT-containing lipid emulsions that contain fish oil activate the immune system similar to MCT and is this MCT effect mediated by TLR-4?

Previous studies by our group and others have shown that saturated medium chain triglycerides (MCT) as part of mixed LCT/MCT emulsions, activate the immune system *in vitro*.[15-26] We found in **Chapter 5** that in line with the expected immune activation by LCT/MCT we found similar effects in any MCT-containing emulsion irrespective of the presence of anti-inflammatory n-3 fatty acids, whereas MCT-free emulsions lacked this effect. Interestingly, addition of the key n-3 PUFAs EPA or DHA could not undo the MCT-induced immune activation. This finding suggests that the mechanism(s) of action of MCT is not modulated by the presence of n-3 fatty acids and therefore does not share common signaling pathways/mediators. Saturated FA-induced immune activation via a toll-like receptor (TLR)-4 mediated mechanism has been described before[27-29], and therefore we also investigated whether TLR-4 was implicated in the MCT-induced immune activation. Since inhibition of TLR-4 by two differently acting TLR-4 inhibitors, TAK-242 or *Bartonella quintana* LPS, did not by any means prevent cell activation by MCTs, we conclude in **Chapter 5** that MCT-induced immune activation is not mediated by TLR-4. Although this *in vitro* setting has its drawbacks concerning time of exposure to lipids and artificial cell environment, the major advantage of this *in vitro* setting is that we used primary human cells and compared the effects of different lipids or of different TLR-4 inhibitors on the immune cells of the same healthy individual. Our hypothesis that parenteral MCTs with regard to their immune activating effects share a common signaling pathway with anti-inflammatory n-3 PUFAs, and that the effect of MCT is mediated by TLR-4 in an *in vitro* setting was therefore rejected.

Do HPN patients who fully depend on parenteral lipids for their EFA intake have an adequate EFA status and immune function compared to healthy controls?

Essential fatty acids by definition cannot be synthesized endogenously, which renders the parenteral nutrition formulation, and more specifically the lipid emulsion, the single source of these nutrients for patients who fully depend on parenteral nutrition. This

urged us to investigate whether EFA intake is sufficient to prevent the development of deficiencies in patients who long-term depend on PN for their EFA intake. EFA intake was presumed to be adequate based on a biochemical criterium; a mead acid/arachidonic acid ratio in plasma phosphatidylcholine (Holman index) below 0.2, given that a Holman index above 0.2 is considered as biochemical proof to establish a diagnosis of EFA deficiency. In Chapter 6, we found no evidence whatsoever for essential fatty acid deficiency in HPN patients and controls, since none of the patients (and controls for that matter) had a Holman index above 0.2. In agreement with this finding, none of the patients and controls had a scaly rash, which is seen as the most prominent clinical feature of this deficiency. On a cellular level, we found no differences in functional immunological parameters between groups, with the exception of some evidence for increased inflammation parameter; increased TNF-alpha production by peripheral blood mononuclear cells (PBMCs) in patients. Immune function in PBMCs was however not correlated with the mead acid/ arachidonic ratio in these cells. Our hypothesis that patients who are fully dependent on long-term OO-based PN have an adequate intake of essential fatty acids was therefore accepted.

Is the function of the innate immune system of long-term HPN patients different from that of healthy controls?

Parenteral lipids derived from olive oil have previously been described as immune-neutral in nature[14], which seems highly relevant for patients who long-term depend on parenteral nutrition. In order to confirm this characteristic we extensively studied the innate immune system of 20 patients on HPN who did not have an active underlying immune-mediated disease and who received OO-based parenteral nutrition. In Chapter 7, we found no differences in various aspects of immune function between patients and healthy controls. More specifically, the capacity of neutrophils to kill the pathogen *Streptococcus pneumoniae*, the expression of activation markers on leukocytes, and stimulus-induced oxygen radical production by neutrophils was similar between patients and controls. No differences in (anti-)oxidant status were found, except for higher concentrations of oxidized glutathione and lower plasma selenium and vitamin C in patients on HPN compared to healthy controls which were not interpreted as serious adverse effects. Although the sample size of our study precludes the detection of minor differences between patients and controls, we conclude that overall we did not find any evidence for the presence of a compromised immune status in these HPN patients. Our hypothesis that the function of the innate immune system is not affected in patients on olive oil-based HPN compared to healthy controls was accepted.

IMPLICATIONS AND FUTURE PERSPECTIVES

In general, HPN care has changed in many ways since the initial implementation of this therapeutic strategy in the '60's. This includes technical improvements such as the development of structurally different mixtures of lipids to provide (essential) fatty acids

and fuel calories and, more recently, the implementation of catheter lock solutions to prevent the development of catheter-related infections.

Taurolidine as catheter locking agent

Taurolidine is a rather new kid on the block when it comes to catheter locking that has shown promising effects and appears to effectively prevent both infectious catheter-related complications as well as occlusions when compared with the anticoagulant heparin (**Chapter 2**). On the other hand, although the short-term safety prophile of taurolidine at this point seems favorable we need long-term outcomes to draw any conclusions with respect to patient tolerability and the development of microbial resistance. Another issue that remains to be addressed in future investigations is to what extent the favorable results that were obtained with taurolidine are caused by adverse effects of heparin. That saline locking may to some extent be superior to heparin was suggested by a recent Belgian randomized, non-inferiority, open trial in oncology patients were a lower incidence of functional complications and CRBSI was found in the saline compared to the heparin group.[30] In this respect we eagerly await the results of an ongoing European randomized clinical trial in HPN patients that compares taurolidine and saline as catheter locks (ClinicalTrials.gov number, NCT01826526). Obviously not all patients who start on HPN have a similar risk for the development of catheter – related infections and we currently cannot clearly identify high-risk patients beforehand. With respect to cost-effectiveness and patient safety an important issue therefore is whether catheter locks should be used in all HPN patients or rather in high-risk patients (i.e. those who have experienced one or more catheter-related infections).

It remains to be seen whether the increased catheter survival that is the result of effective catheter locking eventually will lead to an increased rate of extra-luminal infections that for instance originate from the exit site and subcutaneous catheter tunnel. Such findings would emphasize the need for additional antiseptic measures such as nasal and perineal eradication of *Stapylococcus aureus* carriage.

Concerning the use of available locking agents it is not completely clear at this point what the implications should be of the *in vitro* differences in growth inhibition that we found between the various formulations, moreover because spilling of the catheter lock solution over time into the bloodstream via the catheter tip can be expected to occur in the CVC of HPN patients.[31] The frequency of PN use (and hence of catheter lock change) therefore seems another important issue in this respect. Based on the combined results of our *in vitro* investigation and the retrospective analysis we find no evidence to support the use of anticoagulants (heparin, citrate) in taurolidine-containing catheter locks. Since it still is not clear whether adaptation to taurolidine does occurr (**Chapter 3**), it would be most interesting to grow pathogens *in vitro* in medium with extremely low concentrations of taurolidine for extended periods of time to see whether taurolidine MIC_{50}'s change over time. Another interesting issue

is whether taurolidine affects the development of biofilms. Since we only studied the effect of taurolidine after 60 hours, during the stationary/ plateau stage of the growth curve (maximum number of bacteria under the experimental condition) (**Chapter 4**), we do not know what the effect of taurolidine on biofilm development over time (before the stationary/ plateau stage is reached) is in the setting of PN. A first step might be to study the effect of taurolidine on biofilm formation during submaximal growth of pathogens, for instance after 15-25 hours of growth. Secondly, it would be interesting to see if this biofilm can be broken down by taurolidine in a high concentration: such findings would be relevant for patients who at the start of taurolidine locking therapy already have a catheter with a biofilm.

Lipids

Currently, a choice can be made from several lipid emulsions as part of parenteral nutrition regimens. Apart from a preference for a fish oil-containing mixed lipid emulsions for surgical ICU patients to lower infection rates and limit the length of stay[32] there are no clear indications in adult patients to guide the choice for any emulsion other than that pure soybean emulsions nowadays are considered less favorable due the high content of linoleic acid, a potentially pro-inflammatory n-6 PUFA. We have previously shown *in vitro* that the presence of MCT results in immune cell activation, as exemplified by increased oxygen radical production, adhesion and activation marker expression and decreased cell migration.[15-26] So far, these clear *in vitro* effects have not been corroborated in *in vivo* investigations. The mechanism behind the MCT effects remains unclear, but does not seem to interfere with the signal transduction pathway of lipids and lipid-derived mediators in fish oil, as was shown in this thesis (**Chapter 5**). For another mixed-type lipid emulsion, consisting of soybean and olive oil we were able to show that i) patients who long-term depend on this emulsion as part of their PN show no signs of impaired immune function (**Chapter 7**) and ii) no evidence was found for a deficient essential fatty acid status or impaired immune functions after long-term use of olive oil-based PN (**Chapter 6**). Overall we conclude that the increased risk for CRBSI in patients receiving parenteral nutrition cannot be explained by effects of olive oil based parenteral nutrition on the immune system.

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9

Summary

This thesis focuses on catheter-related complications, like infections and occlusions, experienced by patients on long-term (total) parenteral nutrition in the home setting (home parenteral nutrition, HPN). For one, it remains unclear whether and, if so, which type of catheter lock solution is optimal to prevent these infections. When looking from the perspective of the quality of the immune defense system of the patient, it remains uncertain whether the structurally different lipid emulsions that are available as part of parenteral nutrition formulations affect the host immune function and thus contribute to the increased infection risk seen in HPN patients. These issues led to the research questions that are the basis for the present thesis.

In the first part we studied catheter lock solutions. Since a randomized open-label trial in our own tertiary HPN referral center found a dramatic decrease in the incidence of catheter related bloodstream infections (CRBSI) when using taurolidine compared to low-dose heparin for catheter locking purposes, our HPN center switched from using heparin to 2% taurolidine to lock the catheters in late 2008. In **Chapter 2**, we provide further evidence that taurolidine is more effective in preventing catheter-related complications in the setting of HPN compared to heparin in a retrospective dynamic cohort study. We found an impressive six times increased risk for developing CRBSI and a two times higher risk for developing catheter occlusions in heparin-locked compared to taurolidine-locked catheters. This decrease in catheter complications during taurolidine catheter locking was accompanied by a 60% decrease in the number of hospital admission days per catheter day.

The fact that some patients still develop CRBSI while using 2% taurolidine catheter lock solution might point toward selective growth of microorganisms with a phenotypical adaptation to taurolidine. We therefore studied the susceptibility to taurolidine in clinical isolates of patients with CRBSIs. In **Chapter 3** we found no differences between the susceptibility to taurolidine of microorganisms found in cultures of patients with CRBSI and the susceptibility to taurolidine of these microorganisms described before in the literature. Thus, we found no evidence for relevant adaptation of microorganisms to taurolidine in patients who use long-term taurolidine catheter lock solution.

Several taurolidine-containing lock solutions are commercially available, some of which also contain anticoagulants (citrate, heparin) besides taurolidine. We evaluated whether one of these solution was superior over the others. In **Chapter 4** we found that the 2% taurolidine solutions inhibited the microbial growth and biofilm formation of a yeast, a Gram positive and Gram negative bacterium more when compared with 1.34% taurolidine-citrate-(heparin) at dilutions over 20 times of the lock solutions. It appeared that this difference could be attributed to the 1.5 times difference in taurolidine concentration. We therefore concluded that, at least in the *in vitro* setting, the 2% taurolidine lock solution was superior to the other formulations.

In the second part of this thesis we focus on lipids present in parenteral nutrition. Previous studies have shown that saturated medium chain triglycerides (MCT) as part of mixed long chain triglycerides (LCT)/MCT emulsions, activate the immune system

in vitro. We found in Chapter 5 that similar immune-activating effects were seen in any commercial available MCT-containing emulsion irrespective of the presence of anti-inflammatory n-3 fatty acids, and that MCT-free emulsions lacked this effect. Since saturated FA-induced immune activation via a toll-like receptor (TLR)-4 mediated mechanism has been described before, we investigated whether TLR-4 signaling was implicated in the MCT-induced immune activation. We found that inhibition of TLR-4 could not prevent immune activation by MCTs. In conclusion it seems that immune activation by MCTs is therefore not modulated by either n-3 lipids or by TLR-4 signaling.

Essential fatty acids, by definition, cannot be synthesized endogenously. The lipids present in a total parenteral nutrition formulation are therefore the single source of these nutrients for patients who fully depend on parenteral nutrition. Since our HPN patients are long-term dependent on this type of nutrition we wanted to assess whether they consume an adequate amount of essential fatty acids and whether the function of the immune system of these patients is affected. In our center we use a lipid emulsion that is based on a mixed 80% olive-oil and 20% soybean oil that is rich in the immune neutral mono-unsaturated fatty acid oleic acid. When we compared the essential fatty acid status and the immune function in HPN patients to that of healthy controls, in Chapter 6 we did not find any evidence for essential fatty acid deficiency and immune functions were not disturbed. Also, in Chapter 7, where we studied the immune function more extensively, we did not find evidence for an affected immune system in HPN patients, except for higher concentrations of oxidized glutathione and lower plasma selenium and vitamin C concentrations in HPN patients compared to healthy controls.

Taken together, our results suggest that the lipid emulsion, which is based on 80% olive oil/ 20% soybean oil and is administered to patients who long-term depend on HPN, contains an adequate amount of essential fatty acids and does not impair the immune function of these patients. Morover we found that locking the catheter with taurolidine decreases the number of infections and occlusions, compared to locking the catheter with heparin. This decrease in catheter complications during taurolidine catheter locking was accompanied by a decrease in hospital admission days.

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Samenvatting

Dit proefschrift richt zich op de complicaties in de vorm van infecties en occlusies, die gerelateerd zijn aan het gebruik van een katheter, door patiënten die langdurig voeding via de bloedbaan (totale parentale voeding (TPV)) in de thuissituatie gebruiken. Het is op dit moment onduidelijk of een katheter lock-vloeistof (een vloeistof die de katheter afsluit als er geen voeding doorheen gaat) infecties die gerelateerd zijn aan het gebruik van een dergelijke katheter kan voorkomen, en zo ja, welk type lock-vloeistof dan het beste kan worden gebruikt. Ten tweede weten we niet of de verschillende vetoplossingen (emulsies), die beschikbaar zijn als onderdeel van parenterale voeding, invloed hebben op de functie van het afweersysteem van de patiënt, en zo een bijdrage leveren aan het verhoogde risico op infecties dat deze patiëntengroep treft. Deze kwesties zetten ons er toe om de genoemde aspecten van katheter lock-vloeistoffen en de vetten in parenterale voeding te bestuderen.

In het eerste deel van dit proefschrift richten we ons op de katheter lock-vloeistoffen. In een gerandomiseerde open-label trial in ons eigen tertiaire TPV-verwijzingscentrum werd bij het gebruik van de lock-vloeistof taurolidine een extreme daling in de incidentie van katheter-gerelateerde infecties gevonden, wanneer dit werd vergeleken met de tot op dat moment gangbare lock-vloeistof heparine. Om die reden zijn de patiënten van ons TPV centrum overgestapt van heparine naar 2% taurolidine aan het eind van het jaar 2008. In **Hoofdstuk 2** leveren we in een retrospectieve dynamische cohort studie verder bewijs dat taurolidine inderdaad beduidend effectiever katheter-gerelateerde complicaties voorkomt dan heparine bij patiënten die thuis TPV gebruiken. Katheters die afgesloten waren met heparine hadden namelijk een zes keer hoger risico op katheter-gerelateerde infecties en een twee keer hoger risico op katheter-gerelateerde occlussies vergeleken met taurolidine. De verlaging van de complicaties bij gebruik van taurolidine ging samen met een sterke verlaging (60%) van het aantal ziekenhuisopnamedagen.

Het feit dat sommige patiënten nog steeds katheter-gerelateerde infecties hebben gedurende het gebruik van de 2% taurolidine katheter lock-vloeistof zou kunnen wijzen op selectieve groei van micro-organismen die zich hebben aangepast aan de blootstelling aan taurolidine. We hebben daarom de gevoeligheid voor taurolidine bepaald van micro-organismen uit bloedmonsters van patiënten die een katheter-gerelateerde infecties doormaakten tijdens het gebruik van taurolidine. In **Hoofdstuk 3** vonden we echter geen verschil in de gevoeligheid voor taurolidine tussen micro-organismen die gevonden waren in de kweken van patiënten met een katheter-gerelateerde infectie en micro-organismen die beschreven staan in de literatuur. Er was dus geen bewijs voor een belangrijke aanpassing van micro-organismen in patiënten die langdurig taurolidine als katheter lock-vloeistof hadden gebruikt.

Er zijn verschillende taurolidine bevattende katheter lock-vloeistoffen commercieel verkrijgbaar, waarvan sommige naast taurolidine ook antistollingsmiddelen (citraat, heparine) bevatten. We hebben onderzocht of toevoeging van één van deze middelen invloed had op de groei van microorganismen onder invloed van deze lock-vloeistofen.

In **Hoofdstuk 4** hebben we bij een gist, een Gram positieve en een Gram negatieve bacterie meer remming van groei en biofilmvorming gevonden bij de 2% taurolidine oplossing vergeleken met de 1.34% taurolidine-citraat-(heparine) vloeistof wanneer deze middelen meer dan 20x verdund werden. Dit verschil bleek geheel te kunnen worden toegeschreven aan de factor 1,5 verschil in de concentratie taurolidine tussen deze lock-vloeistoffen. We concluderen daarom dat in de experimentele situatie de 2% taurolidine lock-vloeistof superieur lijkt te zijn.

In het tweede deel van dit proefschrift richten we ons op de vetten in de parenterale voeding. Eerdere studies hebben aangetoond dat verzadigde, dat wil zeggen vetten die geen dubbele koolstofbindingen bevatten, middellange-keten triglyceriden (MCTs), als onderdeel van een vetemulsie met zowel middel- als lange-keten triglyceriden, het immuunsysteem activeren in een experimentele setting. In **Hoofdstuk 5** hebben we laten zien dat vergelijkbare immuun-activerende effecten te zien zijn in elke vetemulsie die MCTs bevat. Het maakt hierbij niet uit of de vetemulsie daarnaast ook anti-inflammatoire omega-3 vetzuren bevat. Vetemulsies zonder MCTs bleken het immuunsysteem niet op deze manier te activeren. Aangezien eerder is beschreven dat verzadigde vetzuren het immuunsysteem via de toll-like receptor 4 (TLR-4) activeren, hebben we onderzocht of de TLR-4 signaalroute betrokken is bij de immuunactivatie door MCTs. Het bleek echter dat remming van TLR-4 de immuunactivatie door MCTs niet kon voorkomen. Onze conclusie is daarom dat immuunactivatie door MCTs niet beïnvloed wordt door de effecten van omega-3 vetten en dat de TLR-4 signaalroute niet betrokken is bij de immuunactivatie.

Essentiële vetzuren kunnen, zoals de definitie al zegt, niet door het menselijk lichaam geproduceerd worden. De vetten die in de parenterale voeding aanwezig zijn, zijn dan ook de enige bron van deze voedingsstoffen bij patiënten die volledig afhankelijk zijn van deze vorm van intraveneuze voeding. Om deze reden, wilden we onderzoeken of patiënten in deze situatie wel voldoende essentiële vetten binnenkrijgen, en wat, gezien de gevoeligheid voor infecties van deze patiënten, de effecten van de vetemulsie op het immuunsysteem zijn. In ons centrum krijgen de patiënten een vetemulsie die gebaseerd is op 80% olijfolie en 20% sojaolie. Deze vetoplossing is daardoor rijk aan het enkelvoudig onverzadigde vetzuur oliezuur. In **Hoofdstuk 6** hebben we de hoeveelheid essentiële vetzuren in het plasma en in bloedcellen, en de functie van het afweersysteem van patiënten die langdurig thuis-TPV hadden gebruikt vergeleken met gezonde controles. We vonden geen aanwijzing voor tekorten aan essentiële vetzuren en de functie van het immuunsysteem was ook niet verstoord. In **Hoofdstuk 7**, waarin we het immuunsysteem van thuis-TPV patiënten uitgebreider bestudeerden, vonden we geen bewijs voor een afgenomen functie van het afweersysteem in deze groep, Wel vonden we hogere concentraties van geoxideerd glutathion en lagere plasmaconcentraties van selenium en vitamine C in thuis TPV patiënten vergeleken met gezonde controles.

Samenvattend suggereren onze resultaten dat de vetemulsie die gebaseerd is op 80% olijfolie en 20% sojaolie en gebruikt wordt door patiënten die langdurig afhankelijk zijn van TPV, de patiënten van voldoende essentiële vetzuren voorziet zonder de functie van het afweer systeem nadelig te beïnvloeden. Daarnaast laten onze resultaten zien dat het afsluiten van de katheters met 2% taurolidine in vergelijking met heparine tot minder katheterinfecties en minder verstopte katheters leidt. De afname van deze complicaties heeft ook geleid tot een daling van het aantal ziekenhuisopnames.

& Addendum

LIST OF PUBLICATIONS

Olthof ED, Gülich AF, Renne MF, Landman S, Joosten LA, Roelofs RH, Wanten GJ. Immune activation by medium-chain triglyceride-containing lipid emulsions is not modulated by n-3 lipids or toll-like receptor 4. Accepted in Tox In Vit 2015.

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CURRICULUM VITAE

Evelyn Dorothé Olthof werd geboren op 26 oktober 1983 te Almelo en groeide op in Nijverdal. Na het behalen van haar VWO-diploma aan de scholengemeenschap Reggesteyn te Nijverdal, startte zij in 2002 met de studie Biomedische Wetenschappen aan de Radboud Universiteit in Nijmegen. In maart 2008 studeerde zij af met het hoofdvak Pathobiologie en de bijvakken Geneesmiddelenonderzoek en Epidemiologie. Na het afronden van haar hoofdvakstage naar de hepatotoxiciteit van anti-tuberculose middelen heeft zij dit onderzoek vier maanden verder voortgezet bij de afdeling Longziekten, en in het laboratorium van Maag-, Darm-, Leverziekten van het Radboudumc te Nijmegen. Vanaf 1 februari 2009 heeft zij een jaar onderzoek gedaan naar auto-antistoffen bij systemische sclerose bij de afdeling Immunologie van het Erasmus MC in Rotterdam. Op 1 april 2010 begon zij onder begeleiding van Dr. Geert Wanten haar promotieonderzoek getiteld "Complications in home parenteral nutrition patients: from lock solutions to lipids" bij de afdeling Maag-, Darm-, Leverziekten in het Radboudumc te Nijmegen. Sinds 1 juli 2014 is zij werkzaam op de afdeling Vernieuwing test strategieën, binnen het centrum Gezondheidsbescherming van het Rijksinstituut voor Volksgezondheid en Milieu (RIVM) te Bilthoven.

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