Metabolic consequences of non-severe burn injury are associated with increased plasma markers of inflammation and cardiovascular disease risk.

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Keywords

Acute burn injury; Non-severe burns; Thermal injury; Inflammation; Cardiovascular disease risk; Metabolic phenotyping; Lipids; Lipoproteins; Supramolecular phospholipid composite; Nuclear magnetic resonance; Liquid chromatography-tandem mass spectrometry

Abstract

To understand the metabolic consequences following non-severe burns, longitudinal plasma was collected from adults with non-severe burn injuries (n=35) within 48 hours of admission to hospital and at 6 weeks post-surgery, as well as non-burn controls (n=14). Samples were analysed by ^{1}H nuclear magnetic resonance spectroscopy and liquid chromatography-tandem mass spectrometry to quantify 112 lipoprotein, supramolecular phospholipid composite (SPC), GlycB arising from the glycoprotein acetyl residues and 852 lipid species across 20 chemical subclasses. Alterations in lipoprotein and lipid metabolism were evident in samples taken within 48 hours of admission and found to be sustained at the 6-week timepoint, with increases in GlycB (p-value < 0.0001) and the multivariate importance of the apolipoprotein B100/apolipoprotein A1 (ABA1) ratio, as well as decreases in anti-inflammatory phosphatidylcholines, phosphatidylglycerols, phosphatidylinositols and phosphatidylserines. Low circulating high density lipoproteins, and increases in pro-atherogenic low density lipoproteins and monoacylglyceride (20:4) (p-value < 0.0001) are potentially indicative of an increased risk of cardiovascular disease after non-severe burns which requires further investigation.

Introduction

Worldwide, burn injuries remain one of the most traumatic injuries for individuals to experience and, despite treatment advances, are associated with long-term impacts on physical and psychological health[1]. Thermal burn injury has been found to be associated with a plethora of long-term complications, including increased risk of cancer[2], infectious[3], cardiovascular disease[4], diabetes mellitus[5] and musculoskeletal disorders[6]. These systemic consequences of burn injury can occur even when there is little or no apparent scarring following the initial burn injury[7], suggesting that the burn trauma itself may initiate a systemic effect that leads to increased risk of disease.

Cardiovascular disease is of particular concern with population studies clearly showing higher rates of admission for ischaemic heart disease, heart failure and cerebrovascular disease after burn injury[8]. Increased risk of cardiovascular hospitalisations, morbidity and mortality is associated with severe and non-severe injuries occurring at any age from childhood to older adulthood[4,9,10]. Long-term hospitalisations for circulatory disease were 33% higher in children <15 years with a burn compared to non-burn controls, and burn injury was associated with 2.26 times greater length of hospital stay for such diseases[9]. Findings were similar for adults, with burn patients having 50% more admissions for heart failure than non-injured controls[4,10]. Although an increased risk has also been shown for adults suffering other forms of trauma, short-/long-term risks and cardiovascular mortalities were significantly higher following burn injury[10]. A recent study showed that there are physiological changes in cardiac muscle after a burn, with murine models showing significant increases in left ventricular end systolic diameter and ventricular wall thickness 3 months post-injury, changes consistent with hypertrophic cardiomyopathy and plausibly attributable to sustained inflammation[8]. Similar increases in left ventricular diameter were also seen in serial observations over 3 months in a patient cohort after non-severe burn injury[8]. These findings are indicative that even non-severe burns have a sustained impact on the circulatory system, more so than other trauma type, and this warrants research into the underlying mechanisms contributing to the elevated risk of cardiovascular disease post-burn.

Metabolic profiling based on Nuclear Magnetic Resonance (NMR) spectroscopy and mass spectrometry (MS) platforms is a useful tool for understanding the downstream pathophysiological changes associated with disease. Characterisation of patterns of low-weight metabolites and metabolite intermediates in various biofluids[11] following insults or exposures (e.g. injury, infection, disease) can inform on pathogenic mechanisms in granular detail. The use of such platforms has previously shown dysregulation of both protein and lipid metabolism occurs following a burn injury[12].

Lipids have multiple essential biological roles including maintaining cellular structure and modulating inflammation[13]. The liver, adipose tissue, skin and muscles are impacted during the hypermetabolic phase of a burn injury with several severe burn studies documenting changes in lipids. Increasing levels of free fatty acids (FFAs) have been shown in the plasma from triacylglyceride (TAG) breakdown in adipose and muscle [14,15], as well as acyl-carnitines in muscle[16]. In addition, accumulation of TAGs[16] and lipoapoptotic-inducing palmitic and stearic lipids[17] has been demonstrated to promote hepatic steatosis in the liver. Conversely a significant decline in FFAs, monoacylglycerides (MAGs), lysophosphatidylglycerols (LPGs) and lysophosphatidylethanolamines (LPEs) has been noted in burn-affected skin[18].

Perturbations in lipoproteins have also been reported following burn injury. High-density lipoproteins (HDLs) that have known benefits to cardiovascular health were found to be decreased during acute (total serum HDLs)[19] and severe burn injuries[20], whereas levels of potentially atherogenic very low-density lipoproteins (VLDL) and low-density lipoproteins (LDL) were found at higher concentrations in paediatric non-severe[21] and adult severe burns[22]. The changes identified in lipids and lipoproteins may reflect burn-induced hypocholesterolemia, hypophospholipidemia and hypertriglyceridemia, which have been associated with morbidity and mortality following a severe burn injury[23].

As lipid and lipoprotein metabolism perturbations have been identified in severe burns and are associated with long-term complications, the application of metabolic phenotyping is a valuable tool to understanding the underlying mechanisms of a burn pathology. With this knowledge, there is potential to develop metabolic biomarkers predictive in the early stages of long-term complications and to select targets for therapeutic intervention[24]. Most research into metabolic consequences of burns has been targeted towards severe burns[20,25–32], with a small proportion examining changes in non-severe burns[8,19,33]. As the incidence of non-severe injury is much greater than severe, it is important to understand how the metabolic perturbations differ between these groups so that therapeutic interventions can be appropriately targeted across the spectrum of injury.

In order to explore the role of lipid metabolism in the persistent systemic consequences of non-severe burn injury, we applied a multimodal strategy to compare plasma from participants with and without burn injury using liquid chromatography-tandem mass spectrometry (LC-QQQ-MS) to characterise the plasma lipidome and integrate with panels of lipoproteins and inflammatory markers measured using ^{1}H nuclear magnetic resonance (NMR) spectroscopy.

Methods

Patient recruitment

Plasma samples analysed were a part of the randomised placebo-controlled trial of Celecoxib for Acute Burn Inflammation (CABIN) and fever study (Registration number: ACTRN12618000732280). Ethics approval for the use of these samples was granted by the South Metropolitan Health Service Health Research Ethics Committee (Approval number: RGS731). Acute non-severe burn injured adults (n=35) who had total burn surface area (TBSA) less than 15% were recruited within 48 hours of admission to the State Adult Burns Unit at Fiona Stanley Hospital. Adults were aged between 18 to 65 years and able to give consent. Whole blood was collected in gel-free lithium heparin tubes on enrolment into the study and 6 weeks post burn debridement surgery. Samples were centrifuged and plasma aliquots transferred to storage at -80°C within 2 hours of collection. The mean number of days from burn injury at the 6 weeks post burn surgery timepoint was 45.64 ± 7.5 days.

Consenting volunteer non-burn controls (n=14) with no medical history of burn injury requiring hospital admission provided whole blood collected in lithium heparin tubes, with ethics for collection of data and sample granted by Murdoch University Human Research Ethics Committee (Murdoch University Ethics no. 2020/053).

For the burn cohort, the mean TBSA was 4.0% ± 3.6% and age at time of collection was 39.87 ± 16.73 years, with the mean age of the non-burn controls of 40.09 ± 10.7 years (Table 1).

Table 1. Demographics of non-severe burn patients from the CABIN study and recruited non-burn controls. Abbreviations: TBSA – Total burn surface area, SD – Standard deviation.

	Non-severe burn injury		Non-burn controls	
	n	**Mean ± SD**	***n***	**Mean ± SD**
Participant	35		14	
Age (years)	Range 18-63	39.87 ± 16.73	Range 28-64	40.09 ± 10.7
Gender				
Male	28		9	
Female	7		5	
TBSA (%)	Range 0.02-8.3	4.0 ± 3.6		
Hospital length of stay (days)	Range 1-9	5.81 ± 3.67		
Post-surgery in-patient length of stay (days)	Range 0-6	2.55 ± 2.16		
Days from injury (at hospital admission)	Range 0.27-10.72	2.89 ± 2.75		
Days from injury (at 6 weeks post-surgery)	Range 35.8-62.8	45.64 ± 7.5		

^{1}H NMR spectroscopy of plasma

Plasma samples were prepared for ^{1}H NMR spectroscopy analysis using a previously published method[34]. Briefly, analysis was performed on a 600 MHz Bruker Avance III HD spectrometer (Bruker BioSpin, Billerica, Massachusetts, USA) fitted with a BBI probe and a Bruker SampleJet™ robot (Bruker BioSpin, Billerica, Massachusetts, USA) using a cooling system maintaining samples at 5°C. Plasma samples were centrifuged at 13 000 x *g* for 10 min at 4°C and phosphate buffer (75 mM Na_2HPO_4, 2 mM NaN_3, and 4.6 mM sodium trimethylsilyl propionate-[2,2,3,3- $2H_4$] (TSP) in D_2O, pH 7.4 ± 0.1) was added in a 1:1 ratio with the plasma supernatant. Of the mixture, 600 µL was then transferred into the Bruker SampleJet™ with SampleJet 5 mm outer diameter NMR tubes sealed with POM balls. NMR analysis was performed in accordance with the Bruker *in vitro* Diagnostics research (IVDr) methods, in which ^{1}H 1D and Diffusion and Relaxation Editing (DIRE)[35–37] experiments were run subsequently. ^{1}H 1D experiments were run with solvent pre-saturation acquiring 32 scans, 98304 data points with 18028.85 Hz spectral width and experiment time 4 min 3 s[38]; and DIRE experiments acquired 64 scans into 98304 data points and 18028.846 Hz spectral width, experiment time 4 min 25 s[35–37].

Lipoprotein subfraction analysis

The ^{1}H NMR spectroscopic analysis generates 112 lipoprotein parameters were generated for each plasma sample via the Bruker IVDr Lipoprotein Subclass Analysis (B.I.-LISA™), whereby the $-CH_2$

at δ = 1.25 and $-CH_3$ at δ = 0.80 peaks of the 1D spectrum after normalization to the Bruker QuantRef manager within Topspin, were quantified using a PLS-2 regression model[39]. The lipoprotein classes quantified were very low-density lipoproteins (VLDLs), low-density lipoproteins (LDLs), intermediate-density lipoproteins (ILDLs), and high-density lipoproteins (HDLs). VLDL subfractions were classified into six density classes (0.950 – 1.006 kg/L) of VLDL-1, VLDL-2, VLDL-3, VLDL-4, VLDL-5 and VLDL-6. LDL subfractions were assigned to six density classes of LDL-1 (1.019-1.031 kg/L), LDL-2 (1.031-1.034 kg/L), LDL-3 (1.034-1.037 kg/L), LDL-4 (1.037-1.040 kg/L), LDL-5 (1.040-1.044 kg/L), LDL-6 (1.044-1.063 kg/L). HDL subfractions were classified into four density classes of HDL-1 (1.063–1.100 kg/L), HDL-2 (1.100–1.125 kg/L), HDL-3 (1.125–1.175 kg/L), and HDL-4 (1.175–1.210 kg/L). An annotation list of lipoproteins from the B.I.-LISA™ method is provided in the Supplementary Information (**Table S1**). DIRE pre-processing included baseline correction using an asymmetric least-squares routine and the resulting spectra were normalised to the ERETIC signal using R (v4.4.1) with R Studio (v1.4.1)[40]. The GlycB acetyl signal (δ 2.07) arising from the glycoprotein acetyl residues were obtained from the DIRE experiment by integration. Due to overlap of triacylglyceride signals with GlycA (a composite peak of *N*-acetyl signals from five proteins: α-1-acid glycoprotein, α-1-antichymotrypsin, α-1-antitrypsin, haptoglobin, and transferrin), only the integrated signals from GlycB were used in all analyses. Supramolecular phospholipid composite (SPC) signal estimates were calculated from the peak spectral intensities in the regions of SPC_1 δ 3.20–3.236, SPC_2 δ 3.236–3.262, and SPC_3 δ 3.262–3.30[41].

LC-QQQ-MS analysis of plasma lipids

Plasma lipids were analysed using a previously defined LC-QQQ-MS method for detection of 1163 lipid species[42]. Briefly, 10 µL of plasma was vortex mixed with 90 µL of propan-2-ol containing internal standards from Lipidyzer™ Internal Standard kit, SPLASH® LipidoMIX® and Avanti Polar Lipids Lyso PI 17:1, Lyso PG 17:1 and Lyso PS 17:1. Samples were centrifugated at 14000 x *g* for 10 min and supernatant transferred to a 96-well plate. The LC-QQQ-MS method was run on a SCIEX ExionLC™ and QTRAP 6500+ using reversed-phase chromatographic separation and electrospray ionisation with polarity switching. Data were acquired using SCIEX Analyst® (v1.7.1).

Data integration

For lipids analysed by LC-QQQ-MS, raw data were integrated in Skyline (v21.2)[43] and concentrations of the detected lipids were calculated from the analyte to internal standard ratios. Data were pre-processed using R (v4.4.1) in R Studio (v1.4.1)[40] where samples/features were filtered out if >50% missing values were present. Subsequently the remaining samples with <50% missing values were imputed by minimum value/2 and a final filtering out of features that had a >30% relative standard deviation (RSD) across the replicate quality control (QC) plasma samples. Signal drift was controlled for using the Random Forrest method from the statTarget package in the R software environment[44].

Statistical analysis

Separate principal component analysis (PCA) and supervised orthogonal partial least squares discriminant analysis (OPLS-DA) modelling for lipids and lipoproteins were performed using the R *metabom8* package (v0.2) from https://github.com/tkimhofer. OPLS-DA models were cross-validated using the Monte Carlo method on 2/3 of the dataset with the remaining 1/3 used to predict. Visualisation plots and univariate statistics were generated using R (v4.4.1) in R Studio (v1.4.1). Variable importance in projection (VIP) scores from the OPLS-DA models were calculated using SIMCA® (v17, Sartorius, Goettingen, Germany).

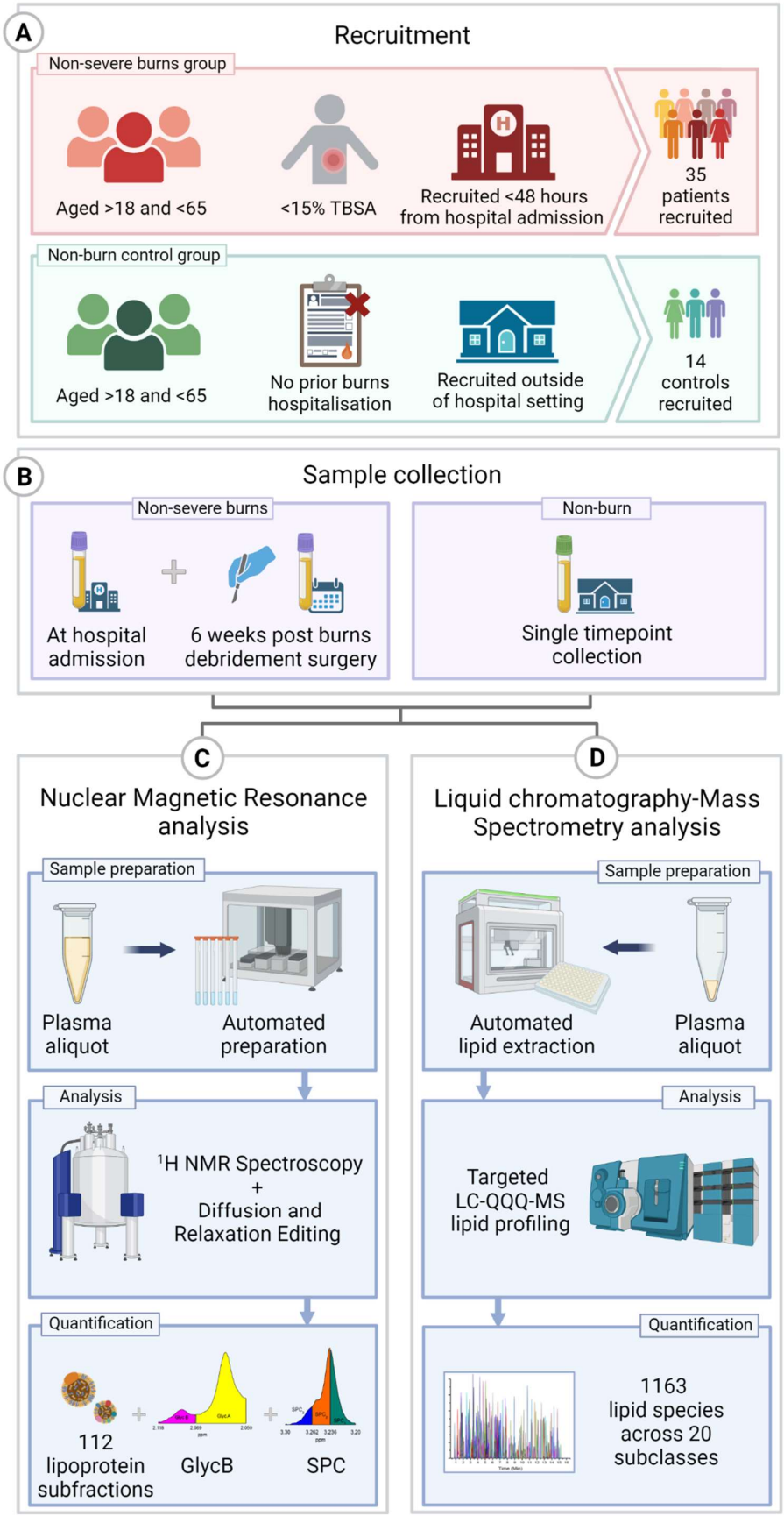
A
Recruitment
Non-severe burns group
Aged >18 and <65
<15% TBSA
Recruited <48 hours from hospital admission
35 patients recruited
Non-burn control group
Aged >18 and <65
No prior burns hospitalisation
Recruited outside of hospital setting
14 controls recruited
B
Sample collection
Non-severe burns
At hospital admission
6 weeks post burns debridement surgery
Non-burn
Single timepoint collection
C
Nuclear Magnetic Resonance analysis
Sample preparation
Plasma aliquot
Automated preparation
Analysis
¹H NMR Spectroscopy + Diffusion and Relaxation Editing
Quantification
112 lipoprotein subfractions
GlycB
SPC
D
Liquid chromatography-Mass Spectrometry analysis
Sample preparation
Automated lipid extraction
Plasma aliquot
Analysis
Targeted LC-QQQ-MS lipid profiling
Quantification
1163 lipid species across 20 subclasses

Figure 1. **Overview of non-severe burn patient and non-burn participant recruitment and experimental workflow. A**) Main selection criteria for non-severe burns and non-burn participants with final recruitment numbers used in the study. **B**) Sample collection timepoints for both groups with single plasma collection timepoint for non-burn controls and two timepoints from hospital admission and 6 weeks post burn debridement surgery for the non-severe burn group. **C**) Basic workflow of non-severe burn and non-burn plasma samples analysed by ^{1}H nuclear magnetic resonance (NMR) spectroscopy and using Diffusion and Relaxation Editing. Data analysed included 112 lipoprotein subfractions, GlycB and the supramolecular phospholipid composite (SPC). **D**) Basic lipidomic workflow of non-severe burn and non-burn plasma analysed by liquid chromatography tandem mass spectrometry to quantify 1163 lipid species across 20 subclasses. Imaged created with BioRender.com

Results and discussion

Plasma lipoprotein patterns indicate persistent inflammation after non-severe burn injury.

^{1}H NMR data were deemed robust, with associated quality control (QC) pooled plasma samples clustering on a PCA scores plot (**Figure S1A**). Non-severe burn injury (total body surface area <15%) was differentiated by a series of changes in the plasma lipoprotein compared with non-burn controls from OPLS-DA modelling that were consistent at both the admission and 6-week time points (**Figure 2**). From OPLS-DA modelling of non-severe vs non-burn cohorts, lipoproteins, GlycB and supramolecular phospholipid composite signals (SPCs) were ranked by their influence on the model using variable importance in projection (VIP) scores (**Figure 2C, D, Table S2**). The plasma profiles from the burn injury participant group were defined by higher concentrations of GlycB, SPC_3 (related to LDLs), and LDL subfraction-2 particles, whereas higher concentrations of HDL cholesterol and HDL apolipoprotein A1 and A2 and lower levels of the phospholipid SPC_1 (related to HDLs) were characteristic of the non-burn controls (**Figure 2C, D**).

GlycB was one of the strongest features defining the burn injury group at both admission and at 6 weeks post-surgery. This acute phase glycoprotein signal at δ 2.07 in the NMR spectra arises from the glycoprotein acetyl residues and has been shown to correlate with the clinical inflammatory marker high sensitivity C-reactive protein (hs-CRP)[45,46]. GlycB has been shown to be associated with a range of acute and chronic inflammatory responses, such as vascular inflammatory conditions[46–49], chronic heart disease[50], prostate cancers[51], rheumatoid arthritis[52–54], SARS-CoV-2 infection[35] and polycystic ovarian syndrome[48,55]. A sustained increase in serum GlycB signals has been reported in a recent study of burn injury in paediatric patients where abnormal levels were found to persist 3 years after the initial burn injury[21]. GlycB in this cohort directly correlated with inflammatory cytokine levels, particularly interleukin-6, tumour necrosis factor-α and interferon-γ[21]. Furthermore, significantly lower plasma concentrations of HDL-4 subfractions (HDL-4 apolipoprotein A1 (H4A1), HDL-4 free cholesterol (H4FC) and HDL-4 cholesterol (H4CH)) and higher LDL-2 subfractions (LDL-2 phospholipids (L2PL), LDL-2 triacylglycerides (L2TG), LDL-2 particle number (L2PN), LDL-2 apolipoprotein B100 (L2AB), LDL-2 free cholesterol (L2FC) and LDL-2 cholesterol (L2CH)) were observed at burns admission and 6 weeks post-surgery when compared to non-burn controls (**Figure 2C, D, Figure 3**). In addition, main fraction HDL-associated apolipoprotein A1 (HDA1) and subfraction H4A1 were observed to be lower in the plasma of the burn injury group compared to non-burn controls. HDL-associated apolipoprotein A1 has been reported to play an anti-inflammatory role in diseased states, due to its ability to carry anti-inflammatory molecules, bioactive lipids and cholesterol[13,56]. Depleted HDL-4 apolipoprotein A2 (H4A2) in plasma have also been associated with poor outcomes and mortality in patients with pulmonary arterial hypertension[57] as well as low H4A1, H4A2 and H4CH were significant lipoprotein markers of SARS-CoV-2 infections[35,58]. As observed with the persistence of altered GlycB signals, the decline in HDL levels was also sustained from admission to the 6 weeks post-injury timepoint, indicating a rapid initiation and persistence of the inflammatory response to acute burn injury.

Multiple LDL-2 subfractions (L2PL, L2TG, L2PN, L2AB, L2FC and L2CH) were elevated in the burn injury group compared with the non-burn control group. Circulating LDL particles contain apolipoprotein B100, which is known to be atherogenic, can be plaque-promoting and linked with increased susceptibility to atherosclerosis[59,60]. An inverse response between elevated LDL cholesterol and depleted HDL cholesterol is commonly recognised to be associated with an increased risk of cardiovascular diseases[61], such as ischemic heart disease[62], myocardial infarction[61] and coronary heart disease[63]. An increase in this LDL/HDL ratio (higher LDL and lower HDL cholesterol) is typically seen in chronic inflammatory conditions[64–66] but has also been noted in survivors of severe burn injury (>20% TBSA) in which levels related to worse healing outcomes, such as longer length of hospital stay and higher infection rates[67]. A recent study into severe burn survivors and cardiovascular health stipulated that survivors with burn-induced hypertriglyceridemia and low HDLs saw a reversal of concentrations over time when engaging in medium to high intensity physical activity after the injury[68]. This may be an important consideration and post-burn intervention to counteract a potentially cardiovascular disease risk lipoprotein profile after non-severe burns.

The results also indicated a higher apolipoprotein B100/apolipoprotein A1 ABA1 ratio from higher apolipoprotein B100 and lower apolipoprotein A1 parameters, (**Figure 3C, 3D**) in the burn injury group compared with the non-burn control group at both the admission and 6 weeks post-surgery timepoints. ABA1 has been reported to be a marker of abnormal lipoprotein changes in response to inflammation [60,69–71]. Apolipoprotein B100 (AB100), a portion of the ABA1 ratio, is an essential structural component to LDLs and is known to be atherogenic[69,70,72]. Contrary to AB100, apolipoprotein A1is a main portion of HDL particles, which is portrayed to be anti-atherogenic, anti-inflammatory and antioxidant[69,70]. Increases in the ABA1 ratio have reported in numerous studies researching into cardiovascular diseases and was developed into a marker for cardiovascular risk[73–75]. Findings involving ABA1 have been additionally documented in other diseases and traumas, such as SARS-CoV-2 infections[41], metabolic syndrome[76], cancers[77,78] and severe burn injury[25,26].

Although the inverse relationship between LDL and HDL cholesterol has been reported in terms of cardiovascular health, to date there is no research on the specific relationship of HDL-4 and LDL-2 subfractions with the risk of cardiovascular disease. Even though this study only has data extending to 6 weeks post-burn surgery, it shows that non-severe burn injuries have altered metabolic responses from sustained systemic inflammation. With the importance of ABA1, HDLs and LDLs to the burns model at admission and at 6 weeks, it indicates that there may be an elevated risk of cardiovascular disease after a non-severe burn injury that metabolically persists more than 6 weeks post injury. To our knowledge, there have been no studies that have characterised lipoprotein profiles in burns in adults.

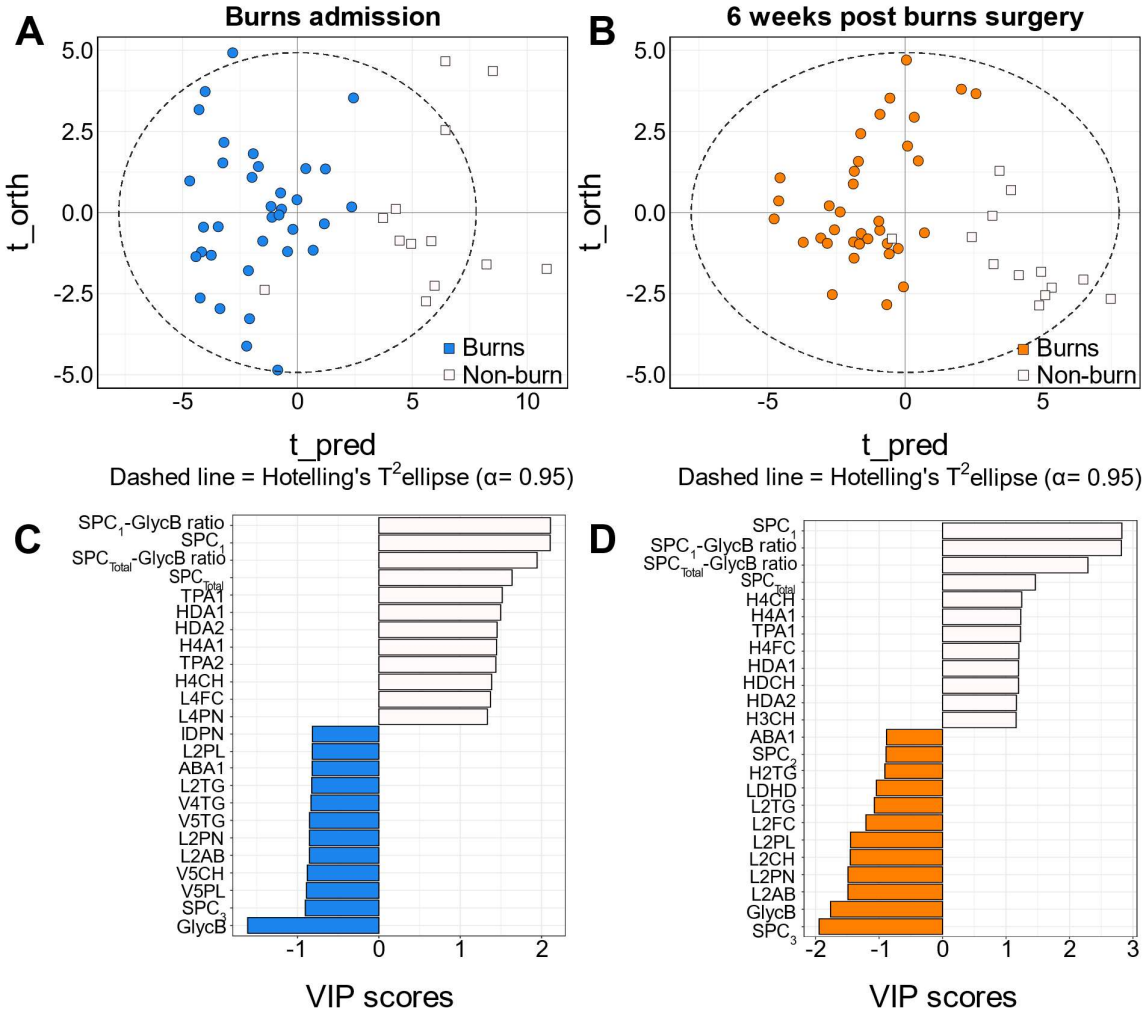

Figure 2. OPLS-DA analysis of lipoprotein profiles from paired burn plasma samples at admission to hospital and 6 weeks post-burn surgery compared to non-burn controls. A) OPLS-DA score plots of admission (n=35; coloured blue) (R^2X = 0.25, Cross Validated-Area under the receiver operating characteristic (CV-AUROC) = 0.95 compared to non-burn controls (n=14; coloured white). **B)** OPLS-DA score plots of 6 weeks post-burn surgery (n=35; coloured orange) (R^2X = 0.12, CV-AUROC = 0.96) compared to non-burn controls (n=14; coloured white). **C)** Bar plot of the top 12 variable importance in projection (VIP) scores, which summarises and ranks the influence of the individual lipoproteins on the OPLS-DA model, representing burns at admission (blue) and non-burn control (white). **D)** Bar plot of the top 12 VIP scores representing the 6 weeks post-burn surgery (orange) and non-burn controls (white).

Recently we have shown that the supramolecular phospholipid composite (SPC) signals in the NMR spectra reflect the trimethylammonium residues in phospholipids that are bound to lipoproteins, with SPC_1 signals related to the phospholipids in HDL-4, SPC_2 related to phospholipids from HDL-1,2 and 3, and SPC_3 related to phospholipids from LDLs[41]. The importance of SPC in reflecting inflammation was discovered recently in a cohort of individuals with SARS-CoV-2 infection and has further become a surrogate to gauge cardiovascular disease risk by analysis of its respective composite sub-regions[35,58]. SPC was first discovered by Diffusion and Relaxation Editing (DIRE) NMR spectroscopy, which allows for deconvolution and integration of the peaks by combining diffusion editing and T_2-relaxation[35]. Both SPC and Glyc were found to be distinguishing features under inflammatory conditions showing a distinctive inverse relationship, particularly in SARS-CoV-2 infections, but also

in the case of burns, with an increase of Glyc and a decrease of SPC (**Figure 3**), which can be expressed as the SPC/Glyc ratio for inflammation assessment[35,41,45]. SPC_1 levels were lower in the burn group at admission and 6-week timepoints when compared to non-burn controls, which is consistent with the lower concentrations of HDL-4 subfraction discussed above. The higher concentrations of the LDL subfractions were additionally confirmed with significantly higher composite levels of SPC_3 at 6 weeks. From a low SPC_1-GlycB ratio (low SPC_1 vs high GlycB), inflammation at burns admission may be evident and remain unresolved by 6 weeks post-surgery. Changes in lipoprotein profiles from burn injury depict an inflammatory response that did not diminish 6 weeks post-surgery and SPC levels that reflect the LDL/HDL cholesterol concentrations could potentially be associated with increased cardiovascular disease risk.

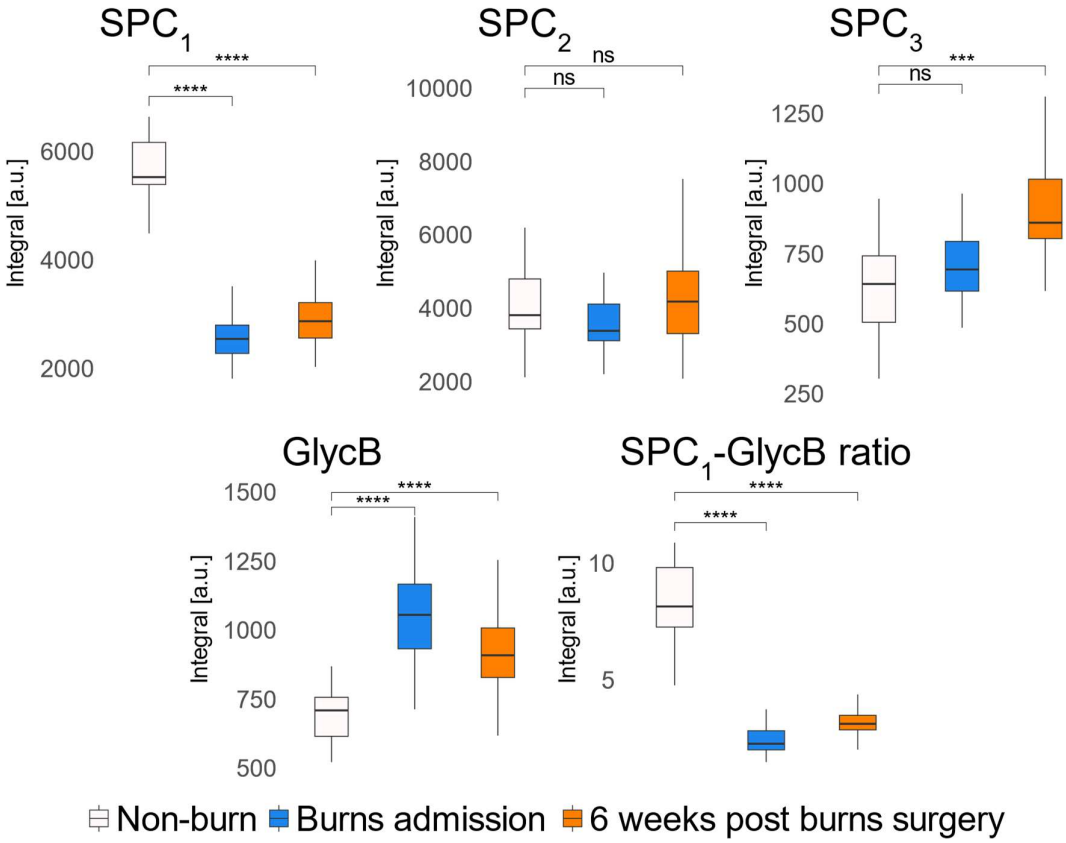

Figure 3. Univariate analysis of SPC_1, SPC_2, SPC_3, GlycB and SPC_1 to GlycB ratio values of non-burn controls compared to burns at admission and 6 weeks post-surgery. Box and whisker plots of non-burn controls (n = 14) (white) compared to burns at admission (n = 35) (blue) and 6 weeks post-surgery (n = 35) (orange) for SPC_1, SPC_2, SPC_3, GlycB and SPC_1 to GlycB ratio values. Significance level from Mann-Whitney U tests between non-burn controls to burns at admission and non-burn controls to 6 weeks post-surgery are shown above the corresponding plots and represented with '*'. Significance: ns = not significant; * = p-value < 0.05; ** = p-value < 0.01; *** = p-value < 0.001; **** = p-value < 0.0001.

Non-severe burn injury causes changes in plasma lipidomic profiles associated with sustained inflammation and increased risk of atherogenesis.

Processed data from the lipid profiling of non-burn and burn cohorts were robust, with intermittently run quality QC pooled plasma samples demonstrating clustering on a PCA scores plot (**Figure S1B**). Alterations in plasma lipid profiles have previously been observed following severe burn injuries, and were reported to be a result of systemic inflammatory responses[13]. Our data indicated differences in

lipidomic profiles between plasma samples collected from the non-burn controls and burn injury group at both admission and 6 weeks post-surgery (**Figure 4**). The VIP plot presents the top 12 lipids contributing most to each of the models, post-burn injury at admission vs non-burn controls (**Figure 4C, Table S2**) and 6 weeks post-surgery vs non-burn controls (**Figure 4D, Table S2**). To visualise whether lipid class and/or sidechain length trends were driving separation of the OPLS-DA models, 2D plots of lipid class (x) and lipid sidechain length (y) were generated presenting the VIP contribution of each lipid variable loading used for the model (**Figure S2**). The 2D plots indicated that there were no specific class and/or sidechain trends, with an overall trend that most plasma lipid species were present in higher concentrations in non-burn controls at both timepoints. However, despite this, the 6-week timepoint vs non-burn control model indicated that more lipid species were present at higher concentrations than at hospital admission, including lipids from diacylglyceride (DAG), lysophosphatidylcholine (LPC), monoacylglyceride (MAG), phosphatidylcholine (PC), phosphatidylethanolamine (PE) and triacylglyercide (TAG) subclasses with side chain lengths of 16:0, 16:1 18:0, 18:1 and 20:4 (**Figure S2**).

Notably, the sidechain length of 20:4, or free fatty acid (FFA) 20:4 (FFA(20:4)) is commonly known as arachidonic acid, a precursor to numerous lipid mediators (eicosanoids) that are fundamental to inflammation and have been implicated in many inflammatory diseases[79]. During the inflammatory response, FFA(20:4) is metabolised into eicosanoid families, mostly in inflammatory cells, through four pathways which yield, but are not limited to, prostaglandins, isoprostanes, thromboxane and leukotrienes[80]. Metabolites within these families are predominately pro-inflammatory, known to enhance vasodilation, oedema formation, chemoattraction of inflammatory cells, and innate lymphocyte migration and activation[80].

OPLS-DA modelling comparing lipid profiles from the burn injury group at admission to the non-burn control group (**Figure 4**) show that MAGs were the primary drivers of separation and significantly higher in concentration in the burn cohort (**Figure 5**), particularly MAG(20:4) (OPLS-DA VIP score at admission = 0.95; Mann-Whitney U p-value < 0.0001). Higher MAG(20:4) concentrations were also key in driving the OPLS-DA model comparing 6 weeks post-surgery (OPLS-DA VIP score = 1.32; Mann-Whitney U p-value < 0.0001) to the non-burn controls (**Figure 5**). The role of MAGs in disease or injury is not well documented and has not been previously observed in burn injury. However, MAG(20:4) is an endogenous lipid that can activate cannabinoid receptors which has importance as an inflammatory modulator[81,82]. MAG(20:4) is able to modulate inflammation as a ligand to the pro-inflammatory cannabinoid 1 (CB1) receptor and the anti-inflammatory CB2 receptor [83]. Evidence has accumulated of the pro-atherogenic impact that elevated levels of MAG(20:4) have *in vivo,* mainly through CB1 activation[84–86]. It is hypothesised that MAG(20:4) stimulates monocytes and macrophages to migrate and promote pro-inflammatory cytokine expression, which can result in local vascular inflammation and atherosclerotic plaque formation[84,85]. High concentrations of MAG(20:4) have been found in patients with different cardiovascular diseases, such as acute coronary syndrome[86], obesity-induced cardiac dysfunction[87] and chronic heart failure[88]. Additionally, a recent lipidomic study into SARS-CoV-2 infections found that MAGs were one of the lipid subclasses associated with the disease, supporting its inflammatory role[89].

The OPLS-DA modelling of burns admission versus non-burn controls also revealed higher concentrations of TAG species in the burn injured group (**Figure 4C**). This complements the observation of higher concentrations of very low-density lipoprotein-5 (VLDL-5) subfractions (VLDL-5 cholesterol (V5CH) and VLDL-5 phospholipids (V5PL)) at burns admission (**Figure 2C**), as VLDLs consist mainly of TAGs (50-70% of the particle mass)[90]. Plasma VLDL remnants are metabolised into IDL and then LDLs through the removal of triacylglycerides[91]. Elevated plasma TAGs and VLDL subfractions 1 and 2 have been correlated in non-burn inflammatory conditions, such as atherosclerotic cardiovascular disease, and has been hypothesised to be a result of impaired clearance by inefficient lipolysis[91].

At the 6-week timepoint, an additional lipid subclass, LPCs, became one of the main drivers of separation between the burn and non-burn cohorts along with MAGs (**Figure 4**). The biological role of the specific lipid species LPC(22:4) and LPC(22:5) is not currently known, but LPCs have been shown to be pro-inflammatory and potentially pro-atherogenic[92]. Promotion of atherogenesis has been associated with an increase in LPCs, through their ability to stimulate immune cell migration, particularly T-cell recruitment for apoptosis[92]. Even though LPCs were not contributors to separation of burn injury and non-burn participants at hospital admission, this change over time may indicate secondary pathways activating after a non-severe burn injury that could increase risk of atherogenesis.

Multiple lipid species from the FFA, PC, PE, phosphatidylglycerol (PG), phosphatidylinositol (PI) and phosphatidylserine (PS) subclasses were significantly lower in the non-severe burn cohort at admission compared to the non-burn controls, as revealed in the OPLS-DA modelling and univariate testing (**Figures 4 and 5**). From the FFA subclass, FFA(22:6) was significantly lower at both admission (Mann-Whitney U p-value < 0.001) and at the 6-week timepoint (Mann-Whitney U p-value < 0.01) compared to the non-burn controls (**Figure 5**), as well as being one of the top 10 drivers of separation in the OPLS-DA models at the 6-week timepoint (**Figure 4**). FFA(22:6) is also known as docosahexaenoic acid, a potent anti-inflammatory lipid mediator[80,93]. This FFA can be metabolised into the eicosanoids resolvin and protectin, which can inhibit neutrophil and dendritic cell migration and modulate surface markers on lymphocytes to contribute to inflammatory resolution[80]. The lower concentration of FFA(22:6) on admission could suggest the increased metabolism into resolvins and protectins (not analysed in this lipid profiling method) but the FFA(22:6) concentrations were unchanged between admission and the 6-week timepoint. Additionally, PCs, PEs, PGs, PIs and PSs were present at lower concentrations in the burn injury group at admission compared with the non-burn control group (**Figure 4C**). PCs, PGs, PIs and PSs have all been reported to have anti-inflammatory properties[94–96], with PEs, PSs and PCs having inter-linking pathways[97]. In particular, PE(P-16:0_18:3), PE(P-18:1_18:2) and PC(18:2_18:2) lipid species were also observed to be low in SARS-CoV-2 infected patients, presenting a similar inflammatory lipid profile to that seen in the non-severe burn cohort at admission (**Figure 4, Figure 5**). At 6 weeks post-surgery, the majority of the significant lipid species from the six subclasses remained at a lower concentration than in the non-burn controls. The lower concentrations of phospholipid subclasses (PCs, PEs, PSs, PIs and PGs) observed is complimentary to observations of lower concentrations of HDL-4 subfractions observed (**Figure 2C, 2D**). Plasma HDLs are predominantly composed of phospholipids (40-60% of particle mass) and are critical contributors to regulating HDL cholesterol efflux and its other anti-inflammatory properties[98]. Lower concentrations of HDL have been previously reported in cardiovascular disease [61], and may be indicative of a systemic inflammatory response from the host. Our data suggests this response is sustained for at least 6 weeks after a burn injury.

When compared to non-burn controls, a distinct panel of lipid species were consistently in the top 12 drivers of separation from the OPLS-DA modelling (**Figure 4**) and significantly different univariately (**Figure 5**) for non-severe burn injury at admission and at 6 weeks. This panel of MAG(20:4), MAG(20:3), MAG(18:2), MAG(18:1), and PS(18:0_18:2) did not change over time for the non-severe burn cohort, while LPC(22:4), LPC(22:5) and FFA(22:6) were found to influence lipid profiles at the 6-week time point. If sustained for months after a non-severe burn injury, this inflammatory profile could be a risk for additional health problems that have been reported after severe burn injuries[7], such as scarring, infectious disease susceptibility, hypermetabolic diseases, cardiovascular strain, tissue and organ damage and mental health impacts.

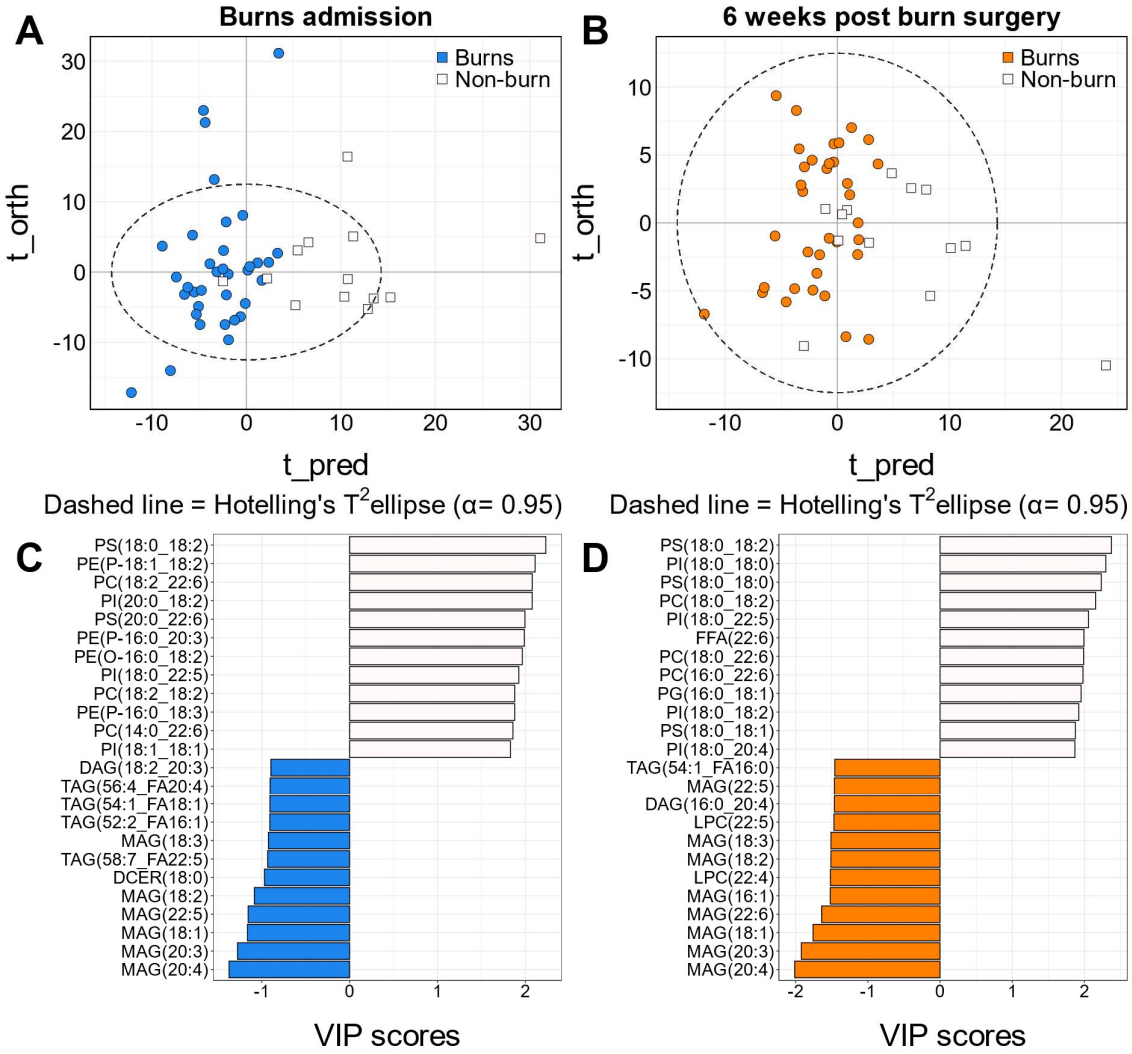

Figure 4. **OPLS-DA analysis of lipids from plasma samples collected from hospitalised patients post-non-severe burn injury both at admission to hospital and 6 weeks post-surgery vs non-burn controls. A)** OPLS-DA score plots of admissions (n=35; coloured blue) vs non-burn controls (n=14; coloured white) (R^2X =0.21, CV-AUROC = 0.95). **B)** OPLS-DA score plots of 6 weeks post burn surgery (n=35; coloured orange) vs non-burn controls (n=14; coloured white) (R^2X = 0.11, CV-AUROC = 0.80). **C)** Bar plots corresponding to the top 12 loading variables of each OPLS-DA model (ranked by variable importance in projection (VIP) score), colours represent burn injury group at admission (blue) vs non-burn controls (white). **D)** Bar plots corresponding to the top 12 loading variables of each OPLS-DA model (ranked by VIP score), colours represent burn injury group at six weeks (orange) vs non-burn controls (white).

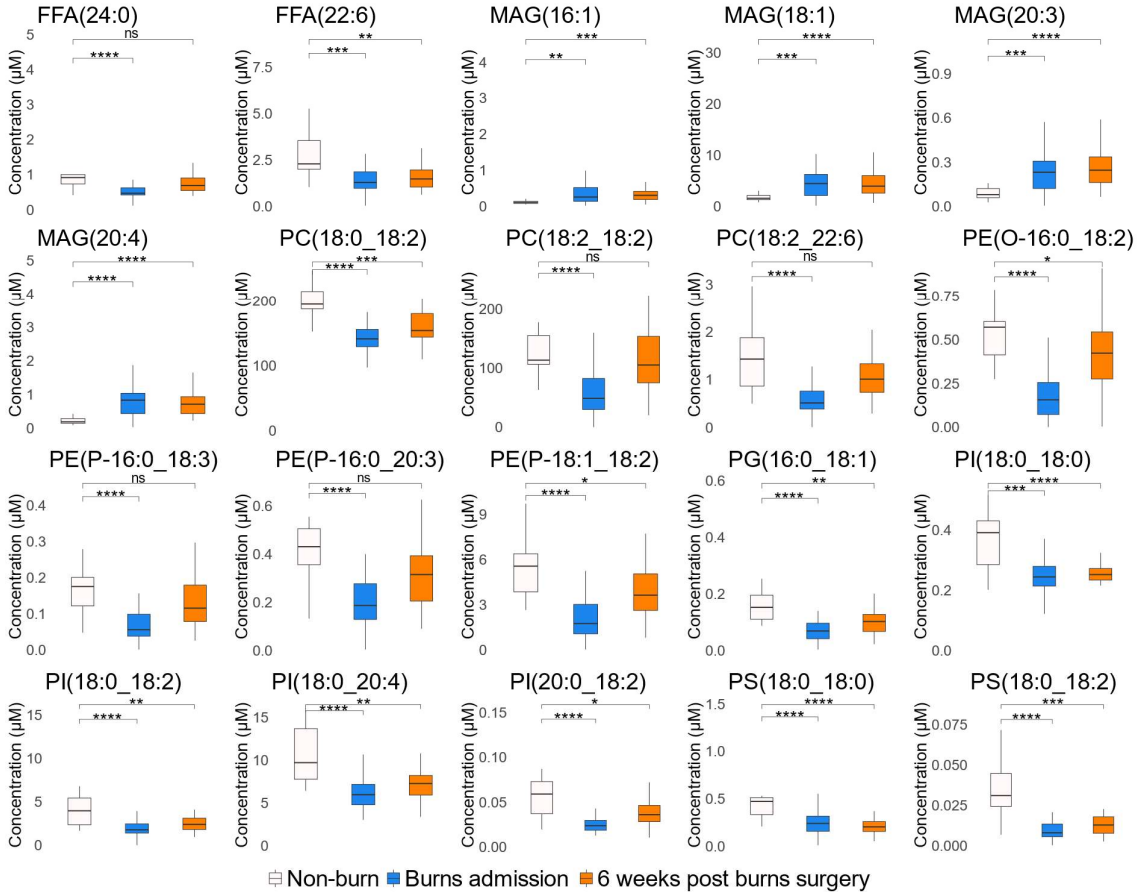

Figure 5. **Top 20 lipids from univariate analysis of lipid profiles from non-burn controls compared to burns at admission and 6 weeks post-surgery.** Box and whisker plots of non-burn controls (n = 14) (white) compared to burn admissions (n = 35) (blue) and 6 weeks post-burn surgery (n = 35) (orange) for the top 20 lipids from univariate analysis of non-burn controls vs the burn injury group at each timepoint. Significance level from Mann-Whitney U tests between non-burn controls to burn admissions and non-burn controls to 6 weeks post-burn surgery are shown above the corresponding plots and represented with '*'. Significance: ns = not significant; * = p-value < 0.05; ** = p-value < 0.01; *** = p-value < 0.001; **** = p-value < 0.0001.

Study limitations

We acknowledge that this study has limitations, particularly with respect to the bias in the sex of participants recruited to the study. The non-severe burn group was mostly comprised of males (80% of the burns cohort), as was the non-burn controls (64.2% of the controls). In Western Australia, adult males are hospitalised for burns at twice the rate of females, so this was expected in the non-severe burn cohort[1]. To assess the influence of sex on the non-burn controls, each lipid and lipoprotein that were important to the OPLS-DA modelling for the burn group were subjected to Mann-Whitney U testing between the sexes (**Figure S3**). No significant difference was found between sex for any of the important lipids and lipoproteins.

Furthermore, due to delays between injury and presentation and again between admission and study recruitment, initial samples in the burn cohort were not collected until several days after injury and so we do not have true baseline or pre-injury profiles for these patients. However, as the observed changes primarily relate to inflammation and because acute burn injury is known to incite an inflammatory response within hours, it seems likely that the changes are truly burn-related.

Finally, hospital admissions for burns patients involve various interventions including surgery and implementation of a multi-modal analgesia treatment strategy, as well as change in diet, activity and sleep. In a mouse model, surgery and the timing of surgery post-injury was seen to alter the cytokine response[99]. Conceptually, burn surgery may reduce the inflammatory stimulus by excising dead and damaged skin or conversely due to its invasive nature, surgery may provide a second inflammatory insult. It is therefore unclear whether increases of lipids and lipoproteins between admission and 6 weeks are solely driven by burn injury or are influenced by surgery. Further research will be required to determine the influence of surgery and the timing of surgery on metabolic profiles after a burn injury.

Conclusions

Non-severe burn injury results in both immediate and persistent impacts on plasma lipid and lipoprotein profiles. Alterations in lipid metabolism reflect a number of differing metabolic processes between burns and non-burn controls, showing an inflammatory profile that is induced post-burn and sustained at a systemic level. At 6 weeks post-surgery for burn injury, the inflammatory marker GlycB remained significantly enhanced and corresponded with a decrease in the supramolecular phospholipid composite, specifically SPC_1 and subsequently HDL-4 subfractions (H4A1, H4A2 and H4CH), that has previously been found as a distinguishing marker of inflammation. The combination of low plasma HDL-4 subfractions, PC, PE, PG, PI, and PS concentrations, and high LDL-2 subfractions, MAGs (particularly MAG(20:4)) and LPCs following burn trauma may be suggestive of an increased risk of sustained inflammation leading to additional health problems and cardiovascular disease, which warrants further long-term investigation. This potential risk and sustained inflammation underscores the value in monitoring lipid and lipoprotein levels following burn injury. Monitoring these profiles may lead to potential interventions that could improve acute burn patient recoveries, such as physical exercise or dietary interventions that would aid in ameliorating altered HDL and LDL plasma levels after a severe burn.

Supplementary Information

Figure S1. Principal component analysis (PCA) of study samples and intermittently run quality control (QC) plasma samples to assess data robustness.

Table S1. Annotation of the keys used by the Bruker IVDr Lipoprotein Subclass Analysis (B.I.-LISA ™) method for each lipoprotein subclass.

Table S2. Combined variable importance in projection (VIP) scores of the 852 lipid species and 112 lipoproteins, SPCs and GlycB detected in both non-severe burns at admission and 6 weeks post-surgery, and non-burn cohorts after orthogonal partial least squares discriminant analysis (OPLS-DA)

Figure S2. Visualisation of the orthogonal partial least squares discriminant analysis (OPLS-DA) model variable important in projection (VIP) scores of each of the loading variables, plotted as lipid class (x-axis) vs lipid side chain (y-axis).

Figure S3. Box and whisker plots to assess the influence of sex on the most important lipoproteins, SPCs, GlycB and lipids using VIP scores and univariate statistics.

Data Requirements

Raw data will be uploaded to the Mass Spectrometry Interactive Virtual Environment (MassIVE) repository and its DOI and identifiers will be submitted with the revised manuscript.

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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Abbreviations

AB100, Apolipoprotein B100; ABA1, Apolipoprotein B100/apolipoprotein A1; B.I.-LISA, Bruker IVDr lipoprotein subclass analysis; CABIN, Randomised placebo-controlled trial of Celecoxib for acute burn inflammation; CER, Ceramide, CV-AUROC, Cross validated-area under the receiver operating characteristic; DAG, Diacylglyceride; DIRE, Diffusion and relaxation editing; FFA, Free fatty acid; H4A1, HDL-4 apolipoprotein A1; HDA1, HDL-associated apolipoprotein A1; HDL, High density lipoprotein; hs-CRP, high sensitivity C-reactive protein; ILDL, Intermediate low density lipoprotein; IVDr, *in vitro* Diagnostics research; L2AB, LDL-2-associated apolipoprotein B; LC-QQQ-MS, Liquid chromatography-tandem mass spectrometry; LDL, Low density lipoprotein; LPC, Lysophosphatidylcholine; LPE, Lysophosphatidylethanolamine; LPG, Lysophosphatidylglycerol; MAG, Monoacylglyceride; MS, Mass spectrometry; NMR, Nuclear magnetic resonance; OPLS-DA, Supervised orthogonal partial least squares discriminant analysis; PC, Phosphatidylcholine; PCA, Principal component analysis; PE, Phosphatidylethanolamine; PI, Phosphatidylinositol; PG, Phosphatidylglycerol; PS, Phosphatidylserine; QC, Quality control; RSD, Relative standard deviation; SM, Sphingomyelin; SPC, Supramolecular phospholipid composite; TAG, Triacylglyceride; TBSA, Total burn surface area; TSP, Sodium trimethylsilyl propionate-[2,2,3,3- $2H_4$]; VIP, Variable important in projection; VLDL, Very low density lipoprotein.

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