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PAULA RENATA BUENO CAMPOS CANELLA

EFEITO DO USO DA EMULSÃO DE LIPÍDEOS (SMOFLIPID®20%) NO PERFIL LIPIDÔMICO PLASMÁTICO EM MULHERES COM ABORTO RECORRENTE E FALHA DE IMPLANTAÇÃO

Bragança Paulista
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Este trabalho é dedicado a meus quatro filhos: três deles no plano divino e à Valentina Campos Canella no plano terrestre pelo amor incondicional.

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EPÍGRAFE

É necessário fazer outras perguntas, ir atrás das indagações que produzem o novo saber, observar com outros olhares através da história pessoal e coletiva, evitando a empáfia daqueles e daquelas que supõem já estar de posse do conhecimento e da certeza.

Mario Sergio Cortella

RESUMO

Dados recentes sugerem a relação entre aborto recorrente e falha de implantação recorrente com diversos fatores inflamatórios e imunológicos. A Terapia com Emulsão de Lipídeos (LET), contendo ácidos graxos das séries n3 e n6 surgiu como uma nova proposta de tratamento e dados preliminares mostraram que LET são capazes de atenuar a resposta inflamatória e imunológica. Embora alguns relatos apontem que os ácidos graxos da emulsão se ligam a receptores das células *natural killers* (NK), diminuindo sua citotoxicidade, ainda há controvérsias sobre a efetividade e sobre o mecanismo que os ácidos graxos exercem tal ação. O presente trabalho teve por objetivo analisar o perfil lipidômico em plasma de mulheres com aborto recorrente ou falha de implantação recorrente de causas inflamatórias antes e depois do uso de LET de acordo com o desfecho e o tempo de uso. Trata-se de um estudo de coorte longitudinal, com coleta prospectiva, sem qualquer alteração de protocolo. Foi realizada análise dos prontuários das mulheres incluídas no estudo, tais como dados bioquímicos e antropométricos. O perfil lipidômico foi analisado através da técnica de cromatografia líquida de ultra eficiência acoplada a espectrometria de massas de alta resolução (UPLC-QTOFMS) utilizando o *MetaboAnalyst* e a correlação de *Spearman*. As mulheres divididas em 2 grupos, *live birth* e *pregnancy loss* apresentaram diferenças significativas nos fosfolipídios plasmáticos. O grupo *pregnancy loss* apresentou abundância relativa significativamente maior das fosfatidilcolinas (PC) 40:8 e PC 36:5, contendo predominantemente ácidos graxos poli-insaturados da série n6, quando comparado ao grupo *live birth*. Também foi observado significativo aumento na abundância relativa de LysoPC 15:0, PC 42:10 e PC 36:6, contendo predominantemente ácidos graxos poli-insaturados da série n3 em uso prolongado de LET no grupo *live birth*. O estudo deverá colaborar para o melhor entendimento do efeito de LET em mulheres com aborto recorrente e falha de implantação recorrente através do fornecimento de possíveis preditores lipídicos na gravidez.

Descritores: Aborto Recidivante. Lipidômica. Fosfolipídeos. Espectrometria de Massas.

ABSTRACT

Recent data suggest a relationship between recurrent pregnancy loss and recurrent implantation failure with several inflammatory and immunological factors. Lipid Emulsion Therapy (LET), containing fatty acids from the n3 and n6 series has emerged as a new treatment proposal and preliminary data have shown that LET is capable of attenuating the inflammatory and immune response. Although some reports indicate that the fatty acids in the emulsion bind to natural killer (NK) cell receptors, reducing their cytotoxicity, there is still controversy about the effectiveness and the mechanism by which the fatty acids exert such an action. The present study aimed to analyze the lipid profile in the plasma of women with recurrent pregnancy loss or recurrent implantation failure due to inflammatory causes before and after the use of LET according to the outcome and duration of use. This is a longitudinal cohort study, with the prospective collection, without any protocol change. An analysis of the medical records of the women included in the study was carried out, such as biochemical and anthropometric data. The lipid profile was analyzed using the ultra-performance liquid chromatography technique coupled with high-resolution mass spectrometry (UPLC-QTOFMS) using MetaboAnalyst and Spearman's correlation. Women were divided into 2 groups, live birth, and pregnancy loss, which showed significant differences in plasma phospholipids. The pregnancy loss group had a significantly higher relative abundance of phosphatidylcholines (PC) 40:8 and PC 36:5, predominantly containing polyunsaturated fatty acids of the n6 series, when compared to the live birth group. A significant increase in the relative abundance of LysoPC 15:0, PC 42:10, and PC 36:6 was also observed, containing predominantly polyunsaturated fatty acids of the n3 series in prolonged use of LET in the live birth group. The study should contribute to a better understanding of the effect of LET in women with recurrent pregnancy loss and recurrent implantation failure by providing possible lipid predictors in pregnancy.

Keywords: Recurrent Pregnancy Loss. Lipidomic. Phospholipids. Mass Spectrometry.

LISTA DE SÍMBOLOS E ABREVIAÇÕES

AA -Ácido Araquidônico

ALA - Ácido α-Linolênico

células *Treg*s - células T reguladoras

COX - Ciclo-oxigenase

DCs - Células Dendríticas

DHA - Ácido Docosaexaenoico

DHET - Ácido diidroxieicosatrienoico

DPA - Ácido Docosapentaenoico

DTA - Ácido Docosatetraenoico

EET - Ácido Epoxieicosatetraenoico

EPA - Ácido Eicosapentaenoico

GPL - Glicerofosfolipídeo

HETE - Ácido Hidroperoxieicosatetraenoico

HPETE - Ácido hidroperoxieicosatetraenóico

IMC - Índice de Massa Corporal

IVF - *In Vitro Fertilization*

LA - Ácido Linoleico

LCPUFAS - Ácidos Graxos Poli-insaturados de Cadeia Longa

LET - *Lipid Emulsion Therapy*

LOX - Lipo-oxigenase

LTS - Leucotrienos

LXS - Lipoxina*s*

LYSO PC - Lysofosfatidilcolina

NK - *Natural Killer*

PC - Fosfatidilcolina

PLA - Fosfolipase

PUFA - Ácidos Graxos Poli-insaturados

RIF - *Recurrent Implantation Failure*

RPL - *Recurrent Pregnancy Loss*

SPMs - Mediadores Especializados em Pró-Resolução da Inflamação

TRA - Técnicas de Reprodução Assistida

TXS - Tromboxanos

uNK - célula *natural killer* uterina

UPLC-QTOFMS - cromatografia líquida de ultra eficiência acoplada a espectrometria de massas de alta resolução

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1. INTRODUÇÃO

1.1. Aborto recorrente e falha de implantação recorrente

A Organização Mundial da Saúde (OMS), considera a infertilidade uma doença, esta consideração é apoiada por diversas associações, dentre elas: *American Medical Association, European Society for Human Reproduction and Embryology, the International Committee for Monitoring Assisted Reproductive Technologies* (ICMART) e *The American Society*. Historicamente, a definição de infertilidade é o insucesso em conceber após 12 meses ou mais de tentativas com relação sexual sem proteção; ou ainda devido a incapacidade de reprodução do casal ou de um dos parceiros (as) (COMMITTEE; SOCIETY, 2020).

Em alguns casos ocorre a concepção e consequente perda de gestação ou aborto. Aproximadamente 30% das concepções humanas são perdidas antes da implantação e, mais de 30% após a implantação, o que corresponde à terceira ou quarta semana de gestação. Estas perdas são denominadas de perdas pré-clínicas. As perdas tardias ocorrem entre a décima segunda e vigésima segunda semana de gestação, sendo esta últimas menos frequentes, representando cerca de 4% dos casos (LARSEN et al., 2013).

A perda de gestação pode ocorrer uma ou repetidas vezes, no último caso é denominado de aborto recorrente (RPL), termo em inglês *Recurrent Pregnancy Loss*, o qual é definido como três ou mais abortamentos espontâneos e consecutivos, ocorrem geralmente no primeiro trimestre em cerca de 0.6 a 1.0% dos casais (MARTINI et al., 2018; SUGIURA-OGASAWARA; OZAKI; SUZUMORI, 2014) . Aproximadamente metade dos RPL são de causas inexplicáveis (TOTH et al., 2020).

Quando a concepção não pode ocorrer de forma natural, pode ser lançado mão das Técnicas de Reprodução Assistida (TRA), como exemplo a fertilização *in vitro* (FIV), que se dá através da união de um espermatozoide e um ovócito em laboratório, ou seja, a fecundação se dá fora do corpo da mulher com posterior implante do embrião formado no útero da mulher. Essa técnica se concretizou em julho de 1978, quando Lesley Brown entrou para a história da medicina ao dar à luz ao primeiro "*bebê de proveta*" do mundo (Louise Brown) marco histórico para a reprodução assistida, que rendeu ao médico Robert Edwards, o prêmio Nobel de Medicina de 2010 (LEWIS, 2004).

A utilização do procedimento FIV tem aumentado, contudo aproximadamente 10% das mulheres submetidas a esse procedimento terminam com falha de implantação recorrente (RIF), termo do inglês *Recurrent Implantation Failure* (CIMADOMO et al., 2021). Embora não existe um consenso sobre a definição de RIF, a mais aceita é uma falha em obter gravidez clínica em mulheres com menos de 40 anos de idade após três ou mais ciclos de transferência consecutivos de pelo menos quatro embriões de boa qualidade em útero sadio (COUGHLAN et al., 2014).Variáveis como idade materna, índice de massa corporal (IMC) elevado, fatores imunológicos, qualidade do esperma, alterações uterinas e condições psicológicas devem ser consideradas para direcionar as abordagens de tratamento no RIF, apesar que sua etiologia não está completamente estabelecida. A taxa de implantação em mulheres que realizam IVF pode variar de 25-40% (BASHIRI; HALPER; ORVIETO, 2018; COUGHLAN et al., 2014).

Fatores genéticos e imunológicos têm se destacado nas últimas décadas, contudo, existe controvérsia entre os autores em relação ao seu valor na pesquisa e ao tratamento de RPL e RIF de causas imunológicas. Dentre os tratamentos de RPL e RIF de causas inflamatórias e imunológicas se destacam: i) imunização ativa com linfócitos paternos, ii) imunização passiva com imunoglobulina intravenosa (IVIg), iii) fatores de crescimento, como G-CSF (Filgrastim®) e iv) Terapia com Emulsão de Lipídeos (LET), visando o crescimento placentário e fetal e com consequente aumento de nascidos vivos (CARP, 2019).

Apesar das controvérsias existentes em relação ao tratamento, eles são bastante promissores. Um estudo preliminar que mapeou as possíveis causas dos abortos espontâneos, conduzido por especialistas do Ambulatório de Perdas Gestacionais do Centro de Atenção Integral à Saúde da Mulher (CAISM) da UNICAMP apontou que a maioria das gestantes apresentavam alterações de ordem imunológica/inflamatória.

Face a novidade do tema, o presente trabalho realizou análise do perfil lipidômico em plasma de mulheres com RPL e RIF de causas imunológicas ou inflamatórias, foi avaliado o perfil lipidômico das pacientes com RPL e RIF de causas imunológicas ou inflamatórias, as quais fizeram uso de LET. Para que uma gestação seja bem-sucedida são necessárias modificações no corpo da mulher através de mecanismos de defesa uterina, tais como imunotolerância aos espermatozoides e ao feto ou intolerância aos microrganismos invasores que contaminam o ambiente uterino.

Diferentes células fazem parte do sistema imunológico de defesa do organismo e manutenção de uma gestação.

1.2. Fatores imunológicos envolvidos em RPL e RIF

1.2.1. Anormalidade da atividade das células Natural Killer (NK)

As células NK fazem parte do sistema imune inato com capacidade de rápida resposta a patógenos. O termo *Natural Killer cell* é oriundo do inglês, são também conhecidas como células exterminadoras naturais; são células citotóxicas não específicas, importantes na resposta precoce às células tumorais e infecções virais. Oriundas da medula óssea, estão presentes no sangue periférico, no endométrio e em alguns tecidos. As células NK periféricas se diferem fenotipicamente e funcionalmente das células NK uterinas *(uNK*) (KOOPMAN et al., 2003).

São maiores que os linfócitos T e B, apresentam citoplasma granular e marcadores de superfície CD16 e CD56. Diferem funcionalmente dos integrantes da imunidade adaptativa por reagirem de maneira rápida, durante a invasão do organismo por vírus e bactérias. As células NK apresentam algumas diferenças em relação aos linfócitos T, como a ausência do receptor de célula T, molécula central da resposta imunológica do linfócito T que necessita de experiência tímica para ganhar a circulação e mostrar efetividade na vigilância do organismo (PARHAM, 2006). Na resposta imune são ativadas precocemente e não requerem sensibilização prévia para o desenvolvimento de células de memória após a sua ativação, elas são as células citotóxicas da imunidade inata, em contrapartida ao linfócito T citotóxico da resposta imune adaptativa. Diversos estudos demonstram aumento das células NK em pacientes com histórico de abortos (QUENBY et al., 1999; ZENCLUSSEN et al., 2001) bem como falhas de implantação de embriões, em gravidez natural ou FIV (TUCKERMAN et al., 2010).

Foi notado em mulheres com RPL decréscimo no número de células supressoras na decídua e aumento na atividade das células NK, estas últimas agridem o concepto instalado, levando ao aumento de aborto precoce (AOKI et al., 1995). Contudo, não foi evidenciado claramente que alterações quantitativas e ou qualitativas das células NK do sangue periférico estejam relacionadas com RPL e RIF. A porcentagem de células NK no sangue periférico de um indivíduo saudável varia de 5 a 29%; essa variação é dependente dos fatores gênero, stress, etnia e idade. A análise das células NK do sangue periférico, como marcador de risco não é adequada e não deve ser oferecida

como análise na rotina nos casos de RPL (RAI; SACKS; TREW, 2005; TANG; ALFIREVIC; QUENBY, 2011).

Com relação as células *uNK*, é defendido que podem desempenhar papel importante na invasão trofoblástica e angiogênese, além de serem um componente importante da resposta imunitária materna local face aos agentes patogênicos (LE BOUTEILLER; PICCINNI, 2008). As células *uNK* são as principais células do sistema imunológico concentradas no útero, aumentam bastante indo de 10% das células estromais na fase proliferativa a 20% na fase secretora e atingindo 30% no início da gravidez. Em tese, elas regulam a invasão trofoblástica por citotoxicidade direta e produção de citocinas. O estudo das células *uNK* a nível endometrial requer procedimento invasivo, por outro lado pode ser utilizado procedimento não invasivo, estudos recentes apontaram correlação entre as células *uNK* e as NK do sangue periférico, já que elas são recrutadas diretamente do sangue periférico (MATTAR; TRAINÁ; DAHER, 2015).

Contudo, as células NK circulantes mostraram ser diferente em origem, função e fenótipo, de células *uNK* deciduais uterinas, e ainda continuam a ser debatidos sobre altos níveis de células NK no sangue periférico terem significado clínico em RIF (FEYAERTS et al., 2018; MELSEN et al., 2016).

No útero, o endométrio é remodelado ao longo do ciclo menstrual e exibe um curto período de receptividade na fase lútea média, conhecida como “janela de implantação”, que é crucial para implantação e gestação (GELLERSEN; BROSENS; BROSENS, 2007). Nesta fase conhecida como “período de receptividade uterina”, ocorre um influxo muito peculiar de células do sistema imunológico, é observada mutação quase completa da imunidade local do adaptativo Th1 para o perfil do tipo inato Th2 (LEE; LEE; LEE, 2011). Nesse período, 65 a 70% das células imunes no endométrio são células *uNK*. Macrófagos e células dendríticas também são detectadas em conjunto com células T imunes adaptativas, como células T regulatórias (*Treg*s) (HANNA et al., 2006; LIU et al., 2017).

Foi demonstrado que 15 a 25% das mulheres com RPL têm altos níveis de células NK. Apesar desses relatos, ainda não se conseguiu provar que os níveis de células NK sejam a causa do aborto, desta forma a contagem de células NK não deve ser oferecida como parte da rotina de investigação de RPL, se realizado, deve ser informado de sua natureza experimental (KING et al., 2010). Embora parcialmente determinadas as mudanças que ocorrem na implantação, as mais importantes

conhecidas até agora nas células do sistema imunológico endometrial envolvem: macrófagos, *uNK*, células dendríticas (DCs), células T, especialmente células T citolíticas (CTLs) (TICCONI et al., 2019).

1.2.2. Macrófagos

Macrófagos são células de extrema importância da imunidade inata, possuem alta plasticidade e capacidade de diferenciarem-se conforme diferentes estímulos. Existem dois subtipos opostos: tipo M1 e M2. Os M1 são macrófagos clássicos, ativados por ligantes dos receptores TLR (LPS e INF-y) com alta capacidade de produção de citocinas pró-inflamatórias, como IL-1-beta, TNF, IL-12, IL-18 e IL-23, conhecidos por suas características microbicidas, inflamatória e tumoricida. Os M2 são conhecidos como macrófagos de via alternativa, estimulados pela IL 4 e IL10. Podem ser divididos em M2a M2b M2c e M2d, são responsáveis pelo reparo tecidual. A polarização dos macrófagos irá depender dos estímulos locais presentes, sendo na presença de ligantes TLR e interferon-y promoverá uma ativação de M1 e IL-4 e IL-13 ativação M2. Ambos coexistem sinergicamente para balancear a resposta imune, enquanto M1 combate o antígeno causando danos tecidual, o M2 tem função reparadora para manter a homeostase (SHAPOURI-MOGHADDAM et al., 2018; WANG et al., 2019). Os macrófagos aumentam progressivamente da fase folicular para fase lútea, apresentando densidade máxima antes menstruação e na gravidez, adquirindo fenótipo tolero gênico, 20% a 25% do total dos leucócitos na decídua são macrófagos.

Estão por toda parte do endométrio; principalmente ao redor das glândulas e no local da implantação, secretam as TGF-β, IL-10, IDO, PGE2. Tem como funções sugeridas a manutenção do corpo lúteo, implantação do blastocisto, remodelação das artérias espirais, controle de invasão do trofoblasto, proteção do feto contra infecção intrauterina (TICCONI et al., 2019).

1.2.3. Células Dendríticas (CDS)

São células especializadas na captura e apresentação de antígenos. As CDs imaturas migram da medula óssea através da corrente sanguínea, atingindo tecidos periféricos como a pele, onde se tornam residentes (células de *Langerhans*). As CDs são as primeiras a chegarem a um sítio infeccioso, antes mesmo dos neutrófilos; tornam-se ativadas após contato com o antígeno, então migram pelos vasos linfáticos até atingirem órgãos linfoides secundários. Os sinais de maturação podem ser recebidos a partir de células NK, NK/T e LT, de moléculas pró-inflamatórias, como

citocinas, prostaglandinas e interferons e dos *pathogen-associated molecular pattern* (PAMPs) (BANCHEREAU et al., 2000).

O antígeno pode ser retido nos órgãos linfoides pelas CDs por longos períodos, fato que pode contribuir para a memória imunológica. Através de secreção de quimiocinas as CDs orquestram migração de outras células imunes dentro dos linfonodos. Também regulam a diferenciação, a maturação e a função de LT de modo contato-dependente e por secreção de fatores solúveis, portanto são fundamentais para o início organização da resposta imunológica adquirida (BANCHEREAU et al., 2000).

No endométrio a densidade de CDs imaturas é mais alto do que os CDs maduros; 1%-2% de células imunológicas na decídua são DC; ocorre aumento de CDs imaturas da FP para LP (TICCONI et al., 2019).

1.2.4. Células T, Células Reguladoras (Treg) e Citocinas

Na década de 1980, a tolerância materna ao feto foi explicada pela predominância da imunidade do tipo célula auxiliar Th2 durante a gravidez, o que protegia o feto do ataque das células Th1 maternas, então associou-se o efeito predominante da imunidade célula auxiliar Th1 ao aborto. O paradigma Th1/Th2 é insuficiente para explicar o mecanismo pelo qual o feto não é rejeitado por células do sistema imunológico materno. Agora, o paradigma Th1/Th2 foi expandido às células T reguladoras (Treg) e às células Th17. As células Th17 produzem a citocina pró-inflamatória IL-17, e desempenham um papel importante na indução da inflamação. Em contraste, as células Treg desempenham um papel central para a imunorregulação e indução de tolerância imunológica. As células T além de poderem estar envolvidas no aborto, também podem estar envolvidas na gênese da pré-eclâmpsia e do parto pré-termo (SAITO et al., 2010). O balanço adequado de células Th1, as quais são importantes na imunidade mediada por células e protegendo contra infecções virais, Th2 é fundamental para a implantação do embrião. Tanto a ausência como excesso de citocinas estimuladas por Th1 são consideradas prejudiciais para implantação e placentação, como a ausência de citocinas estimuladas por Th2 (CHAOUAT, 2003, 2007). Este interruptor imune com uma ativação adequada de *uNK*, regulação de células T e células dendríticas, tem se demonstrado fundamental para possibilitar o estabelecimento de uma tolerância materna local e a sobrevivência do feto (CHAOUAT, 2003).

As células T auxiliares podem se diferenciar em células auxiliares Th1 na presença de citocina pró-inflamatória IL12 e aumento quantidade de antígeno, mediando a rejeição do aloenxerto ou Th2 na presença de IL4 e IL10 e baixo nível de antígeno, são importantes em respostas alérgicas. As células Th1 e Th2 inibem-se mutuamente visando manutenção da homeostase. O trofoblasto extraviloso (EVT) secreta *histocompatibility antigen* G (HLA-G) solúvel induzindo células T reguladoras do tipo 1 (TR1) a produzir IL10, promovendo a tolerância materna. A Figura 1 mostra o mecanismo de tolerância ou rejeição do embrião (LORENZETTI, 2021).

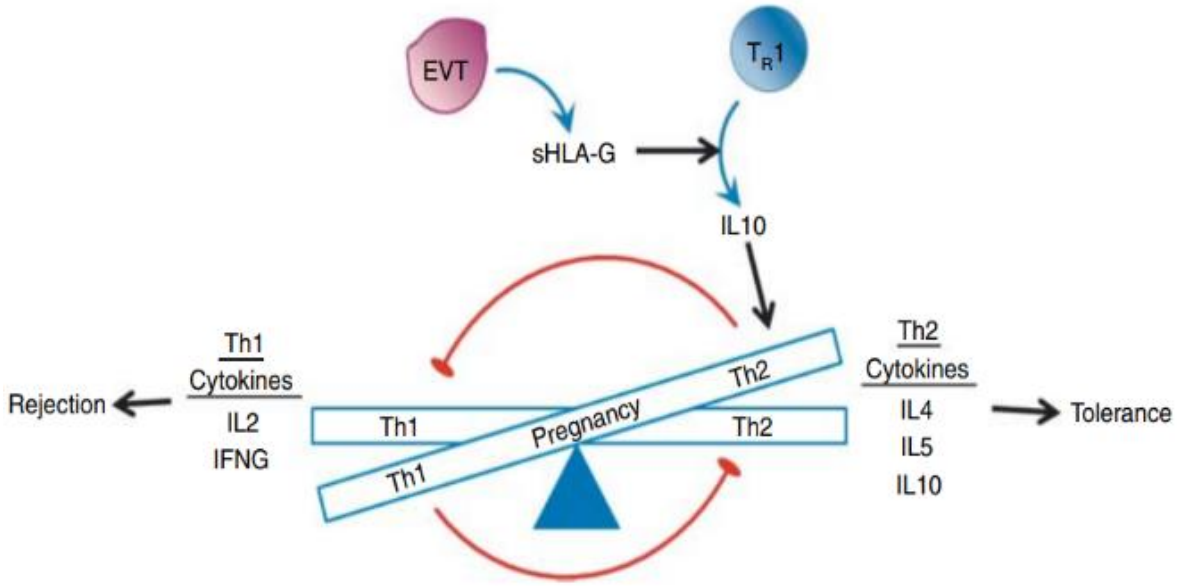

FIGURA 1. Equilíbrio das células T auxiliares Th1 e Th2 na rejeição ou tolerância do embrião na gravidez. EVT (trofoblasto extraviloso), sHLA (antígeno de histocompatibilidade G solúvel), IL (interleucina), TR: células T reguladora, IFNG: *interferon gamma*. Fonte: (LORENZETTI, 2021) modificado.

As células Th1 maximizam a eficácia fagocitária, macrófagos e proliferação de células citotóxicas, enquanto as células Th2 exercem seu comportamento pró-inflamatório através da produção de anticorpos (SARAIVA et al., 2009). Ambos podem ser inibidos pela ação imunossupressora das células Treg, uma interação aparentemente vital para a gravidez. A maior parte dos abortamentos é causada por embriões com anormalidades genéticas, que tornam o embrião incompatível com a vida. No entanto, o papel fundamental do sistema imunológico da mãe em "rejeitar" um feto é um conceito também de muitos especialistas (SARAIVA et al., 2009).

Estudos recentes estabeleceram a crítica importância das células Treg na gravidez humana normal, enquanto também demonstrando que a função Treg prejudicada aparece marcada em RPL e RIF (GUERIN; PRINS; ROBERTSON, 2009).

Diante disso, sugere-se que o aumento da imunidade do tipo Th1 têm efeitos nocivos sobre uma gravidez viável, e que perfis de citocinas podem ser indicadores confiáveis. O papel fundamental das células Treg em gravidez surgiu pela primeira vez a partir de estudos com animais exibindo um acúmulo dessas células dentro do endométrio, na decídua de ratos prenhes (BETTINI; VIGNALI, 2009). Posteriormente confirmou-se que a decídua no início da gravidez continha uma abundância de células Treg em humanos (LOUTEN; BONIFACE; DE WAAL MALEFYT, 2009). A observação de que a frequência de células T CD4 + no sangue periférico em mulheres grávidas aumentou durante o primeiro e segundo trimestre de gravidez também acrescentou peso para a teoria de que as células Treg desempenham um papel significativo na tolerância imunológica durante início da gravidez (HEIKKINEN et al., 2004).

Um tratamento de primeira linha para RPL e RIF de imunoterapia frequentemente prescrita são os corticoides, por ser anti-inflamatório potente de amplo espectro com propriedades imunossupressoras, com objetivo de impedir um envolvimento do útero em um estado não receptivo ou terminação pós-implantação precoce através de processo semelhante à rejeição. No entanto, existem pacientes resistentes aos corticoides, 29% dos pacientes testados tiveram piora dos parâmetros do sistema imunológico endometrial, mesmo que tenha sido documentado ativação local imune excessiva (LÉDÉE et al., 2018).

Tendo em vista o aumento exponencial das taxas de infertilidade, até mesmo quando é lançado mão de Técnicas de Reprodução Assistida (TRA), muitas vezes ocorre a falha de implantação, especula-se que a causa de infertilidade possa ser de origem imunológica. Nesse cenário a Terapia de Emulsão de Lipídeos (LET), vem se destacando como alternativa terapêutica, associada ou não as TRA em tratamentos de RPL ou RIF de causas inflamatórias ou imunológicas, um benefício importante de LET é ser um procedimento menos invasivo, defende-se que tal tratamento melhora o ambiente inflamatório uterino, proporcionando um ambiente favorável para uma gestação saudável bem como buscando aumentar a taxa de nascidos. Na literatura, ainda não existe um consenso nesse tratamento, sendo alguns autores favoráveis e outros desfavoráveis ao tratamento diante de resultados obtidos (CANELLA et al., 2021).

1.3. Terapia com Emulsão de Lipídeos (LET)

Falhas de implantação embrionária repetidas e inexplicadas necessitam de elucidações, principalmente em casos das transferências dos chamados “embriões de boa qualidade”. Diferentes mecanismos de ação têm sido propostos para alcançar um ambiente imune “ideal” (SHIRLOW et al., 2017).

A etiologia da RPL e RIF pode ser multifatorial e, em cerca de 50% dessas pacientes não há causa identificável, sendo, portanto, classificada nessa condição como idiopática ou inexplicada. Estudos conduzidos nas últimas décadas vêm propondo uma explicação imunológica para a ocorrência de ambas as intercorrências. A compreensão sobre o autorreconhecimento humano de um embrião ou feto ainda é bem limitada, sugerindo-se alterações nesse processo contribuintes para o fracasso na obtenção de uma gravidez clínica. Diversos fatores bioquímicos foram implicados, incluindo mediadores inflamatórios locais, antígenos de leucócitos humanos e níveis elevados de células NK circulantes (DRIVER et al., 1989). Logo moduladores imunológicos direcionados foram propostos para contornar esse problema, no entanto, sua capacidade de melhorar os resultados da gravidez em pacientes com RPL ou RIF foi contestada em diversos ensaios clínicos randomizados (GRANATO et al., 2000).

A terapia LET surgiu como opção alternativa para casos de aumento da ativação imune local para tratamento de RPL e RIF de causas imunológicas, principalmente quando houver aumento das células *uNK* no endométrio e NK no sangue periférico. As repercussões desta terapêutica são descritas como similares à imunoglobulina e o corticoide, mas na prática clínica, ressalta-se que do ponto de vista econômico se apresenta mais viável (LIANG et al., 2008). Trata-se de uma emulsão de lipídeos contendo ácidos graxos poli-insaturados (PUFA), dentre eles os ácidos graxos essenciais das séries ômega 3 e 6. LET é administrada por via intravenosa durante 3-4 horas e tem ação supressora no sistema imune materno, fato que pode aumentar a taxa de implantação embrionária. Pode conter óleo de soja, peixe, glicerina e fosfolipídios de ovo, é usado como um componente de nutrição parenteral em pacientes incapazes de tolerar uma dieta enteral (DRIVER et al., 1989).

Enquanto o mecanismo exato em que a modulação imunológica obtida por LET permanece incerto, seu ingrediente ativo, óleo de soja, é capaz de inibir mediadores pró-inflamatórios, especificamente células Th1(GRANATO et al., 2000). Em 2008 um estudo comparou a

suplementação com AGPI em paciente pós cirúrgico com as marcas comerciais Intralipid® e Omegaven® e obteve como resultado diminuição da inflamação e modulação sua resposta imune através de significativa diminuição da interleucina 6 (IL-6), do aumento da razão dos linfócitos CD4+/CD8+ no grupo que recebeu Omegaven® (LIANG et al., 2008).

No contexto das falhas de reprodução, foi relatado que Intralipid® tem propriedades imunossupressoras nas células NK circulantes (ROUSSEV et al., 2008; ROUSSEV; NG; COULAM, 2007). Foi postulado que os ácidos graxos dentro da emulsão servem como ligantes para ativar receptores ativados por proliferadores de peroxissoma expressos pelas células NK. A ativação de tais receptores nucleares diminuiria a citotoxicidade das células NK favorecendo a implantação (ROUSSEV et al., 2008). Posteriormente foi relatada uma diminuição da citotoxicidade, da circulação e recrutamento de células NK relacionada sob Intralipid® (MENG et al., 2016). Contudo, a maioria dos autores afirmaram que são necessários estudos confirmatórios para comprovar a eficácia do Intralipid® antes de qualquer recomendação para seu uso na rotina (SHREEVE; SADEK, 2012).

O papel do tratamento LET para melhorar os resultados reprodutivos é uma área promissora, a maioria dos autores concluem que esse tratamento deve ser usado no contexto da pesquisa e não deve ser usado na prática clínica de rotina para melhorar os resultados reprodutivos. Um dos objetivos deste estudo é demonstrar através da análise dos metabólitos obtidos e seu enriquecimento de vias metabólicas seja confrontado com a literatura buscando entender o mecanismo de ação de LET exercido sobre o sistema imune na gestação, correlacionado metabólitos que favorecem e os que não a gestação.

Existe um crescente número de casais com infertilidade que passam por RPL e RIF, levando em consideração que grande parte desses casos de infertilidade são de causas imunológicas/inflamatórias e mesmo com auxílio de tratamentos de TRA não são solucionados, o que podem causar ainda mais frustração, ansiedade e até mesmo problemas psicológicos aos casais. LET têm se destacado, visando proporcionar um ambiente imunológico favorável para gestação, com o benefício de não ter custo muito elevado bem como não ser invasivo. Atualmente o mercado disponibiliza diferentes marcas de emulsão parenteral de lipídeos contendo PUFAs, que se diferem pela razão da série n6/n3.

1.3.1. Ácidos Graxos Poli-insaturados (PUFAS)

Os ácidos graxos podem ser divididos em saturados (SFA) e insaturados, esse último pode ser subdividido em monoinsaturados (MUFAs), representados pelas séries n7 e n9 e poli-insaturados, representados pelas séries n3 e n6. Mamíferos podem sintetizar SFA e MUFA, por outro lado não sintetizam os PUFAs da série n6 e n3, sendo os mesmos adquiridos através da dieta, por isso PUFAs n3 e n6 são denominados de ácidos graxos essenciais (EFA). A incapacidade de sintetizá-los se explica pela falta das enzimas delta 12 e delta 15 desaturase. O ácido linoleico (LA) representa a série n6, pode ser encontrado em óleos de soja, girassol e milho, o ácido α-linolênico (ALA) representa a série n3, pode ser encontrado em óleos de linhaça, canola e peixes (CALDER, 2003). A Figura 2 mostra a classificação dos ácidos graxos.

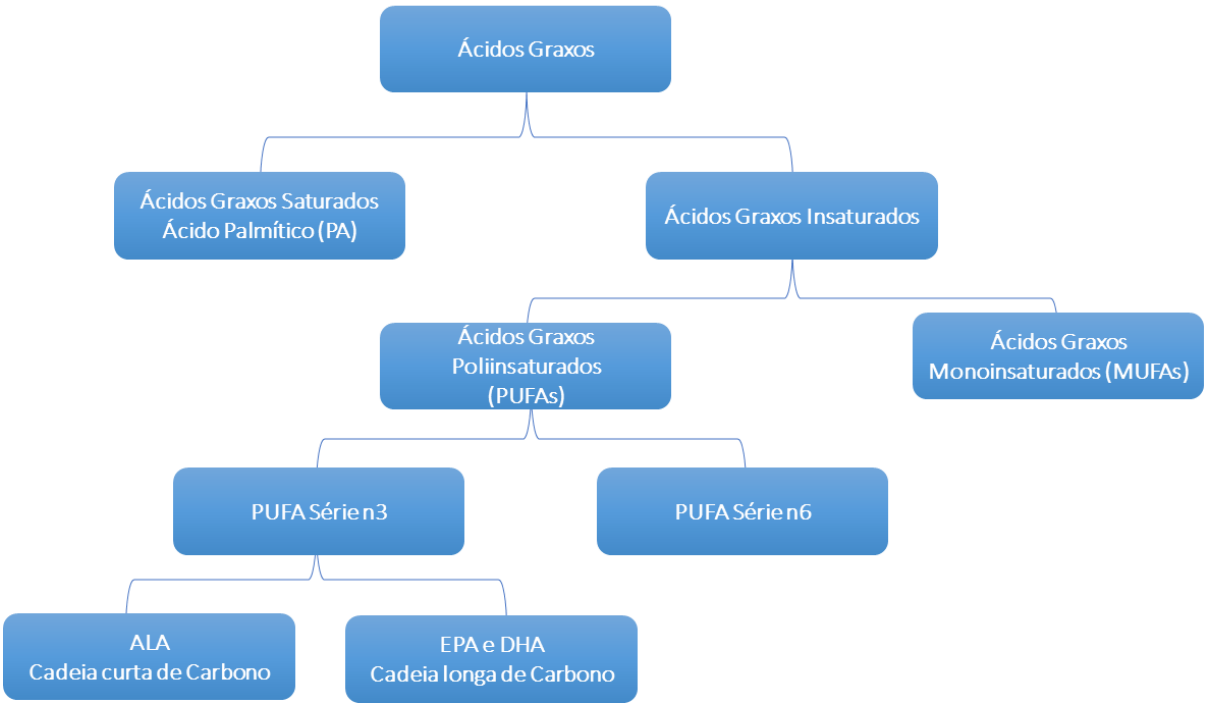

FIGURA 2. Classificação dos ácidos graxos. Fonte: (LORENZETTI, 2021) modificada.

Os PUFAs são constituintes estruturais do núcleo hidrofóbico das bicamadas fosfolipídicas de membrana, com sua propriedade biofísica fornecem o andaime ideal para proteínas de membrana, como receptores, transportadores, proteínas de canal, proteínas de adesão e enzimas. Além disso, os PUFAs servem como precursores de uma variedade de ligantes lipofílicos em vias de sinalização divergentes (STOFFEL et al., 2020).

Os PUFAs incluem os (EFAs): ácido linoleico (LA) da série n6 e ácido α-linolênico (ALA) da série n3, essenciais para viabilidade celular. São utilizados como precursores para a síntese de PUFAs de cadeia longa (LCPUFAs), menos de 24 carbonos na cadeia, e cadeias muito longas, entre 24 e 36 carbonos na cadeia, contendo de duas à seis duplas ligações cis (STOFFEL et al., 2020). O principal LCPUFA da série n6 é o ácido araquidônico (AA) enquanto os principais LCPUFAs da série n3 são ácidos eicosapentaenoico (EPA) e docosahexaenóicos (DHA), estes LCPUFAs são sintetizados a partir dos EFA LA e ALA, respectivamente (SERHAN; LEVY, 2018).

A biossíntese dos PUFA da série n6 inicia-se com a desaturação de LA, a qual consiste em inserção de ligações duplas na cadeia acil formando o ácido γ-linolênico, que sofre elongamento da cadeia carbônica dando origem ao ácido dihomo-γ-linolênico, que sofre desaturação e dando origem ao ácido araquidônico (AA) série n6. Os PUFAs da série n3 passam pelo processo de elongação e desaturação pelas mesmas enzimas, originando os ácidos eicosapentaenóico (EPA) e docosahexaenóico (DHA). As séries n6 e n3 concorrem pelas mesmas enzimas. Contudo, sabe-se que a enzima Δ6 desaturase tem maior afinidade pelo ALA, pertencente à série n3(TEITELBAUM; ALLAN WALKER, 2001). A Figura 3 mostra o metabolismo dos PUFAs n3 e n6 e produção dos mediadores de modulação da resposta inflamatória (eicosanoides).

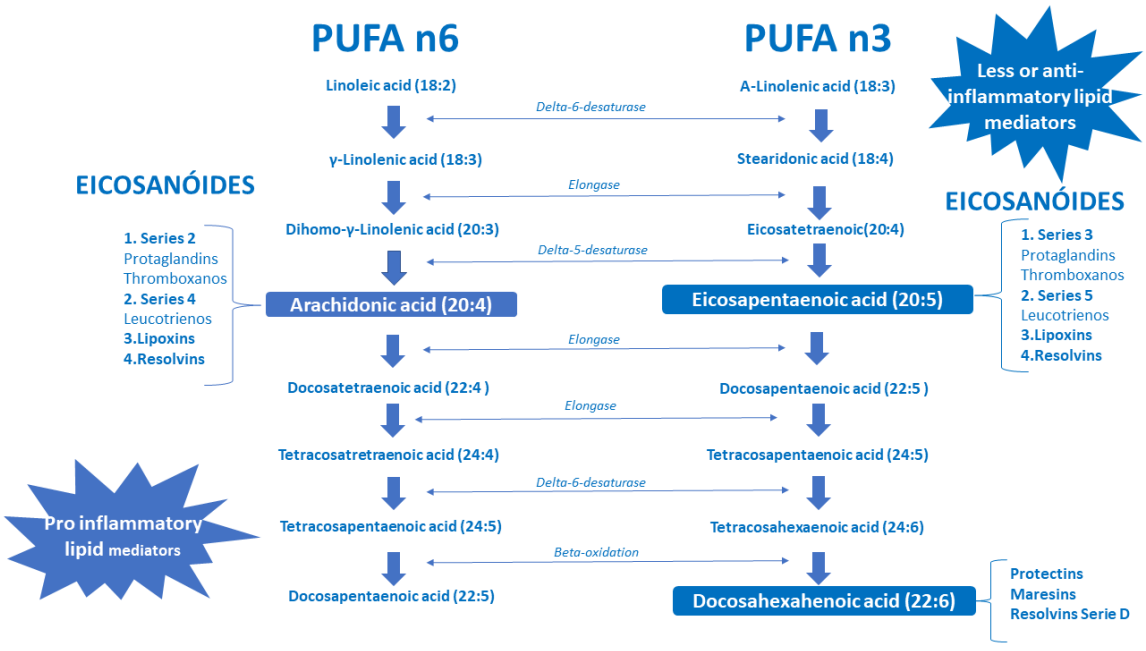

FIGURA 3: Metabolismo de PUFA n6 e n3 e vias no metabolismo ativo de eicosanoides. Fonte: (SALAS-CORONADO et al., 2017) modificado.

Os eicosanóides estão cada vez mais reconhecidos como importantes moléculas vasorreguladoras e biomarcadores envolvidos na fisiopatologia da inflamação, stress oxidativo, patologias ou adversidades da gravidez, incluindo pré-eclâmpsia, parto prematuro e distúrbios fetal do crescimento (WELCH et al., 2020). Sua biossíntese pode ocorrer através das enzimas ciclooxigenase (COX), citocromo P450 (CYP) e lipoxigenase (LOX), ou pela não enzimática. O EPA e AA concorrem pelas mesmas enzimas (LOX e COX). AA formam os eicosanoides pró-inflamatórios: prostaglandinas, tromboxanos, lipoxinas, leucotrienos (MCCARTHY et al., 2014).

O AA, EPA e DHA podem ser alterados através da aquisição de EPA e DHA da dieta. Sendo seus efeitos sobre a expressão do gene inflamatório devido, pelo menos em parte, à redução da ativação de NFkB que parece estar relacionada com eventos mediados por membrana, incluindo a inibição da formação de cascata lipídica em resposta a gatilhos inflamatórios (DĄBEK; KUŁACH; GĄSIOR, 2010). A Figura 4 mostra a formação dos eicosanoides a partir do AA.

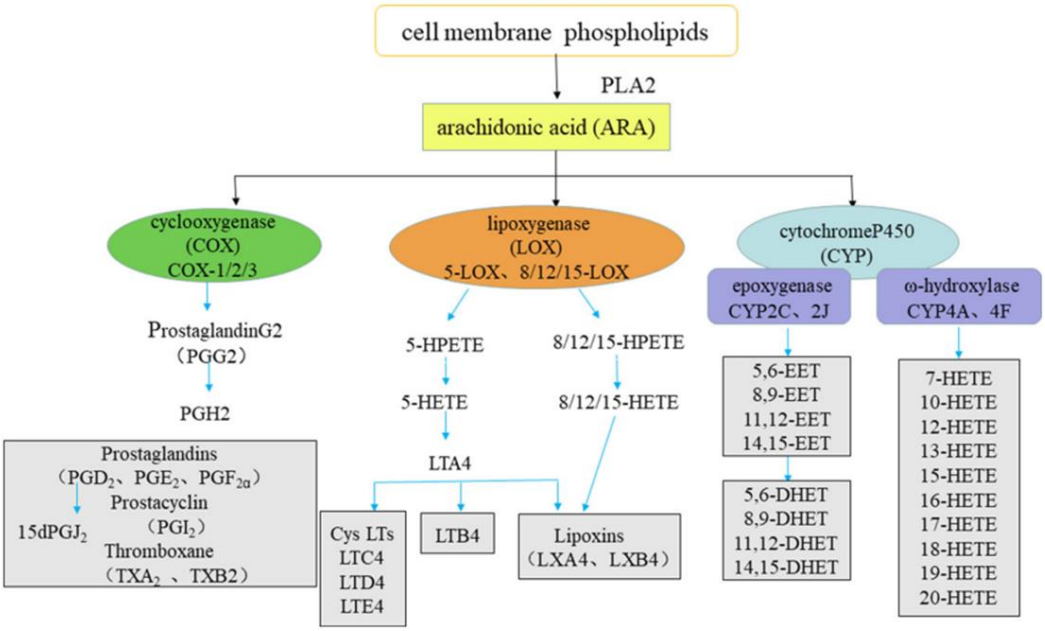

FIGURA 4. Diferentes vias enzimáticas para a produção de eicosanoides a partir de AA. AA, ácido araquidônico; PLA2, fosfolipase A2; PG, prostaglandina; TX, tromboxano; LT, leucotrieno; HPETE, ácido hidroperoxiéicosatetraenóico; HETE, ácido hidroxieicosatetraenoico; LX, lipoxinas; EET, ácido epoxieicosatrienóico; DHET, ácido diidroxieicosatrienóico. Fonte:(HUANG et al., 2020) adaptada.

Enquanto AA é substrato para mediadores lipídicos pró-inflamatórios, EPA e DHA são substratos para a resolução da resposta inflamatória. Os dois últimos originam mediadores especializados em pró-resolução da inflamação (SPMs): resolvinas, protectinas e maresinas. Sendo

que EPA origina mediadores bioativos chamados resolvinas da série E e DHA origina as protectinas, resolvinas da série D e maresinas (SERHAN; CHIANG; DALLI, 2015). Os SPMs lipoxinas podem ser sintetizados a partir do AA (VIRÁG et al., 2019). A Figura 5 mostra a formação de eicosanoides a partir do EPA e DHA.

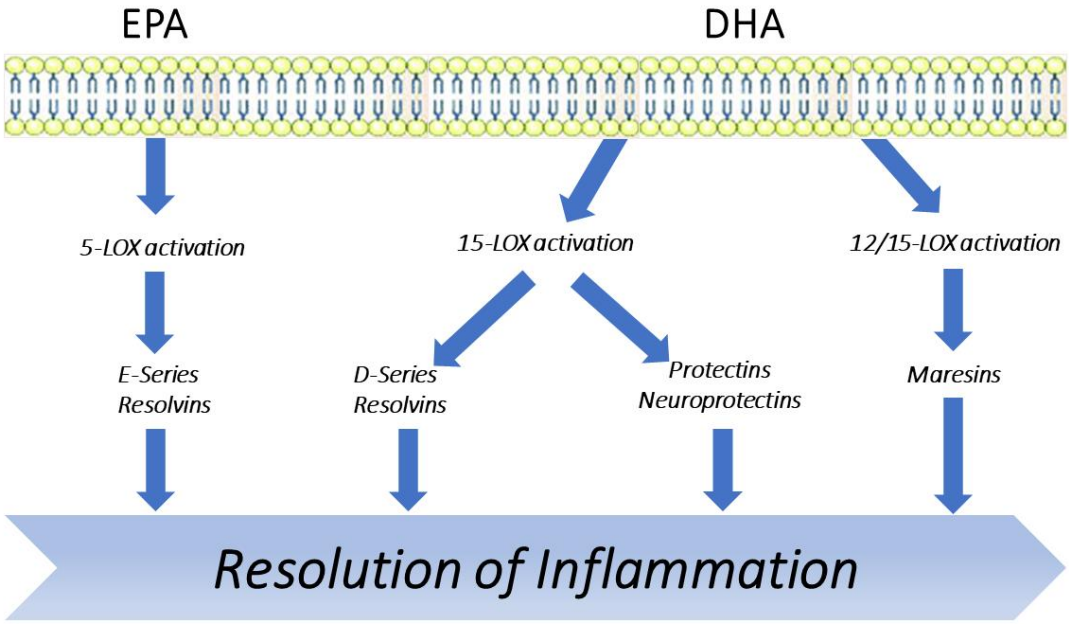

FIGURA 5. Metabólitos biossintéticos de mediadores especializados pró-resolução (SPMs) a partir de EPA e DHA da série n3 PUFA. Fonte:(VIRÁG et al., 2019) modificada.

Os mediadores químicos a partir de PUFAs são conhecidos por desempenhar um papel na iniciação, perpetuação e término de respostas inflamatórias, alterações na composição de ácidos graxos podem modificar lipídios formação da cascata de sinalização celular levando à expressão genética alterada e um padrão alterado de produção de mediador lipídico (AMHERST; CORE, 2020) .

Na fertilidade PUFAs são citados como benéficos para mulher na síntese de prostaglandinas e esteroidogênese (LEYROLLE et al., 2021). Um estudo concluiu que a ligação molecular baseada em estrutura de membrana não reconhecida anteriormente entre PUFAs n3 e n6 e estruturas de membrana gonadal e fertilidade feminina e masculina podem fomentar estudos do papel fundamental dos PUFAs na fertilidade humana. Atividade enzimática de Δ-6-desaturase tem

grande importância em estudos, deficiência de síntese de PUFA causa interrupção da foliculogênese, atresia de oócitos e infertilidade em camundongos fêmea e interrupção da espermatogênese em macho(STOFFEL et al., 2020). Em gestantes com síndrome do ovário policístico foi demonstrada diferença no índice de desaturação MUFA e PUFA especialmente de PUFAs da série n3 no tecido adiposo de gestantes com e sem SOP (EMAMI et al., 2020). Os PUFA n3 podem ser ingeridos através da alimentação embora em fontes mais escassas estão presentes em óleos de canola, linhaça e peixes. Também foi reportado que baixa ingestão materna de PUFA n3 piora a disfunção intestinal induzida pela ativação imune materna com maior reatividade inflamatória local (LEYROLLE et al., 2021).

Além de benefícios na fertilidade, estudos demonstraram benefícios de PUFAs n3 em tempestades de citocinas, possivelmente através de mediadores especializados de pró-resolução (SPMs)(CALDER, 2020). Benefícios também na proteção contra a inflamação vascular, neuro inflamação, hipertensão e trombose, que são parcialmente mediadas por sua conversão em metabólitos epóxidos endocanabinóides n3, docosahexanoil etanolamida (DHEA) e eicosapentaenoil etanolamida (EPEA), originários da *cross-talk* entre endocanabinóide e a via metabólica citocromo P450 (CYTP450). Os epóxidos endocanabinóide n3 têm sido mostrados por diminuir a produção dependente de dose da citocina pró-inflamatória, IL6, enquanto aumenta a produção da citocina anti-inflamatória, IL10. Além disso, n3 epóxidos endocanabinóides podem exercer efeitos anti-angiogênicos em células endoteliais microvasculares humanas. Desta forma os epóxidos endocanabinóides recém-descobertos são esperados para desempenhar um papel crítico durante inflamação *in vivo* (SCAIOLI; LIVERANI; BELLUZZI, 2017).

Existe um consenso científico sobre a importância fisiológica de AGPI n3 de cadeia longa, todavia debates sobre seu potencial mecanismo para curar ou prevenir doenças continuam. A inflamação aguda acontece de forma benéfica visando proteger o organismo de agressões ou invasões por patógenos diversos, contudo, o excesso de marcadores inflamatórios aumenta danos colaterais do tecido e inflamação(SERHAN; LEVY, 2018). Propriedades anti-inflamatórias, em intervenção nutricional com PUFA n3, podem acontecer através de três principais mecanismos: alteração no metabolismo de eicosanoides, fluidez da membrana celular e modulação da expressão genética (MARION-LETELLIER; SAVOYE; GHOSH, 2015).

Ingestão aumentada de PUFA n3 resulta em aumento do teor de EPA e DHA nas membranas celulares, bem como aumento nas concentrações de oxilipina e endocanabinóide derivadas de n3, como amidos de ácido graxo e glicerol-ésteres, essas mudanças (em parte) podendo explicar os efeitos farmacológicos e anti-inflamatórios dos PUFAs n3. Recentemente descobriu-se que endocanabinóides derivados do PUFA n3, podem ser metabolizados pelas enzimas oxidativas CYP-450, LOX e COX, semelhantes aos endocanabinóides derivados de PUFA n6; os endocanabinóides derivados PUFA n3 eicosapentaenoyl etanolamida (EPEA) e docosahexaenoyl ethanolamida (DHEA) têm maior potencial anti-inflamatório e anti-proliferativo do que seus precursores (DE BUS et al., 2019).

O presente trabalho analisou o plasma de mulheres com RPL e RIF de causas imunológicas submetidas ao tratamento com LET para as possíveis alterações lipidômicas relacionadas ao aborto e a gestação.

2. OBJETIVOS

2.1. Objetivo Geral

Avaliar o perfil lipidômico plasmático (classes de lipídeos) em mulheres com RPL e RIF antes e após tratamento com a infusão de lipídeos (SMOFlipid ®20%).

2.2. Objetivos Específicos

Avaliar a alteração do perfil lipidômico plasmático de acordo com desfecho (aborto ou nascimento), antes e após a gravidez.

Avaliar a alteração do perfil lipidômico plasmático de acordo com uso prolongado de SMOFlipid ®20% no grupo nascimento após confirmação da gravidez.

Analisar dados antropométricos e bioquímicos das mulheres submetidas a LET.

3. CAPÍTULO I

3.1. Artigo de revisão

Canella PRBC, Barini R, Carvalho P de O, Razolli DS. Lipid emulsion therapy in women with recurrent pregnancy loss and repeated implantation failure: The role of abnormal natural killer cell activity. **J. Cell. Mol. Med**., v. 25, n. 5, p. 2287-2735, 2021.

Este trabalho teve como objetivo realizar um levantamento de artigos publicados na última década a respeito do uso de LET em mulheres com RPL e RIF com atividade de células natural killers aumentada, analisá-los sobre o uso de LET nesses casos e consequente desfecho. Uma busca sistemática nas bases de dados *PubMed* e *Web of Science* foi realizada para recuperar artigos relatando o uso de LET em RPL e RIF e atividade de células NK no período de 2008 a 2020. Os estudos selecionados foram resumidos com relação ao uso e desfecho, o que permitiu analisar os resultados obtidos com uso da infusão de lipídeos, o que disponibilizou um apanhado de achados para pesquisadores interessados na área.

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REVIEW

WILEY

Lipid emulsion therapy in women with recurrent pregnancy loss and repeated implantation failure: The role of abnormal natural killer cell activity

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Abstract

Altered immune and/or inflammatory response plays an important role in cases of recurrent pregnancy loss (RPL) and repeated implantation failure (RIF). Exacerbation of the maternal immune response through increased NK cell activity and inflammatory cytokines can cause embryo rejection leading to abortion or embryo implantation failure. Immunosuppressors or immunomodulators can help or prevent this condition. Currently, lipid emulsion therapy (LET) has emerged as a treatment for RPL and RIF in women with abnormal NK cell activity, by decreasing the exacerbated immune response of the maternal uterus and providing a more receptive environment for the embryo. However, the mechanisms by which the intralipid acts to reduce NK cell activity are still unclear. In this review, we focus on the studies that conducted LET to treat patients with RPL and RIF with abnormal NK cell activity. We find that although some authors recommend LET as an effective intervention, more studies are necessary to confirm its effectiveness in restoring NK cell activity to normal levels and to comprehend the underlying mechanisms of the lipids action in ameliorating the maternal environment and improving the pregnancy rate.

KEYWORDS

lipid emulsions, NK cell activity, recurrent pregnancy loss, repeated implantation failure

1 | BACKGROUND

Recurrent pregnancy loss (RPL) was first defined by the Royal College of Obstetricians and Gynecologists as three or more consecutive miscarriages before the twentieth week of pregnancy, excluding ectopic, molar and biochemical pregnancies. More recently, RPL was redefined as two or more spontaneous losses of clinical pregnancies before completing 22 weeks of gestation, affecting around 1%-2% of women.[1,2]

Some cases of RPL can benefit from assisted reproduction techniques, among them in vitro fertilization (IVF), an approach where fertilization is performed outside of the body and then the embryo is transferred to the uterus; even so, the in vitro transfer can be unsuccessful. Repeated implantation failure (RIF) is a failure to achieve a clinical pregnancy in women under 40 years old after three or more consecutive transfer cycles of at least four good-quality embryos.[3]

Although the RIF aetiology is not completely established, variables such as maternal age, elevated BMI, immunological factors,

sperm quality, uterine alterations and psychological conditions should be considered to direct treatment approaches. The implantation rate in women under IVF can vary from 25% to 40% depending on the embryo transfer protocol, and about 10% of patients under IVF are affected with RIF.[3,4] Studies conducted in recent decade have been suggesting that immunological abnormalities such as self-recognition of an embryo or foetus could contribute to the implantation failure and thus explain the occurrence of RPL. The abnormal inflammatory response in RPL and RIF includes increased expression of pro-inflammatory markers, human leucocyte antigens and circulating natural killer (NK) cells.[5] Given that, several randomized clinical trials have assessed immune modulators as an approach to address the RPL and/or RIF conditions.[6,7]

Other recent studies discuss the effectiveness in RPL treatment of some immunomodulatory agents such as paternal leucocyte immunization (PLI), intravenous immunoglobulin (IVIg), filgrastim and intralipid.[8-11] Among these, the lipid emulsion therapy (LET) has emerged as a possible new intervention therapy for women stricken by RPL and RIF. The cellular mechanisms by which intralipid acts are not completely understood, but some authors believe that the lipid emulsion restores the NK cells' abnormal activity to normal levels thereby improving embryo implantation.[6,11,12]

In this review, we included only studies using LET to treat patients with a history of RPL and/or RIF conditions as a result of increased NK cell activity. The Medical Subject Headings (MeSH) “NK cells”, “NK cell activity”, “natural killer”, “lipid emulsion therapy”, “intralipid”, “intralipid therapy”, “recurrent implantation failure” and “recurrent pregnancy loss” were used in different combinations for searching in the MEDLINE/PubMed electronic database. The period from 2008 to 2020 concentrates most of the publications matching both the selected MeSH and the scope of the review.

2 | THE ROLE OF NATURAL KILLER CELLS IN RPL AND RIF

Natural killer (NK) cells are a type of cytotoxic lymphocyte involved in the early, innate, immune response against tumour cells and viral infections.[13] NK cell activity is independent of prior activation and triggers the secretion of cytokines such as TNF-alpha and INF-gamma.[14] NK cells are able to lyse virus-infected cells and non-expressing human leucocyte antigen (HLA) cells, inducing cell death through apoptosis.[15] NK cells also undergo interaction with the human G-leucocyte antigen HLA-G gene, which is highly expressed in the trophoblast to prevent the activity of NK cells and the self-recognition of foetal antigens by the maternal immune system suggesting that abnormal expression of the HLA-G gene is involved in recurrent abortions.[16]

There are different types of NK cells classified according to their surface antigen expression markers that include CD16 and CD56. CD16 is responsible for the antibody-dependent cytotoxic action, and CD56 can differentiate into two subpopulations with $CD56^{dim}$ being the most cytotoxic one and $CD56^{bright}$ the less cytotoxic one, producing pro-inflammatory markers such as IFN-gamma and TNF-alpha.[17] In recent years, studies have confirmed that abnormal expression of NK cells surface markers in peripheral blood, endometrial and uNK cells is involved in RPL and RIF, suggesting that NK cells activity is involved in the achievement and maintenance of pregnancy.[18-22]

NK cells in the peripheral blood of healthy individuals range from 5% to 29% depending on the gender, stress, ethnicity and age. Confirming RIF or RPL based on the levels of NK cells in patients' peripheral blood is a controversial issue. Although the analysis of uterine NK cells (uNK) instead of peripheral blood is more robust, this method requires an invasive procedure.[23,24] Although a positive correlation between NK cells from peripheral blood and uNK cells has been observed, some authors have shown non-correlation suggesting that peripheral NK cells and uNK cells have completely different phenotypes and functions.[23,25,26] Recently, it was shown that women with RIF presented an increased percentage of NK cells in their blood compared to the control group, evidence of a positive correlation between peripheral blood and endometrial CD56 cells, suggesting NK cell activity as a potential marker of RIF.[27]

A study performed by Mariee et. al. detected NK cells in the peripheral blood and directly from the endometrium. The authors observed a positive correlation between the number of uNK cells and interleukin 15 (IL-15) in stromal cells suggesting that IL-15 may play a role in the control of uNK endometrial function or cell proliferation.[28] Using endometrial biopsy analysis, a study evaluated uNK abundance in the endometrium of women with idiopathic recurrent miscarriage (IRM) compared to fertile women and found that uNK was increased in IRM patients, suggesting a uNK role in the pathophysiology of recurrent miscarriage.[29]

Control of the immunological response, especially the regulation of NK cell cytotoxicity, is important to ensure embryo implantation success. The embryo triggers the implantation and invasion of trophoblasts that produce the preimplantation factor (PIF). It was shown that synthetic PIF is able to inhibit NK cell-mediated cytotoxicity by reducing NK CD69 expression to comparable levels in intralipid and intravenous gamma-Ig therapies for patients with RPL.[30] Strengthening the hypothesis of NK cell activity involvement in RPL or implantation failure, one study found increased CD69 expression in different peripheral NK cell subtypes in women with unexplained RPL at least two months after the second consecutive miscarriage compared to a control group, suggesting peripheral NK as a marker of altered immune response.[31]

The unexplained aetiology of RPL and RIF increases the interest in seeking new targets and treatments for those conditions. More recently, LET has emerge as a potential intervention to prevent those conditions, especially when NK cells display increased activity in peripheral blood and/or the endometrium and uNK cells. The LET studies and their findings are described in detail in the next section.

3 | LIPID EMULSION COMPOSITIONS AND APPLICATIONS

Lipid emulsions (LEs) are a mixture of fatty acids (FAs), including the essential linoleic and α-linolenic unsaturated fatty acids, which are not produced by the organism. In 1920, Yamahakawa was the first to administer intravenous LEs to humans, and in 1945, McKibbin et al established the use of lipid emulsion for parenteral nutrition. The LEs solutions only became commercially available in the 1950s.[32] Currently, the commercial lipid emulsions are constituted of n-3, n-6 and n-9 long-chain triglycerides isolated or in association with medium chain triglycerides.[33]

Since the 1960s, LEs have excelled in parenteral nutrition therapy. The first generation of LEs was composed exclusively of soy oil (SO), containing a high percentage of n-6 polyunsaturated fatty acids. In the 1980s, the second generation of LEs was elaborated with a lower percentage of n-6 fatty acids compared to the first one. This second generation was composed of 50% coconut oil (CO) which is rich in medium chain triglycerides (MCT). In the 1990s, olive oil (OO) was introduced to LEs, giving rise to the third generation. Currently studies have shown the importance of MUFA/PUFA, and from the 2000s onwards, the n-3 fatty acids family have been included in LEs, represented by the addition of fish oil (FO) ensuring the desired n-6:n-3 proportion.[34]

Isolated or mixed FO emulsion is a source of n-3 and has anti-inflammatory properties.[35] Bae et al published a meta-analysis showing reduced mortality and hospital stay in surgical patients receiving LEs with fish oil compared to patients receiving LEs without fish oil.[36] Another study evaluated the effects of LEs from the n-3 fatty acids family in septic patients and observed a reduction in arachidonic acid (AA) compared to the amount of EPA + DHA and that was associated with improved survival in those patients.[37]

In view of its anti-inflammatory effect, LET has emerged as a potential candidate to ameliorate the RPL or RIF conditions in women. Among the commercially available LEs, the most used is Intralipid®; it consists mainly of purified soya bean oil (10% or 20%) and egg yolk phospholipids (2.25%) emulsified with glycerine and water. Table 1 shows all the commercially available LEs.

4 | LIPID EMULSION THERAPY IN RPL AND RIF

Taking into account the increased activity of NK cells reported in patients with RPL and RIF, and considering the possible anti-inflammatory action of the polyunsaturated fatty acids from the lipid emulsions, the intralipid infusion is emerging as a possible therapy. For this review, we selected studies published from 2008 to 2020, where LEs were used as an intervention to treat RPL and RIF in women with abnormal NK cell activity.

From 2008 to 2020, five studies meeting the abovementioned criteria were published. Among them, three studies observed a significant decrease of NK cell activity when LET was administered, and two did not report any significant effect. Although the studies mention that women with high NK cell activity were chosen for the intervention, some authors fail to present complete data for the NK cell activity before and/or after treatment. Here, we describe in detail the main findings of the studies summarized in Table 2.

Roussev et al (2008), Meng et al (2016) and Lédée et al (2018) observed a decrease in NK cell activity and share the idea that LET is an option to treat RPL and RIF conditions. In the opposite direction, Dakhly et al (2016) observed an increase in the rate of ongoing pregnancies and live births; however, there was no increase in the chemical pregnancy rate. In addition, the authors mentioned that

TABLE 1 Composition of commercially available lipid emulsions

Commercial name	Lipid source	Linoleic (%)	α-Linolenic (%)	α-Tocopherol, mg/L	Phytosterols, mg/L	ω-6: ω-3 ratio
Intralipid® 10%, 20%, 30%	100% soya bean oil	44-62	4-11	38	348 ± 33	7:1
Structolipid® 20%	64% soya bean oil 36% MCT	35	5	6.9	NA	7:1
Lipofundin® MCT/LCT 10%, 20%	50% soya bean oil 50% MCT oil	27	4	85 ± 20	NA	7:1
ClinOleic® 20%	20% soya bean oil 80% olive oil	18.5	2	32	327 ± 8	9:1
SMOFlipid® 20%	30% soya bean oil, 30% MCT, 25% olive oil, 15% fish oil	21.4	2.5	200	47.6	2.5:1
Omegaven® 10%	100% fish oil	4.4	1.8	150-296	0	1:8

Note: Description and composition of the main lipid emulsions. Data supplied by the manufacturers.

Abbreviations: LCT, long-chain triglyceride; MCT, medium-chain triglyceride; NA, not available; ω-6, ω-3 Ratio, ratio of ω-6 fatty acids to ω-3 fatty acids.

TABLE 2 Summary of the analysed studies

Authors and year of publication	Aim	Participants and Methods	Results
Roussev et al,[12] 2008	To establish the duration and efficacy of Intralipid® 20% infusion treatment in suppressing NK cell activity in patients with reproductive failure	50 women with abnormal NK cell activity received three Intralipid® 20% infusions.	In the third Intralipid® infusion, all participants showed normal NK cell activity. The suppressive effect lasted mostly 6-9 weeks.
Dakhly et al,[6] 2016	To determine the efficacy of Intralipid® 20% infusion in women with recurrent spontaneous abortion and abnormal NK cell activity submitted to in vitro fertilization (IVF)/ intracytoplasmic sperm injection (ICSI) cycles	296 women with increased NK cell activity participated in a randomized, double-blind, controlled trial, of which: n = 144 received three Intralipid® 20% infusions and n = 152 received placebo	Chemical pregnancy was achieved in 58.3% of women from Intralipid® group and 50.0% of women from the control group, suggesting that Intralipid® infusions did not increase the frequency of chemical pregnancy
Meng et al,[7] 2016	To determine whether intralipid can be used as an alternative treatment to the intravenous immunoglobulin (IVIG) treatment in women with abnormal natural killer cell activity	154 women distributed in 2 groups, of which: n = 76 received intralipid infusion and n = 78 received intravenous IVIG infusion. Both intralipid and IVIG were infused at different times.	There was no statistically significant difference in successful pregnancy rates between the two groups (92.1% versus 88.2%, $P = .415$).
Lédée et al,[19] 2018	To investigate whether Intralipid® 20% therapy has NK cell immunosuppressive properties	Total of 94 patients with a history of RIF and NK cell over-immune activation between 2012 and 2017.	The intralipid showed a 54% of live birth rate in women after embryo transfer. Also, a reduction of the over-immune endometrial markers (CD56; IL-18/ TWEAK; IL-14/FN-14) was observed
Martini et al,[5] 2018	To determine whether intralipid infusion improves live birth rate in RPL and RIF women with elevated peripheral NK cells activity and to confirm whether intralipid is a cost-effective therapy.	A retrospective cohort study was performed with 127 patients who underwent intralipid therapy from 2012 to 2015 compared to n = 20 from historical cohort data.	Intralipid infusion did not improve live birth rates and was more expensive compared to control group in patients with RIF or RPL.

Note: Description of the studies included in this review, according to the authorship and year of publication, aim, participants included, methods performed and the main findings.

women with abnormal NK cell activity were included in the study, but the data for NK cell activity were not shown. Martini et al (2018) did not observe any increase in the live birth rate in a retrospective intralipid cohort (n = 127) study compared with the historical control cohort (n = 20) data.[5-7,12,19]

Roussev et al analysed fifty patients with abnormal NK cell activity. The patients selected for 20% intralipid solution infusion had NK cell activity checked weekly. The authors showed that 78% of patients presented suppression of NK cell activity in the first week after infusion. However, for 22% of the patients, second and third infusions were necessary to attain normal levels of NK cell activity. In 47 patients, the suppressive effect of intralipid in NK cells lasted 6-9 weeks, and in 3 patients, the suppression lasted 4-5 weeks. The authors stated that the main advantages of LET are the low cost and long-lasting effects when compared to other therapies. They suggested that the fatty acids present in the lipid emulsions can act as ligands to activate peroxisome proliferator-activated receptors (PPARs) expressed by NK cells, reducing their activity. Activation of PPARs has been shown to decrease the cytotoxic activity of NK cells, so it is possible that the intralipid modulates the immune system favoring embryo implantation and pregnancy maintenance.[12]

Meng et al conducted a prospective, randomized, clinical trial between December 2010 and December 2012 and investigated whether intralipid is an immunosuppressive treatment as effective as intravenous immunoglobulin (IVIG), which is expensive and has many side effects. The participants were divided into an intralipid group and an IVIG group. The first intralipid 20% infusion in 250 mL of saline was administered on the third day of the menstrual cycle for at least 2 hours. Thereafter, infusions were administered every 2 weeks before and once a week after pregnancy until the 12th week of pregnancy. The primary outcome was the successful pregnancy rate. In addition, percentage comparisons of peripheral NK cell activity were performed by flow cytometry and were compared before and after each treatment. The results obtained showed

non-significant differences in successful pregnancy rates between intralipid and IVIG (92.1% vs 88.2%, $P = .415$). The decreased NK cell concentrations revealed the cytotoxic effects of treatments in both groups; the authors affirm that LET can be used with the same effectiveness as IVIG to treat patients with elevated NK cell activity.[7]

Lédée et al carried out a randomized control trial between 2012 and 2017 where 94 patients with a history of unexplained RIF and endometrial over-immune activation received a slow infusion of LE (Intralipid®) before embryo transfer. The analysis of NK cells in this study was through endometrial biopsy performed by aspiration with a Cormier pipette in the middle luteal phase. The gene expressions of IL-15/Fn14 and IL-18/TWEAK were determined by quantitative RT-PCR, and uNK CD56 + positive cells were verified by immunohistochemistry. An association of three biomarkers was used to define the uterine immune profile. The proportion of IL-18/TWEAK reflects the locally immuno-regulated Th1/Th2 balance and local angiogenesis; the proportion of IL-15/Fn-14 reflects the maturation of uNK cells and the number of CD56 positive cells. The activated immune profile was characterized by a high ratio of IL-18/TWEAK and/or a high proportion of IL15/Fn-14.[19]

The infusion was administered around day 8 of the embryo transfer cycle (100 mL Intralipid® 20% in 400 mL of saline for 90 minutes). When pregnancy was confirmed, new infusions were performed on the fifth and ninth weeks of amenorrhoea. Among the 94 patients with over-immune activation, 60% had a local excess of Th-1 cytokines (high IL-18/TWEAK ratio); 57% showed uNK cell over activation via IL-15 (high proportion of IL-15/FN-14); and 37% had an excessive recruitment of CD56. In patients who received intralipid emulsion, the authors found a significant decrease in the three biomarkers used to confirm over-immune endometrial activation. The decrease in IL-18/ TWEAK is mainly induced by the decrease in pro-inflammatory cytokine IL-18. The significant decrease in IL-15/FN-14 is mainly caused by a decrease in IL-15 expression. Intralipid® appears to decrease the over activation of uterine NK cells through regulation of the recruitment and expression of the pro-inflammatory cytokines.[19]

Dakhly et al performed a double-blind, randomized, controlled trial at Cairo University from February 2013 to April 2015. The study included women with unexplained infertility and increased NK cell activity (>12%) undergoing in vitro fertilization/intracytoplasmic sperm injection. The women were divided in n = 144 for the intralipid group and n = 152 for the control group. The intralipid group received 2 mL of intralipid infusion 20% in 250 mL of saline on the day of the embryo transfer or insemination. After positive pregnancy, the intralipid infusion was repeated every 2 weeks until the end of the first trimester. The authors observed a significant frequency of ongoing pregnancies and live births rate (P value of .005 for both) in the intralipid group; however, in chemical pregnancy, the effect was not observed. The study mentions that the women presented an increase in NK cell activity, but they did not include or mention the number, percentage or expression of NK cells.[6]

Martini et al performed a retrospective cohort study at a large private infertility clinic from 2012 to 2015. For the study, they selected 127 patients with increased peripheral NK cell activity and a history of RPL and RIF and they received Intralipid. The analyses of NK cell activity were performed by flow cytometry at different timepoints, the first at least 2 weeks before the intervention and then repeated weekly. The authors considered that values above 19% were high for NK activity (aNK), and the intralipid infusion aimed to reach aNK below 10%. Over a period of 90 to 120 minutes, 4 mL of intralipid infusion 20% plus 250 mL 0.9% saline solution was administered 7-10 days before embryo transfer or insemination. After pregnancy was achieved, the infusion was repeated at approximately 6 and 10 weeks of gestation. The authors are against LET as a treatment for RPL; however, they recommend that research should focus on the standardization and the development of a secure method to confirm the NK cell activity, as currently there is no standard for analysis. Although the authors chose patients with increased NK cell activity and history of RPL and RIF for the study, they do not show or comment on whether there was any decrease in NK cell activity in the treated patients.[5]

Lipid emulsion therapy has been proposed as a valid and promising alternative for the treatment of RPL and RIF in women with abnormal NK cells activity. When compared to IVIG therapy, intralipid infusion did not show any significant difference in the rate of live births in women with a history of embryo implant failure, recurrent abortion and high NK cell cytotoxicity.[7,38] It has been shown that 200 patients with RPL and RIF with increased NK cell activity had 61% of live births after LET, which did not differ significantly from the 52% observed for intravenous therapy with immunoglobulin.[38] In other words, LET has been shown to be as effective as immunoglobulin but with the advantage of not being derived from blood and having a lower cost.[38] Nevertheless, such findings should be considered with caution, as more studies are necessary to confirm the results and explain the mechanisms by which lipid emulsions suppress NK cell activity in RPL and RIF.[12,39]

Although the mechanism by which intralipids regulate NK cells function is still unclear, the fatty acids present in intralipid can be recognized by peroxisome proliferator-activated receptors (PPARs), G-protein-coupled receptors (GPCR) and cluster of differentiation (CD1) receptors. Once intralipid particles enter NK cells, they activate signalling pathways involved in immune response, fatty acids activation and transportation. Furthermore, intralipids have been shown to stimulate the reticuloendothelial system to remove 'danger signals' that can lead to pregnancy loss.[11]

According to the reviewed papers, the infusion protocols and results obtained with LET are not consensual and more studies are necessary to confirm its efficacy in improving live birth rates. The beginning of intralipid treatment can vary from the day of oocyte retrieval, the third day of menstrual cycle, 7-10 days before embryo transfer or insemination to day 8 of the embryo transfer. Most authors used around 3-12 infusions of a 20% LEs administered over 30-120 minutes diluted in saline to reach the aimed concentration and guarantee the slow infusions recommended for LEs. Before pregnancy, the intralipid infusion is given every 2 weeks and, once pregnancy is confirmed, the infusion protocol differs among the

authors from once a week to every 2-4 weeks. Most of the authors end the treatment by the 12th week of gestation. Moreover, large-scale studies, double-blind placebo-controlled trials need to be performed in different populations to test the efficacy of LET before it can be recommended for routine use.[19,40,41]

To sum up, sixty per cent of the reviewed studies obtained satisfactory results demonstrating that LET contributed to decreasing NK cell activity in patients with RPL and RIF. Twenty per cent mentioned that they achieved satisfactory and significant results with the LET, although the data for NK cell activity were not addressed in the results and discussion, that would have been interesting to confirm whether there had been a significant reduction of those cells. Twenty per cent did not obtain satisfactory results with LET and mentioned that studies are necessary to define secure protocols for the analysis of NK cell activity, whether peripheral or endometrial, as well as the protocol for intralipid dilution and infusion.

5 | CONCLUSION

Currently, RPL and RIF caused by inflammatory or immunological abnormalities are increasingly common. Although there are only a few studies published on the field, lipid emulsion therapy has been proposed as an immuno-suppressor of the activity of NK cells and other inflammatory biomarkers which could contribute to a viable pregnancy in patients with a history of RPL and RIF. Some studies have observed an increase in implantation and live births and a decrease in the activity of NK cells after intralipid infusions. However, more studies are necessary to verify the mechanism by which LEs acts to decrease the activity of NK cells. Another important factor that must be considered is the composition of the lipid emulsions, as the n-6:n-3 ratio is essential to promoting increase or reduction in the immune inflammatory response. In conclusion, the LEs are a promising option to treat patients with RPL and RIF but studies focusing on NK cell activity must be performed in order to understand the LEs mechanism in RPL and RIF and promote a better comprehension of the pathophysiology of these conditions.

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CONFLICT OF INTEREST

The authors have declared no conflict of interest.

AUTHOR CONTRIBUTIONS

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DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analysed in this study.

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4. Capítulo II

4.1. Artigo experimental

Canella PRBC, Vinces SS, Silva ÁAR, Sanches PHG, Barini R, Porcari AM, Razolli DS, Carvalho PO. Altered profile of plasma phospholipids in woman with recurrent pregnancy loss and recurrent implantation failure treated with lipid emulsion therapy.

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Este trabalho teve como objetivo analisar o perfil lipidômico plasmático de mulheres com aborto recorrente e falha de implantação recorrente de causas inflamatórias/imunológicas tratadas com LET. Foi observada alterações significativas na classe dos glicerofosfolípideos (GPL), com destaque para as fosfatidilcolinas: PC 40:8, PC 36:5 quando realizada comparação de acordo com o desfecho (aborto ou nascimento); e para lysofosfatidilcolinas LysoPC 15:0, PC 36:6 e PC 40:10 quando realizada comparação do uso de LET a longo prazo no grupo nascimento. O estudo deve colaborar para a importância da composição no perfil lipidômico em mulheres com RPL e RIF podendo fornecer possíveis preditores lipídicos relacionados aos abortos ou nascimentos.

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ORIGINAL ARTICLE

Altered profile of plasma phospholipids in woman with recurrent pregnancy loss and recurrent implantation failure treated with lipid emulsion therapy

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Abstract

Background: Recurrent Pregnancy Loss (RPL) and Recurrent Implantation Failure (RIF) are highly heterogeneous condition and many of the mechanisms involved still require elucidation. The aim was to analyze the lipidomic profile in plasma of women with RPL and RIF before and after receiving the Lipid Emulsion Therapy (LET) containing 10% fish oil (SMOFlipid® 20%).

Methods: This study included twenty-six women with RPL or RIF from immunological or inflammatory causes, with elevated natural killer cell levels and divided into a Pregnancy Loss or a Live Birth group according to the outcome. The women received intravenous LET and sample collecting was done before the first, third and fifth dose of LET in the pregnant women. Ultra-performance liquid chromatography quadrupole time of flight mass spectrometry (UPLC-QTOF MS) and multivariate statistical methods were performed to evaluate the profile of phospholipids present in the women's plasma.

Results: An increase of phosphatidylcholines (PC) 40:8 and 36:5 levels with predominance of n6 polyunsaturated fatty acids (PUFA) was observed in plasma lipids of the Pregnancy Loss Group compared to Live Birth Group. We also observed an increase in the relative abundance of n3 PUFA-PC species (42:10 and 36:6) and LysoPC 15:0 with the long term use of LET.

Conclusion: The greater availability of n3 PUFA in plasma of the pregnant women stemming from LET use can be considered advantageous regarding the alteration of the phospholipid profile and its postulated anti-inflammatory and immunomodulatory role.

KEYWORDS
lipid emulsion therapy, lipidomic analyses phospholipids, recurrent implantation failure, recurrent pregnancy loss

1 | INTRODUCTION

Recurrent Pregnancy Loss (RPL) is defined as the failure of two or more clinically recognized pregnancies before 20–24 weeks of gestation and includes embryonic and fetal losses.[1] There is no consensual definition of Recurrent Implantation Failure (RIF), however, the preimplantation genetic diagnosis consortium of the European Society of Human Reproduction and Embryology (ESHRE) defines RIF as more than three failed embryo transfers with high quality embryos, or transfer failure of ten embryos in multiple transfer.[2] Chronic inflammation of endometrial cells (endometritis) is often associated with a high risk of RPL and RIF and affects 15%–50% of women.[3]

During the pregnancy process, inflammatory changes are necessary in the woman's body to enable trophoblast invasion, angiogenesis and placental growth. However, when it is continuous and uncontrolled, it can jeopardize the pregnancy and even maternal health.[4] Immune cells are present in implantation, such as: decidual natural killer cells, T helper cells (Th1, Th2, Th17) and regulatory T cells (Treg), natural killer cells (NK), macrophages, and dendritic cells. The decidual natural killer cells produce the cytokines interleukin-4, interleukin-5, and interleukin-13 and they constitute 70% of immunocompetent cells in the endometrium, controlling angiogenesis and trophoblast invasion. Imbalance in decidual NK cell activity may lead to pregnancy termination or gestational trophoblastic disease.[5]

Considering the importance of a balanced response of the woman's immune system's (anti-inflammatory x pro-inflammatory) to the embryo and its association with metabolism in pregnancy disorders (such as RPL and RIF), treatment generally focuses on modulating the initial inflammatory response. Cell membrane lipids are also responsible for the regulation of various biological processes, and changes in membrane lipid homeostasis are linked to several diseases.[6] Lipid emulsion therapy (LET) is a lipid emulsion for intravenous infusion for the treatment of critically ill or surgical patients unable to acquire adequate nutrients orally or enterally.[7] LET has been used in RPL and RIF treatments as it provides a more receptive environment to the embryo implantation by decreasing the exacerbated immune response[8] However, although the direct effect of LET on fertility is still not clear, it seems to depend on NK cytotoxicity inhibition, possibly mediated through short fatty acids stimulating peroxisome proliferator-activated receptor γ. It has been shown that there is a decrease in NK cell cytotoxicity for several weeks after a single infusion of LET.[9]

The LET SMOFlipid® 20% (Fresenius Kabi, USA), among others, contains in its formulation a mixture of soybean oil, medium chain triglycerides, olive oil, and fish oil; that is, among the fatty acids, the polyunsaturated fatty acid (PUFA) α-linolenic acid (ALA) from the n3 series and linoleic acid (LA) from the n6 series. These two PUFAs represent the essential fatty acids that are not synthesized by the body and are obtained through the diet. In the last two decades, efficacy evaluation studies have indicated SMOFlipid® as having the probable optimal proportion of PUFA n6 to n3 series, and it would seem to provide immunological, anti-inflammatory and pro-regenerative capabilities, in addition to the fact that SMOFlipid® may be less toxic to the liver than soybean oil or olive oil.[7] Through endogenous synthesis, PUFAs of the n3 series, ALA, and the n6 series, LA, can give rise to lipid molecules with 20 carbons like arachidonic acid (AA) of the n6 series, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) of the n3 series and to other so-called eicosanoids that can act as signaling molecules or local hormones. Many eicosanoids have effects on inflammation and immunity, with prostaglandin E2 being the most widely studied and many have functions in the regulation of the female reproductive systems.[10] Also, from PUFA n3 series (EPA and DHA) important specialized pro-resolution lipid mediators are formed such as resolvins, maresins and protectins. Many studies have elucidated the crucial roles of pro-resolution lipid mediators in the resolution of inflammation.[11]

Although, the LET has been proposed as an immune suppressor of the activity of NK cells and other inflammatory biomarkers which could contribute to a viable pregnancy in patients with a history of RPL and RIF,[12] to our knowledge, there are no studies on the lipidomic profile under these conditions, here we analyzed the changes in the lipid profile of women using LET. We have performed a longitudinal study, with prospective collection, to compare Live Birth and Pregnancy Loss groups (separated according to the outcome) as well as the effect of long term use of LET (SMOFlipid® 20%) in the Live Birth group on specific lipid levels. An untargeted metabolomics approach based on ultra-performance liquid chromatography quadrupole time of flight mass spectrometry (UPLC-QTOF-MS) was performed to assess the women's plasma lipid profiles. This high-resolution platform integrates full MS with MS/MS fragmentation for all precursor ions simultaneously, enabling high-throughput acquisition of data and simultaneous annotation of diverse groups of secondary metabolites.[13,14]

2 | MATERIALS AND METHODS

2.1 | Study groups

The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Research Ethics Committee of the São Francisco University (USF) under CAAE number 03731518.7.0000.5514. Patient recruitment was done at Barini Institute (City of Campinas, São Paulo state Brazil) from March 2019 to March 2021 and no changes were made to clinic's protocol conducted in the institute by the responsible medical team. The women were divided into: Pregnancy Loss and Live Birth groups. The following inclusion criteria were adopted for the groups: women with RPL (three or more spontaneous, unexplained miscarriages—Pregnancy Loss group), from 30 to 45 years old, increased levels of NK cell activity 50:1 effector-to-target ratio, measured in peripheral blood (above 20%) and capable of understanding the nature and purpose of the study confirmed by signing the voluntary informed consent form. The exclusion criteria were women with miscarriages due to genetic, anatomical, infectious or unknown causes, and women with associated chronic diseases such as cardiovascular disease, hypertension, diabetes, cancer, and autoimmune diseases. Women were included after assessment of

TABLE 1 Fatty acid composition of LET SMOFlipid® 20% (Fresenius Kabi, USA)

Fatty acid[a]	Family	Grams (g)/L
Caproic acid (C 6:0)	–	0.2
Caprylic acid (C 8:0)	–	32.3
Capric acid (C 10:0)	–	22.7
Lauric acid (C 12:0)	–	0.3
Miristic acid (C 14:0)	–	1.9
Palmitic acid (C 16:0)	–	18.2
Palmitoleic acid (16:1)	n-7	3.3
Estearic acid (C 18:0)	–	5.5
Oleic acid (C 18:1)	n-9	55.3
Linoleic acid (C 18:2)	n-6	37.2
Arachidonic acid (C 20:4)	n-6	1
Estearidonic acid (C 18:4)	n-3	0.9
α- Linolenic acid (C 18:3)	n-3	4.7
Eicosapentaenoic acid (C 20:5)	n-3	4.7
Docosapentaenoic acid (C 22:5)	n-3	0.7
Docosahexaenoic acid (C 22:6)	n-3	4.4
α-tocopherol	–	∽200 mg/L
Ratio n6/n3	–	2.48

[a]Information provided by the manufacturer Fresenius Kabi, EUA.

their health status by clinical evaluation and laboratory tests. Sixty-six women with RPL and RIF from inflammatory or immunological causes undergoing LET treatment were selected for this study. Forty were excluded for the following reasons: 21 did not become pregnant, four abandoned treatment and 15 did not have a sufficient number of samples for analysis.

2.2 | Lipid emulsion

SMOFlipid® 20% is intravenous lipid emulsion containing fatty acids from the omega 9, 7, 6, and 3 series. In relation to the unsaturated fatty acids profile, there is a predominance of oleic acid (n9 series), LA (n6 series), ALA (n3 series), EPA (n3 series), DPA (n3 series), and DHA (n3 series) (Table 1).

2.3 | LET protocol

Spontaneous pregnancy: the first dose of LET was administered between the 6th and 8th day of the menstrual cycle and every 60 days. In vitro fertilization: the first dose was administered between the second and fourth day of the ovulation-inducing medication. After a positive beta-HCG result (pregnant), five doses were administered 30 days apart until the 20th week of gestation for the above two cases. Twenty-six women participated in the study. Initially 19 women were categorized into Live Birth ($n = 9$) and Pregnancy Loss ($n = 10$) groups by outcomes. Ten women went to the pregnancy loss group after the second dose (T2), corresponding to the 30–60 days period. Seven women entered the study already pregnant (T3) thus totalizing $n = 16$ (Live Birth group) who continued using the infusion. The mean period of T0 to T3 in preconception treatment group was approximately 150–180 days. The first blood sample was collected before starting the LET treatment (T0). Analyses were performed at four different times: (i) non-pregnant women: before starting LET treatment (Time 0-T0, $n = 19$) and (ii) pregnant women (Live Birth): before the first LET during pregnancy (Time 3-T3, $n = 16$), the third LET during pregnancy (Time 5-T5, $n = 9$), and fifth LET during pregnancy (time 7-T7, $n = 7$). In this study, we included patients who received up to 2 LET doses before T3 (at times designated as T1 and T2), which were not analyzed. Before each LET infusion, a peripheral blood sample was collected for analysis. Figure 1 illustrates the blood collection scheme.

2.4 | Sample collection

Peripheral blood samples were collected into tubes containing ethylenediamine tetraacetic acid (EDTA), from women submitted to lipid emulsion therapy at different (pre and post) gestational stages and before each LET dose. After collection the samples were centrifugated and the plasma and erythrocytes fractions were separated then transported to the Multidisciplinary Research Laboratory of the USF in Bragança Paulista-SP-Brazil, where they were stored at −80°C until the lipidomic analyses could be performed. The NK cell activity was performed only prior to initiation of LET (Sollutio Soluções Diagnósticas Integradas, Campinas São Paulo state, Brazil). The test result is expressed as a percentage and should be interpreted as the % lysis of target cells by NK cells in the ratio of effector cell to target cell of 50:1. The test methodology was developed and validated by the aforementioned laboratory in accordance with current legislation, based on previously published studies.[15–17]

2.5 | Liquid chromatography—Mass spectrometry (LC-MS) analysis

Plasma samples (75 μl) were extracted by adding 800 μl of a MeOH/MTBE/$CHCl_3$ solution (1, 33:1:1, v/v/v). Afterward, vortex (30 s), shaker (2000 RPM, 30 min a 22°C) and centrifugation (13 000 RPM, 5 min, 4°C) were carried out and 500 μl of the bottom organic layer were collected and dried under nitrogen flow. Dried samples were stored at −20°C until analysis. For analysis, samples were reconstituted in 300 μl of a solution of IPA/ACN/water (2:1:1 v/v/v). To analyze in positive mode the samples were diluted 5x to decrease signal strength.

For the positive ionization mode, samples were 5-fold diluted. Samples were randomly analyzed to observe the biological variation and minimize instrumental bias. Aiming to monitoring the stability of the analytical analysis, equal amounts of all samples were pooled as a quality control (QC) sample and splinted in 16 equal fractions that were placed after every 10 samples. The method was based on previously

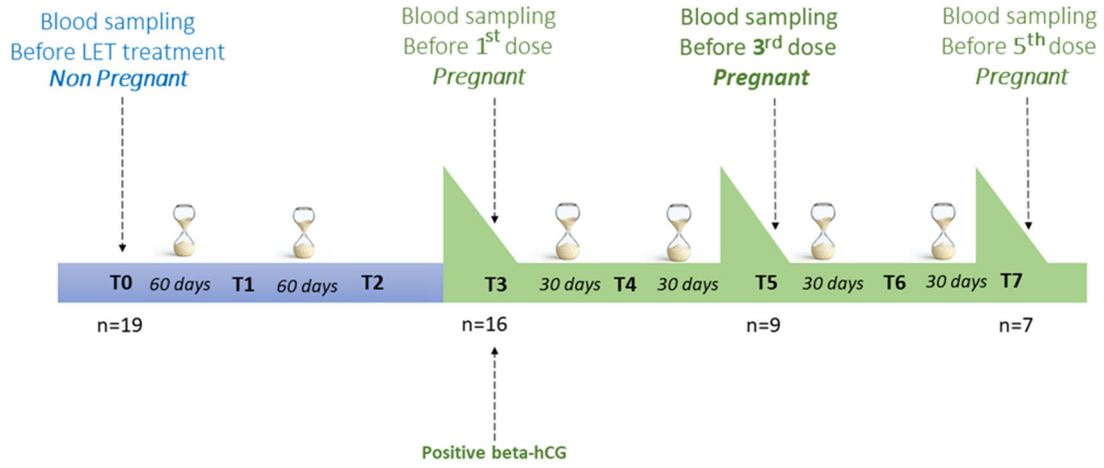


FIGURE 1 Times of blood collections and LET infusion

published works from our group.[18,19] An ACQUITY UPLC coupled to a XEVO-G2XS QTOF mass spectrometer (Waters, Milford, MA) equipped with an electrospray ion source was used. Liquid chromatography was performed using an SUPELCO Titan® C18 column (2.1 mm × 100 mm, 1.7 μm, Waters). The Mobile phase A was composed of a solution of 10 mM ammonium format with .1% formic acid in ACN/water (60:40, v/v), while mobile phase B was composed of a solution of 10 mM ammonium formate with .1% formic acid in IPA/ACN (90:10, v/v). The flow rate was .4 ml/min. The column was initially eluted with 40% B, increasing to 43% B during 2 min and subsequently to 50% within .1 min. Over the following 9.9 min, the gradient was further ramped to 54% B, and then to 70% of B in .1 min. In the final part of the gradient, the amount of B was increased to 99% over 5.9 min. Solution B finally returned to 40% in .1 min, and the column was equilibrated for 1.9 min before the next injection. The total run time was 20 min. Positive and negative ion modes were recorded (separately) and the instrument was operated in MS^E mode in the m/z range of 50–1200, with an acquisition time of .5 s per scan. Other parameters were as follows: source temperature = 140°C, dessolvation temperature = 550°C, dessolvation gas flow = 900 L/h, capillary voltage = 3.0 kV (+)/2.5 kV (–), cone voltage = 40 V. The acquisition scan range was from 50 to 1700 Da and the data were acquired using the high-energy mass spectrometry (MSE) approach. Leucine encephalin (molecular weight = 555.62; 200 pg/μl in 1:1 ACN: H2O) was used as a lock mass for accurate mass measurement.

Data Preprocessing. The selection of possible adducts and compound annotation based on MS^E experiments were obtained using Progenesis QI 2.0 software (Nonlinear Dynamics, Newcastle, UK). The parameters established for putative annotation were mass error of 5 ppm for the precursor and 10 ppm for the fragment. Putative identification was defined by fragmentation scores, mass precision, and isotope similarity. LIPID MAPS and the Human Metabolome (HMDB) Databases were used for the compound annotation according to the Metabolomic Standards Initiative (MSI) guidelines.[20] In brief, ions with some matching level with MS/MS database achieved level 2, while compounds supposedly identified by their exact mass, using the mummichog algorithm, achieved level 3. Progenesis QI software was used to generate ion intensity tables per sample and ions were labeled according to their retention time and mass-to-charge ratio (*m/z*).

2.6 | Statistical analysis

MetaboAnalystR 3.0,[21] Systematical Error Removal using Random Forest (SERRF) code implementation and Bioconductor package manager using R programming language,[22] were used to perform data treatment and statistical the metabolites. A quality control-based signal correction was performed using a SERRF method.[23] The features of all collection times were ranked using multivariate statistical analysis (SVM with AUC > .75 accuracy >75%), comparing the abundances of the sample features according to the outcome for each collection time (T0, T3, T5, and T7). According to the "80% rule" peaks present in more than 80% of the samples of each group were kept for further analysis. Then, the corrected data were log-transformed and scaled using the Pareto scale.[24] The missing values were replaced by half of the minimum positive values detected. Features were considered for further statistical analysis when they met the criteria of Relative Standard Deviation (RSD) in pooled QC <20% and not-low variance based on the interquartile range.[23] Univariate, Fold Change and t-test, and multivariate statistical models, Support Vector Machines (SVM), were used to compare the groups of the most relevant variables

Univariate statistics and multivariate models were performed for the feature selection of the most relevant variables comparing the study groups. For univariate analyses, features with *p*-values <.05 (or -log(*p*-value) >1.3) using *t*-test and 2-fold (or |log2(fold change) >1) intensity between groups, for each feature were considered. For multivariate analyses, the biomarker analysis module implemented in the MetaboanalystR package was used for detecting relevant features for each desired classification. The SVM model produces a reduced list of features ranked by their importance. All the features obtained here were then used in the annotation stage.

TABLE 2 Baseline anthropometric and biochemical parameters of women of live birth and pregnancy loss groups

	Live birth	Pregnancy loss	Reference value*	*p*-value
N	16	10	–	–
N RPL/ N RIF (T0)	5/4	5/5	–	–
Miscarriage				
<10 week	–	6		–
<20 weeks	–	4		–
Age (years)	34.4 ± 2.6	39.2 ± 4.1	–	0.29
BMI	23.1 ± 4.1	24.1 ± 3.6	–	0.39
Glucose (mg/dl)	84.3 ± 5.6	85.6 ± 7.1	–	0.71
NK cytotoxicity				
(%, effector to target cell ratio 50:1)	26.3 ± 7.8	25.5 ± 9.2	<20%**	1
Total cholesterol (mg/dl)	189.5 ± 12.8	193.5 ± 17.3	<190 mg/dl*	0.86
HDL-cholesterol (mg/dl)	51.5 ± 6.5	58.4 ± 9.1	>45 mg/dl*	0.68
LDL-cholesterol (mg/dl)	116.8 ± 7.0	142.8 ± 7.4	<115 mg/dl*	0.52
Triglycerides (mg/dl)	79.8 ± 18.2	97.1 ± 9.4	<150 mg/dl*	0.46

Dates are analyzed by means ± SD;
Abbreviations: BMI, body mass index; *N*, number of women measured for each characteristic; HDL, high-density lipoprotein; LDL, low density lipoprotein; NK, natural killer; RIF, recurrent implantation failure; RPL, recurrent pregnancy loss.
*(25); **(15–17).

3 | RESULTS

3.1 | Clinical characteristic

Table 2 shows a statistical comparison of the two groups for the baseline characteristics of the women involved in this study. Nonparametric tests were carried out and the values for each characteristic represent the mean of the group. No significant differences were found between the characteristics of the groups. The activity of NK cells considered normal is less than 20%, however, in both groups, these values were higher than normal, which was used as an inclusion criterion. The reference values adopted were considered according to previously reported data.[15–17,25]

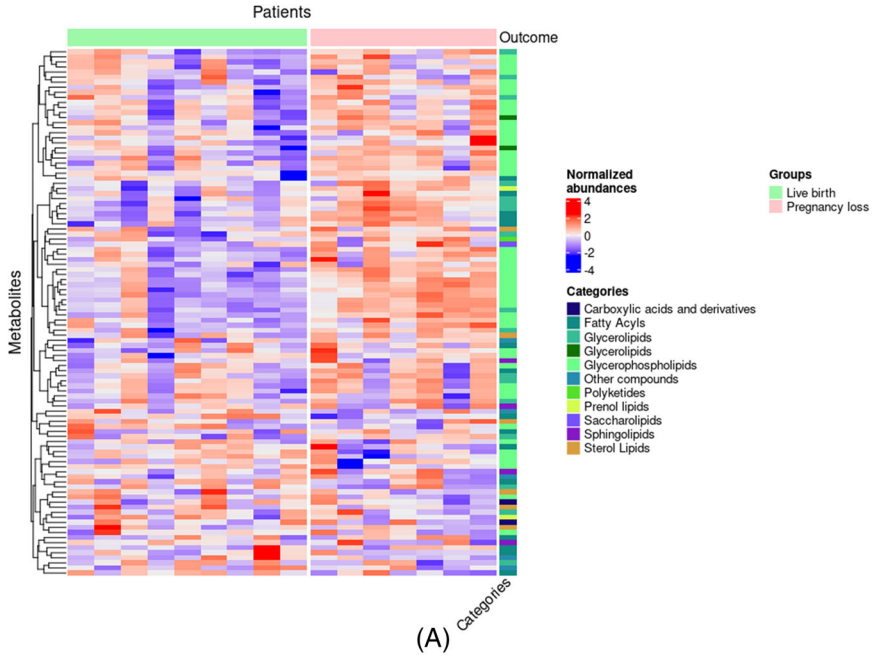
Analysis of lipids Signatures for Outcome: Live Birth or Pregnancy Loss. UPLC-QTOFMS was performed and, after applying 2134 features in negative mode and 601 in positive ion mode were obtained. Matching the obtained list of features from the multivariate SVM model for negative and positive mode with Human Metabolome Database (HMDB) and LIPID MAPS databases resulted in the annotation of 123 significant features as possible candidates as predictors for pregnancy loss or live birth outcomes.

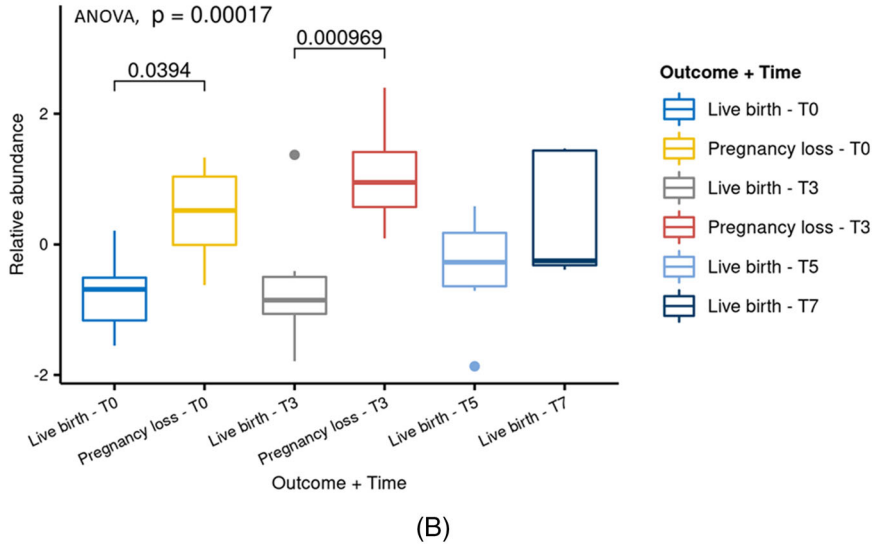
The outcome comparisons revealed two altered PCs. The first, PC 40:8, composed by two fatty acids which are both the AA, 20:4 n6 series/20:4 n6 series. According to fragmentation ions the C = C locations in fatty acid 20:4 was assigned at Δ5, 8, 11, 14, and no C = C location isomers were observed. The second, PC 36:5, formed by LA 18:2 n6 series/gamma-linolenic acid 18:3 n6 series or palmitic acid 16:0/EPA 20:5 n3 series. Based on this supposed composition of PCs, we observed a greater predominance of n6 fatty acids series possibly related with pro-inflammatory molecules such as prostaglandins and leukotrienes as previously reported.[26,27]

Differences between groups were significant for PC 40:8 and PC 36:5 compounds, as illustrated in the heatmap (Figure 2A) and the significant molecules shown in boxplots is displayed in Figure 2B,C. The pregnancy loss group presented up regulation of some metabolites and in the boxplot display this group demonstrates significantly higher relative abundance of both PC 40:8 (P = .00017) and PC 36:5 (P = .011). The differences are noted when the data are evaluated between the groups before starting LET treatment, both for the non-pregnancy woman in T0; PC 40:8 (P = .0394) as well as after pregnancy confirmation in T3 (P = .000969). Interestingly, the lipid profile of women who entered the study already pregnant (*n* = 7, T3) did not show differences in relation to women who became pregnant during the study (Live Birth, *n* = 9, T3) which received only two doses of LET (data not shown). This corroborates the results that altered metabolites (PC 36:5 and PC40:8) are related to the outcome.

All enriched pathways show in supplementary material (Table S1), the importance of the compound within the metabolic pathway evaluated was related with the higher impact. The molecules with statistical difference between the groups were the glycerophospholipids (GPL) with False Discovery Rate (FDR) .0013524.

The metabolites considered in pathway enrichment according to statistical selection shows in Table 3 and the representative metabolic pathways most associated with the lipids found to be relevant according to KEGG code can be viewed in supplementary material (Figure S1). Represented in red square are the metabolites PE, LysoPC 15:0, PC 36:5 and sn-glycero-3-phosphocholine, the last three with highest impact, which is why they are more at the center of representation.





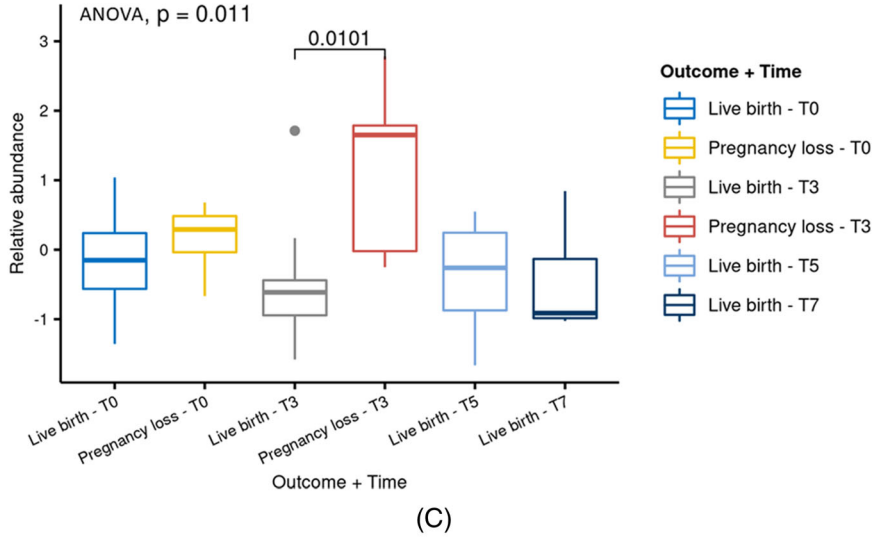


FIGURE 2 (A) Heatmap of standardized intensities of relevant categories clustered on rows obtained by feature selection. The upper bars indicate the live birth and pregnancy loss group to which the samples belong (columns). (B) Relative abundance of metabolite PC 40:8, *p*-values were obtained using ANOVA and Tukey pos-test. (C) Relative abundance of metabolite PC 36:5, *p*-values were obtained using ANOVA and Tukey pos-test

TABLE 3 Metabolites considered in pathway enrichment

Lipid assignment	Measured m/z	SVM ranking	Log2 (Fold Change)	*T*-Test-log (*p*-value)	KEGG code	Paths
PE (32:4)	1.14_718.4208	0.12	15.731	0.1289	C00350	GPI/GPL
PC (42:10)	5.68_898.5594	–	1.1014	1.8481	C00157	GPL/AA/LA/ALA
PC (40:8)	7.38_810.5429	–	2.1052	3.0125	C00157	GPL/AA/LA/ALA
PC (36:5)	6.30_802.5392	0.32	1.7991	2.762	C00157	GPL/AA/LA/ALA
PC (36:6)	5.37_822.5284	–	1.6805	1.6484	C00157	GPL/AA/LA/ALA
LysoPC (15:0)	9.53_480.3080	–	2.0528	1.3336	C04230	GPL
L-Arginine	0.54_175.1192	0.1	0.66031	0.5256	C00062	AG /AGP/Aacyl
Glycero-3 -phosphocholine	0.54_241.1062	0.44	0.084543	0.0878	C00670	GPL/EL
Glucosylceramide (d18:1/16:0)	6.06_736.5121	0.18	−0.36123	0.2169	C01190	SP

Abbreviations: AA, arachidonic acid metabolism/LA, linoleic acid metabolism/ALA, alpha-linolenic acid metabolism; AG, arginine biosynthesis; AGP, arginine and proline metabolism; Aacyl, aminoacyl-tRNA biosynthesis; EL, ether lipid metabolism; GPI, glycosylphosphatidylinositol-anchor biosynthesis; GPL, glycerophospholipid metabolism; LysoPC, lysophosphatidylcholine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; SP, sphingolipid metabolism.

3.2 | Analysis of lipids signatures for long term use analysis of lipids signatures for long term use

With the 123 features assigned from the databases as possible discriminatory biomarkers for outcomes (pregnancy loss or live birth), we also performed the analysis on the woment's lipidome plasma using LET over the time, that is, women in the "Live Birth group" who had blood collected (Time 3-T3), third (Time 5-T5), and fifth (time 7-T7) dose of LET. Descriptive analyses of clustered intensities considered in pathway analysis were performed for all samples and show in heatmap. The GPL pathway was found to be significantly altered between the times (T0, T3, T5, and T7) in the Live Birth group, as previously observed for the comparison based on the outcome. The metabolites PC 36:6 (T0 vs. T5, T0 vs. T7, T3 vs. T5, and T3 vs. T7), LysoPC 15:0 (T3 vs. T7 and T3 vs. T7) and PC 42:10 (T0 vs. T7 and T3 vs. T7) presented greater abundance in long term use of LET in the Live Birth group (Figure 3). The altered PC 36:6 was composed of the fatty acids 16:1/20:5 or of the 14:1/ 22:5 and PC 42:10 which can be composed of the fatty acids 20:5/ 22:5, showing the effect of the use of LET in increasing the levels of PC species containing n3 PUFA. Considering that there was no cohort of women with pregnancy loss in the samples collected at T5 and T7, these points were only used to assess the long term LET use.

4 | DISCUSSION

Our findings show that the GPL pathway was significantly altered when comparing both the outcomes (Live Birth or Pregnancy Loss) and long-term LET use. The normal regulation of GPL has been importantly related to successful pregnancy and positively correlated with *Lactobacillus* abundance that can lead to successful embryo implantation.[14] Increases of PC 40:8 and PC 36:5 containing predominance of n6 fatty acids (LA and AA) were observed in plasma lipids of the Pregnancy Loss Group as compared to the Live Birth Group.

The AA is involved with pro-inflammatory pathways, which may perhaps explain its significant relative abundance in the Pregnancy Loss group, that is, in the group in which pregnancy was interrupted by abortion. AA is mainly produced in the liver being obtained directly from the diet or synthesized from LA (n6 series) through the action of the enzymes Δ6-desaturase, elongase and Δ5-desaturase. The AA and LA derivatives play important roles in human fertility and the course of pathological pregnancies.[28] However, their role in the reproductive cycle is limited. The 12/15-lipoxygenase may be a key modulator of uterine activity during the implantation process. In implantation, the morula enters the uterine cavity, adheres to the uterine lining, and invades the uterine endometrium. Under cyclooxygenase-2 deficiency conditions, incorrect embryo implantation and decidual reaction occur.[29]

AA is a precursor of lipid mediators that are involved in physiological and pathological processes due to the enzymatic activity that metabolizes AA, that is, lipoxygenase, cyclooxygenase, and cytochrome P450 pathways. Hydroperoxyl-eicosatetraenoic acid (HETEs) and leukotrienes are formed by the action of lipoxygenase, while epoxyeicosatrienoic acids (EETs) and HETEs are formed by cytochrome P450 action. Prostaglandins and thromboxanes, which are pro-inflammatory mediators, are formed by the action of cyclooxygenase.[29] Investigation of cytochrome P450-derived eicosanoids has focused primarily on their role in inflammation, following two major routes that can be taken in the cytochrome P450 pathway: ω-hydroxylases, which convert AA to HETEs, and epoxygenases, which convert AA to EETs. It is worth remembering that less is known about these molecules compared to prostanoids and leukotrienes.[30]

Studies have underscored the importance of metabolic dysregulation of AA pathways in pregnancy metabolism. Elevated levels of plasmatic AA may be present years before the onset of diabetes and is revealed during the early postpartum period, preceding the progression to type 2 diabetes among women with gestational diabetes mellitus.[31] The production of eicosanoids has been associated with the

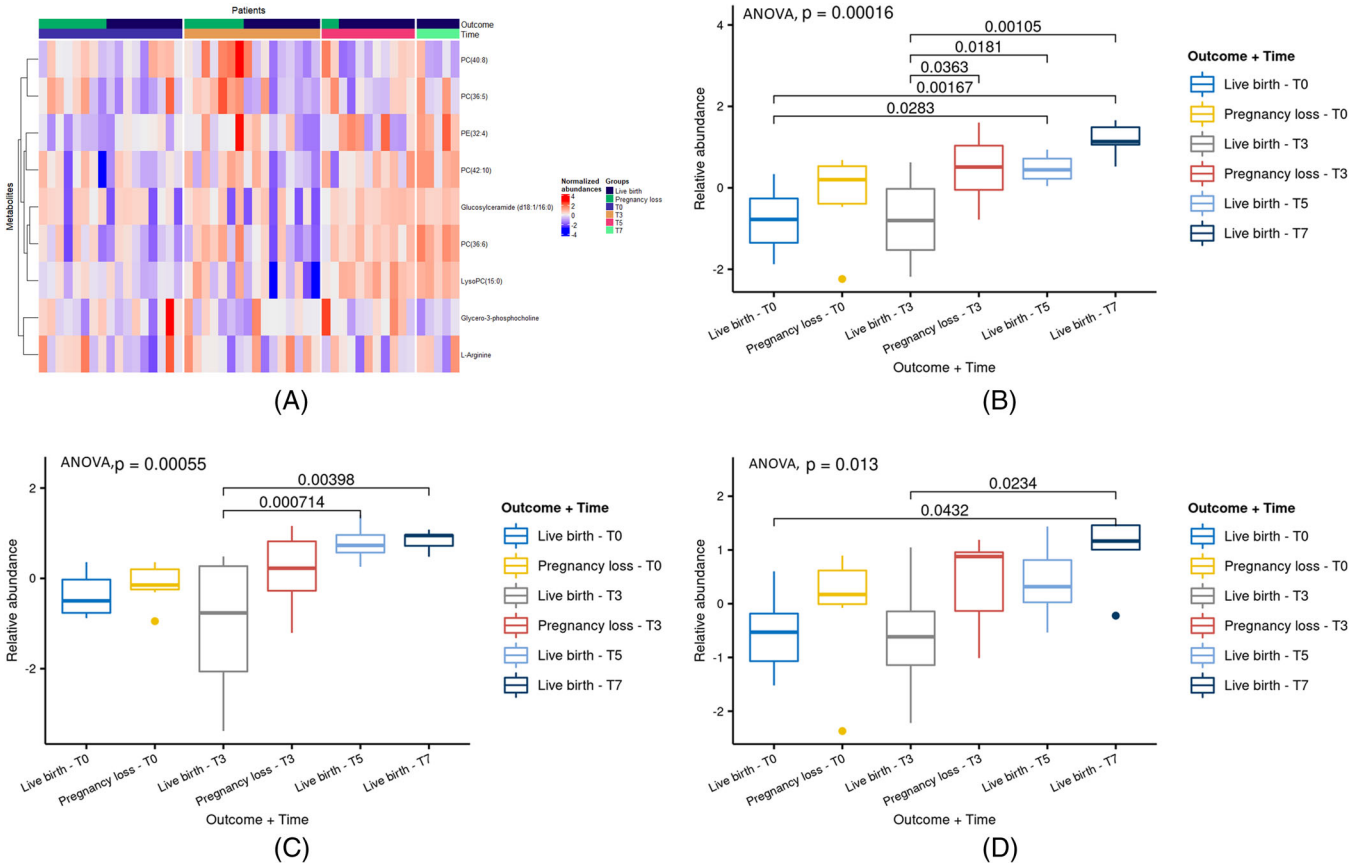


FIGURE 3 (A) Heatmap of standardized intensities of relevant categories clustered on rows obtained by feature selection. The first upper bars indicate the group live birth and pregnancy loss which the samples belong (columns) and the second bar the gestation time. (B) Relative abundance of metabolite PC 36:6, *p*-values were obtained using ANOVA and Tukey pos-test. (C) Relative abundance of metabolite Lyso PC 15:0, *p*-values were obtained using ANOVA and Tukey pos-test. (D) Relative abundance of metabolite PC 42:10, *p*-values were obtained using ANOVA and Tukey pos-test

activity of PLA2, which has access to one of the main easily mobilized PUFA reservoirs in the sn-2 position of GPL.[26] A study of plasma levels of PLs in pregnant women showed that PLA2 mobilizes AA and DHA from the membrane to meet the high fetal need for these nutrients resulting in lower levels of AA and DHA and higher levels of PA and oleic acid in their plasma PLs. The reduction in DHA and total n3 series fatty acids in those pregnant women was accompanied by an increase in docosatetraenoic acid (DTA) PC 22:4 n6 series and DPA PC 22:5 n3 series and in the DPA/DTA ratio. In the PLs of the red blood cells of the pregnant women, the acids DTA and DPA from the n6 series and the DPA/DTA ratio did not increase with the decrease of the n3 metabolites (EPA, DPA and DHA) and n3 series total.[32]

In the placentas of aborted fetuses with neural tube defects, significantly higher concentrations of AA and higher C20:4 /EPA C20:5 ratios were found, and those proportions were positively correlated with thromboxane B2/6-keto- prostaglandin F1α.[26] One study even concluded that altered lipid expression in pre-eclampsia or RPL includes those implicated in immune response, coagulation, oxidative stress and apoptosis, resulting in significantly up-regulated total PS levels in women with pre-eclampsia and RPL while PI, PA and ganglioside mannoside 3 were significantly down-regulated.[33]

An association has also been reported between altered PL metabolism in pregnancy disorders and lipid molecules such as endocannabinoids, anandamide and lysophosphatidic acid, and prostaglandins are important mediators in early pregnancy, with regulatory functions during implantation, decidualization, and feto-placental development.[34] Effects on immune cells in the umbilical cord and their responses have been observed in studies with pregnant women supplemented with n3 PUFAs.[35] Prostaglandins E2 and F2α are particularly abundant during the implantation window and serve as important biomarkers to define the receptive phase of the endometrium.[27]

Analyzing the lipid profile of the women in the Live Birth group who received LET we observed an increase of n3 PUFA-PC levels with long term LET use (at both T5 and T7). A possible explanation for that is the amount of n3 PUFA in the emulsion used in the work. SMOFlipid® has been identified in studies over the last 20 years as probably having the ideal n6/n3 PUFA ratio, providing immunological, anti-inflammatory effects associated with clinical benefits.[7]

The greater relative abundance of PLs related to anti-inflammatory pathways found in the in the Live Birth group may explain the improvement in the anti-inflammatory response of the uterine environment,

enabling a successful pregnancy. Pregnancy seems to have no effect on the observed PUFA levels change, since no significant differences were observed in the n6PUFA/n3 PUFA ratios or in the n3 index between pregnant compared to non-pregnant women.[36]

The high intake of AA and DHA suggests that bio attenuation of DHA during pregnancy and postnatal antagonism between AA and DHA is the physiological standard for humans throughout the life cycle.[37] An association between changes in PLs and the hormone progesterone was also reported in the peri-implantation period and in embryo implantation, insofar as the lipid profile of PC, PE, LysoPC, diacylglycerol, ceramide, PI and PS was significantly lower with the premature elevation of progesterone.[38]

To our knowledge, this is the first study conducted in women with consecutive miscarriages and it evaluated the plasma profile of PLs revealing the relation between pregnancy loss and significantly higher relative abundance of PCs composed by fatty acids participating in pro-inflammatory pathways such as AA. Also, women who received the LET long term demonstrated greater significance for PLs with a predominance of n3 series, that is, composed by fatty acid associated with the anti-inflammatory pathway. It seems to be an interesting way to go deeper into the pro and anti-inflammatory pathways linked to fatty acids in pregnant women.

A possible limitation to the study is related to the absence of immunological data, in particular the NK cell activity follow-up and at the end of the study as well as small sample size. Another limitation was the absence of a group of pregnant women who did not use the infusion in order to show, in a clearer way, whether the lipid profile effects on pregnancy are associated or not to the use of the lipid infusion. The contribution of PLs to miscarriage as a cause or effect is still unclear; however, our differential PL plasma abundance results represent another step in advancing our understanding of pregnancy complications. The data evaluated in the study do not allow us to assess the effect of LET in pregnancy outcome. Further work is needed to validate these findings in independent pregnancy cohorts.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Data access available under request to the author.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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5. CONCLUSÃO

No Capítulo I foi realizada revisão de literatura sobre o uso de LET em mulheres com RPL e RIF com atividade inflamatória aumentada, com atividade de células NK acima das referências. Embora alguns estudos observaram diminuição na atividade das células NK com o uso de LET e aumento na taxa de implantação e de nascimentos, ainda não existe um consenso entre os autores, sendo que os dados obtidos não foram suficientes para demonstrar um efeito favorável de LET em RPL e RIF. Dessa forma mais estudos são necessários para verificar que LET favorece implantação e nascimentos em mulheres com RPL e RIF, bem como através de qual mecanismo LET atua.

Os resultados demonstrados no Capítulo II, teve como principal limitação a perda de um grande contingente de casos devido ausência de informações importantes tais como o estadiamento. Esta limitação pode ser contornada com estímulo ao preenchimento completo das informações nos prontuários e nos sistemas de informações. Outra limitação é que não podemos fazer a inferência dos achados. Ou seja, os resultados aqui descritos se referem apenas à população aqui estudada.

Apesar das limitações citadas podemos considerar que essa pesquisa foi pioneira apresentando alterações significativas no perfil lipidômico plasmático de fosfolipídeos (PL) em mulheres com RPL ou RIF sob uso de LET. Foram obtidos possíveis preditores lipídicos que podem estar relacionados ao aborto (PC 40:8 e PC 36:5 apresentaram abundância relativa significativamente maior no grupo *Pregnancy Loss*), assim concluímos que esses PCs podem favorecer ao aborto, pois eles parecem estar relacionados a vias pró-inflamatórias; ou ao nascimento (LysoPC 15:0, PC 42:10 e PC 36:6 apresentaram aumentada abundância relativa quando comparamos em relação ao uso prolongado de LET nos tempos (T3-T5-T7), no grupo *Live Birth*), uma vez que esses parecem estar envolvidos com vias anti-inflamatórias. Os dados desse trabalho poderão ser utilizados como importante referência por pesquisadores interessados na área.

O número de estudos sobre o assunto é escasso. Embora nossos dados não sejam conclusivos é necessária a realização de mais estudos sobre o tema, tais como ensaios clínicos randomizados, duplo-cego, controlados.

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