



The metabolomics of a protein kinase C delta (PKC δ) knock-out mouse model

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Received: 26 June 2022 / Accepted: 29 October 2022 / Published online: 13 November 2022
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Abstract

Introduction PKC δ is ubiquitously expressed in mammalian cells and its dysregulation plays a key role in the onset of several incurable diseases and metabolic disorders. However, much remains unknown about the metabolic pathways and disturbances induced by PKC deficiency, as well as the metabolic mechanisms involved.

Objectives This study aims to use metabolomics to further characterize the function of PKC from a metabolomics standpoint, by comparing the full serum metabolic profiles of PKC deficient mice to those of wild-type mice.

Methods The serum metabolomes of PKC δ knock-out mice were compared to that of a wild-type strain using a GCxGC-TOFMS metabolomics research approach and various univariate and multivariate statistical analyses.

Results Thirty-seven serum metabolite markers best describing the difference between PKC δ knock-out and wild-type mice were identified based on a PCA power value > 0.9, a t-test p-value < 0.05, or an effect size > 1. XERp prediction was also done to accurately select the metabolite markers within the 2 sample groups. Of the metabolite markers identified, 78.4% (29/37) were elevated and 48.65% of these markers were fatty acids (18/37). It is clear that a total loss of PKC δ functionality results in an inhibition of glycolysis, the TCA cycle, and steroid synthesis, accompanied by upregulation of the pentose phosphate pathway, fatty acids oxidation, cholesterol transport/storage, single carbon and sulphur-containing amino acid synthesis, branched-chain amino acids (BCAA), ketogenesis, and an increased cell signalling via N-acetylglucosamine.

Conclusion The characterization of the dysregulated serum metabolites in this study, may represent an additional tool for the early detection and screening of PKC δ -deficiencies or abnormalities.

Keywords Auto-immune diseases · Diagnostic biomarker · Metabolomics · Protein kinase C · Metabolic disorder · Serum samples · Non-communicable disease

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1 Introduction

Incurable diseases, metabolic disorders (such as diabetes, hypertension, dyslipidemia, newborn inborn errors of metabolism, and ethylmalonic encephalopathy), infectious diseases, and a plethora of other autoimmune diseases, are becoming increasingly prevalent globally. According to available data, chronic noncommunicable diseases continue to be a global health concern. The world health organization (WHO) estimates that about 41 million deaths are due to non-communicable diseases (NCDs), accounting for 71% of all global deaths per annum (WHO, 2021b). Additionally, the Covid-19 pandemic has recently exacerbated the health-care system's multimorbidity burden. (Azarpazhooh et al., 2020; WHO, 2021a; Wood et al., 2021).

Many enzyme deficiencies/abnormalities have been associated with the aforementioned metabolic disorders or metabolism-related diseases, plaguing humanity, none more so, however, than protein kinase C delta (PKC δ), a member of the PKC serine or threonine kinases family (Brodie & Blumberg, 2003). To date, three categories of ten PKC isozymes/families have been identified (conventional, novel, and atypical), and their activation differs significantly. (Fields & Gustafson, 2003; Zhao et al., 2012). These PKC isozymes frequently perform overlapping, but sometimes opposing, functions (Black & Black, 2013; Dempsey et al., 2000), as they differ in structure, function, and biochemical properties (e.g. requirements for co-factors and calcium) (Brodie & Blumberg, 2003; Newton, 2001). Diacylglycerol, DAG (a lipid second messenger), and calcium, activate the conventional PKCs (PKC α , PKC β I and PKC β , and PKC γ); the novel PKCs (PKC δ , PKC ϵ , PKC θ , and PKC η) are activated solely by DAG, whereas the atypical PKCs (PKC ζ and PKC ι/λ) are neither activated by lipid second messengers or calcium. (Black & Black, 2013; Poole et al., 2004; Zhao et al., 2012). PKCs have been implicated in numerous cellular processes, including transcriptional regulation, membrane structure modulation, immune mediation, and cell growth regulation (Carey et al., 2020; Lien et al., 2021; Poole et al., 2004; Salzer et al., 2016). Although the full functionality of PKC δ is yet to be determined, it is the first identified member of the novel PKC subfamily to be largely expressed in mammalian cells, including macrophages (Wang et al., 2020; Zhao et al., 2012). While PKC δ generally functions as a pro-apoptotic protein during DNA damage-induced apoptosis, it also acts as an anti-apoptotic protein during receptor-initiated cell death (Salzer et al., 2016). PKC δ 's functionality depends on various factors, including its localization, tyrosine phosphorylation, and the presence of other pro- and anti-apoptotic signaling molecules (Basu & Pal, 2010).

PKC δ dysregulation contributes to the onset and aggravation of several non-communicable disease conditions including (1) LPS-induced osteolysis and human lupus [systemic lupus erythematosus (SLE)], (Gorelik et al., 2015; Khor et al., 2013), (2) diabetes and other obesity-related complications (Lien et al., 2021; Sparks et al., 2019), (3) Alzheimer's (AD) and Parkinson's disease (PD), (Du et al., 2018; Gordon et al., 2016). Remarkably, people with autoimmune diseases, such as a PKC δ -deficiency or defect, also typically have other invasive, recurring, and/or severe infections. In particular, PKC δ -deficient patients are more susceptible to viral, bacterial, and fungal infections, mostly affecting the lung, gastrointestinal tract, and lymph nodes (Kuehn et al., 2013; Nanthapaisal et al., 2017; Salzer et al., 2016; Sharifinejad et al., 2020). PKC δ 's conflicting roles in cell survival and cell death signaling (acting either pro-survival and/or pro-apoptotic during cellular activities), makes it a potential

target in the treatment and diagnosis of many disease conditions, including the aforementioned. PKC δ inactivation has been shown *in vivo* to (1) protect organs, (2) improve pathogen clearance, and (3) improve survival during sepsis. (Liverani et al., 2020). Hence PKC δ inhibition may represent a valuable target for innovative therapeutic strategies, for example, in the study by Choi et al., (2013), PKC δ inhibition reversed all pathophysiological symptoms of asthma. Dysregulated PKC δ expression has also been shown to play a regulatory role in tumorigenesis (tumor suppression and cancer proliferation) (Allen-Petersen et al., 2014; Berardi et al., 2016; Chen et al., 2011). Herein, PKC δ isozyme inactivation may also represent a new molecular target for cancer stem cell populations (CSC-like populations).

Most PKC δ research to date (cellular process regulation, interference, inhibition, etc.) has been done using various histological, molecular, genomics, proteomics, and transcriptomics techniques (Berardi et al., 2016; Contreras et al., 2012; Gorelik et al., 2015; Nanthapaisal et al., 2017). The paucity of metabolomics investigations into the functionality of PKC, either independently and/or in conjunction with other omics techniques, however, necessitates this study. This also follows the understanding that the potential use of PKC isoforms, including PKC δ , as a target for therapeutic intervention, still faces diverse challenges. Furthermore, to better understand the effects or role of PKC δ on the mammalian metabolome, the myriad of metabolites and metabolic changes associated with its dysregulation should firstly be evaluated, as was done in the current investigation. Since various host-directed therapeutic approaches continue to show promising advantages in the management of many non-communicable (e.g. PKC δ deficiency and PKC δ -comorbidities) and communicable diseases (e.g. tuberculosis, covid-19, HIV), the identification of new biomarkers is now readily recognized as important not only for diagnostics but also to better disease characterization, and in so doing, directing the development of new therapies against many of these incurable diseases plaguing humanity (Adeniji et al., 2020; Baidara et al., 2020; Bausch et al., 2021; Mahon & Hafner, 2017).

Metabolomics is a rather new, yet well-established research technology, employed towards clinical biomarker discovery, for better elucidating pathological processes (Beyoğlu et al., 2013; Bonte et al., 2019) and the dynamic metabolic responses of substances in the mammalian metabolome (Stander et al., 2020). These important clinical-metabolomic investigations range from elucidating differences between healthy and diseased states (Dahabiyeh et al., 2021; Hao et al., 2017), to the quantitative measurement of endogenous disruptions induced by exogenous substances introduced to a host system (Knoll et al., 2021). Considering this, PKC δ is a host factor important for disease control, while also serving as a marker of disease progression in

humans (Parihar et al., 2018; Wang et al., 2020). This study seeks to use metabolomics in order further characterize the functionality of PKC δ from a metabolomics perspective, by comparing the full serum metabolic profiles of naïve PKC δ deficient mice with that of its wild-type mouse counterpart. The metabolomic analysis presented here not only corroborates the findings of other omics strategies but also reveals novel PKC δ functionality.

2 Methods

The experiment and metabolomics analysis were conducted following the procedures previously described by Williams et al. (2006), Du Preez and Loots (2013), and Parihar et al. (2018). PKC δ deficient and wild-type mice on the 129 Sv genetic background (8–12 weeks), were maintained under specific-pathogen-free conditions in individually ventilated cages. Five animals per group were used to collect serum samples from naïve mice. All experiments were performed in accordance with the South African National Guidelines and University of Cape Town practice for laboratory animal procedures. The protocol (AEC: 012/036) was approved by the Animal Ethics Committee, Faculty of Health Sciences, University of Cape Town, Cape Town, South Africa.

2.1 Reagents and chemicals

Deionised water from a Millipore MilliQ purification system was used in all cases. Acetonitrile, Optima grade, was purchased from Fisher Scientific (Pittsburgh, USA). All other reagents and organic solvents used for this investigation were purchased from Sigma–Aldrich, St. Louis, MO, USA, unless specified otherwise.

2.2 GC x GC-TOFMS metabolome analysis of PKC δ deficient and wild-type mice serum

2.2.1 Sample preparation and extraction

Extraction of the collected serum samples from PKC δ deficient and wild-type mice were performed as previously described by Williams et al. (2006), using 50 μ L of 0.45 μ g/mL 3-phenylbutyric acid (Sigma-Aldrich), as an internal standard, before GC x GC-TOFMS analyses. Briefly, the serum samples (100 μ L) were protein precipitated using 3 volumes of acetonitrile and the sample was then centrifuged at 3000 rpm for 10 min at room temperature, then 100 μ L of each supernatant was evaporated to dryness under vacuum, prior to derivatization. In addition, before analysis, 50 μ L of each sample were pooled to generate a quality control (QC) sample stock solution and, following protein precipitation, 100 μ L of this pooled sample was also evaporated to

dryness. All the dried samples were derivatized with 20 μ L methoxyamine hydrochloride (40 mg/mL in pyridine) at 28 $^{\circ}$ C for 90 min followed by 180 μ L MSTFA at 37 $^{\circ}$ C for 30 min. All experimental samples were randomly assigned to, and analyzed in batches of 10 samples, together with 1 aliquot of pooled QC sample per batch, injected in the beginning, middle and end of each batch.

2.2.2 GC x GC-TOFMS analysis

Chromatographic analyses of the derivatized samples were performed using a Pegasus GC x GC-TOFMS (Leco Corporation), utilizing an Agilent 7890 A GC (Agilent) coupled to a time of flight mass spectrometer (TOFMS) (Leco Corporation) and a Gerstel Multi-Purpose Sampler (MPS) (Gerstel GmbH & co. KG) as described previously, with minor modifications. This included that a split ratio of 1:2 was used, a Restek Rxi-17 (1 m, 0.25 mm i.d., 0.25 mm d.f.) column for the second dimension separation, and the secondary oven programmed with an offset of +15 $^{\circ}$ C, increasing at 4.5 $^{\circ}$ C per min to a final temperature of 300 $^{\circ}$ C. Cryogenic modulations and a hot pulse of nitrogen gas of 0.5 s, every 3 s was used to control the effluent emerging from the primary column onto the secondary column. Detection was achieved by using MS detection in full scan mode (m/z 50–800). Leco Corporation ChromaTOF software (v4.5) was used for peak finding and mass spectral deconvolution at an S/N ratio of 50, with a minimum of 2 apexing peaks. Peak identification and alignment was done as previously described by Du Preez and Loots (2013). To select metabolites, a comparison of mass fragment patterns of metabolites identified with those in the commercially available databases (National Institute of Standards and Technology (NIST) 2020 and Wiley v12), containing previously injected standards was done. All compounds were normalized relative to the internal standard by calculating the relative concentrations of each.

2.3 Statistical analysis

The dataset presented for statistical analysis comprised the relative concentrations of 581 compounds for QC, WT, and KO samples. To more closely match the assumptions made by the various statistical techniques used, the data were pre-treated using a standardized metabolomics data clean-up procedure (Gromski et al., 2014; Sullivan & Feinn, 2012; Wei et al., 2018). As stated above, analytes and compounds with more than 50% zero values in each group were excluded from further analysis. The remaining zero values were replaced by random numbers generated from the left tail of a beta distribution fitted to the data. The data were log-transformed to improve normality for parametric univariate analysis and subsequently converted to z-scores for multivariate analysis, ensuring the abundance of a compound

does not dictate its importance, as abundance does not necessarily imply biological importance (van den Berg et al., 2006). Multivariate models and univariate statistics were used in a complementary manner, as univariate analyses are not affected by uninformative, yet competing variables, while multivariate approaches leverage the joint ability of variables to explain variation (Saccenti et al., 2014).

Univariately, both the statistical (independent t-test p-values adjusted for multiple testing to control the false discovery rate) and practical significance (Cohen's d-value) of group differences were evaluated. However, given the large number of compounds and the necessity to correct for multiple testing, great emphasis was placed on practical significance. Furthermore, the impact of different transformations can be dramatic yet difficult to evaluate, therefore, XERp, a univariate method requiring no pre-processing, was also included (Van Reenen et al., 2017). The primary model for multivariate analysis of group differences was partial least squares–discriminant analysis (PLS-DA). As this supervised method is a known struggle given unseen data, different validation strategies were implemented alongside. Besides a leave-one-out cross-validation of the model's predictive stability, principal components analysis (PCA) (Brereton, 2003) was also performed to identify the compounds associated with the largest amount of variability observed and how that relates to the groups. PCA was also used to evaluate the repeatability of GC x GC-TOFMS analysis by comparing the variability between QC aliquots and research samples. All data preparation and analyses were performed in Matlab using the Statistics toolbox (MATLAB, 2012b) and the PLS packages from Eigenvector (Eigenvector, 2016).

3 Results

3.1 Data overview

The GC x GC-TOFMS system detected on average over 1000 compounds per sample. This data set was cleaned/reduced by removing “unknowns” (compounds with no corresponding mass spectral matches in the libraries), column and/or reagent-related compounds (non-biological), and merging duplicate peaks of putative compounds (e.g. sugars and amino acids), 581 usable compounds remained. This data set containing the 581 compounds (excluding internal standard) were then subjected to the aforementioned processing steps [i.e. quality control coefficient of variation (QC-CV) filter etc.] and further statistical analyses.

3.2 Quality control

A PCA model was constructed for all data including QC repeated observations of a sample. A scores plot labelled as

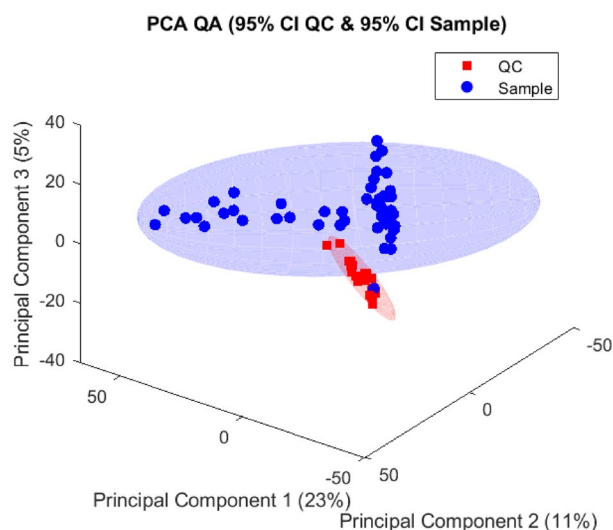


Fig. 1 Principal component analysis scores plot for all experimental and quality control samples

research samples and QC observations was drawn (Fig. 1). A PCA model was constructed for all data including QC repeated observations of a sample. A scores plot labelled as research samples and QC observations was drawn (Fig. 1). The differentiating grouping of the QC observations with respect to the experimental samples is most likely due to the high number of metabolites detected in low abundance, which would have been further reduced due to pooling to construct the pooled QC sample. Almost half of the QC observations were zeroes (49%), which is why zero filtering was approached with care (refer to the section on Statistical Analysis) in this instance. In evaluating the quality of the raw data, we are therefore content to see a good clustering of QC samples, which indicates negligible shifts between batches and machine drift within batches. Furthermore, all samples were randomised to batches as well as within batches, therefore any analytical variation would have been present in both experimental groups, and as such is accounted for.

3.3 Multivariate statistical results

Partial least-squares discriminant analysis (PLS-DA) showed excellent separation (Fig. 2a) and predictive accuracy, however, due to the small group sizes, the model was unstable. Principal component analysis (PCA) showed comparable separation (Fig. 2b) and considering that this is an unsupervised method, the variable rankings underpinning the PCA model was preferred for variable selection. PCA ranks variables in terms of their role in explaining the variability captured by selected latent variables, in these cases, latent variables 1 through 3. The ranking is quantified into a power

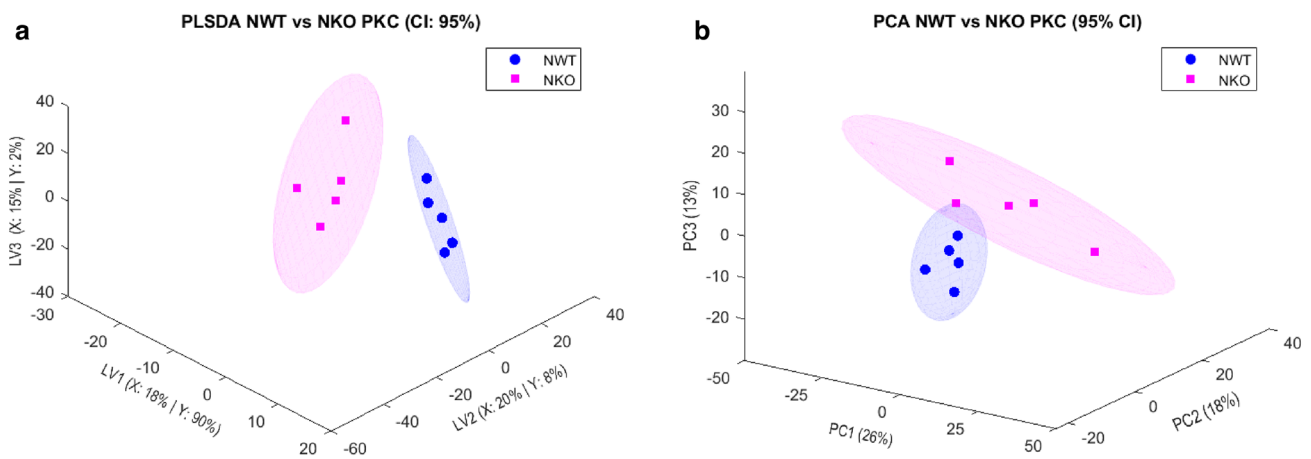


Fig. 2 Multivariate models for wild-type (NWT) versus PKC δ deficient mice (NKO) after gene deletion. **a** Partial least-squares discriminant analysis (PLS-DA) scores plot of the first three latent variables (LV). Percentages represent the proportion of variation explained in the observed data (X) and the group assignment (Y) by the specific

LV. **b** Principal component analysis (PCA) scores plot for the first three principal components (PC). Percentages in brackets represent the proportion of variation explained in the observed data by the specific PC

metric which was used for variable selection. The rule-of-thumb is that variables with power values exceeding 0.5 contribute sufficiently to the model to be of interest. Considering the small group sizes and the fact that a variable's contribution to a PCA model does not necessarily correlate to its predictive ability (PCA models are unsupervised and so do not attempt to explain group membership, but rather general variability in the data), the rule was made stricter (power > 0.9) and the focus shifted to univariate statistics.

3.4 Univariate statistical results

Two univariate approaches were used for variable selection: (1) Cohen's d-value in its strictest form, i.e. using the maximum standard deviation as the denominators, and (2) variables identified using XERp showing predictive potential. The most common approach in this setting would be the independent samples t-test, which was also applied here. However, the design lacked power and so limited significance was retained after correcting for multiple testing over 500 variables (Fig. 3). Effect sizes can still be informative when statistical power is insufficient (Sullivan & Feinn, 2012) and as such was the focus for variable selection. A cut-off of > 0.8 was set for Cohen's d-values which is indicative of a practically significant difference between groups.

Cohen's d-value assumes normality and even though these calculations were done using log-transformed data after zero replacement, it is not possible to ensure this assumption is met. Therefore, Cohen's d was supplemented by XERp (van Reenen et al., 2017), a method making no distributional assumptions with the ability to also assess the

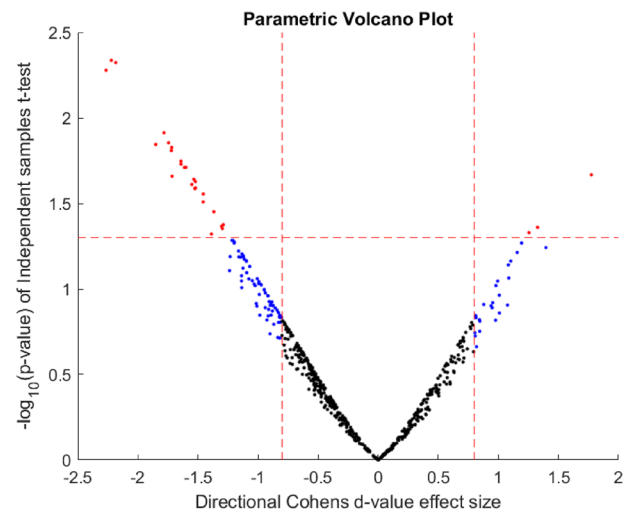


Fig. 3 A scatter plot of log scaled p-values from independent samples t-tests for each compound against Cohen's d-value where the sign is indicative of up- or down-regulating effect. P-values have not been adjusted for multiple testing

importance of differences in the proportion of zeros. XERp follows a leave-one-out approach, selecting variables for each iteration based on their predictive accuracy within each group. Compounds selected in all iterations, with a classification accuracy of at least 80% for both groups, were considered informative.

3.5 Marker selection

The metabolite markers (n = 37) best describing the variance between PKC δ deficient and wild-type mice were selected based on compliance with the following criteria: a

Table 1 Identifiable serum metabolites best describing the variance between PKC δ deficient and wild-type mice at day zero

Metabolite (PubChem ID)	Average concentration (ug/L) (standard deviation)		Fold change (mean)	PCA (power value)	Cohens (effect size: d-value)	XERp classification accuracy %	
	Wild-type mice	PKC δ deficient mice				Wild-type mice	PKC δ deficient mice
Amino acid, ketones, and tricarboxylic acid-associated intermediates							
β -Hydroxybutyric acid (11,266)	0.275 (0.188)	0.948 (0.600)↑	-1.785	0.415	1.122	60	60
α -Ketoisocaproic acid (70)	1.927 (0.950)	3.102 (0.888)↑	-0.687	0.283	1.237	60	60
α -Ketoisovaleric acid (49)	0.860 (0.444)	0.379 (0.050)↓	1.183	0.140	1.083	60	100
Leucinic acid (92,779)	0.706 (0.087)	1.138 (0.316)↑	-0.689	0.708	1.368	80	80
Serine (5951)	0.647 (0.144)	8.811 (9.703)↑	-3.767	0.355	0.841	100	80
Malic acid (525)	1.8623 (0.293)	2.964 (1.132)↑	-0.621	0.657	0.913	90	60
Carbohydrate metabolism and associated intermediates							
Glycolysis and gluconeogenesis							
Glucose (5793)	0.200 (0.083)	0.316 (0.063)↑	-0.659	0.220	1.439	90	70
2-Phosphoglyceric acid (59)	0.237 (0.108)	0.412 (0.170)↑	-0.801	0.583	1.032	60	100
Pyruvic acid (1060)	0.6020 (0.850)	2.607 (1.647)↑	-2.114	0.301	1.217	80	80
Mannose (18,950)	0.195 (0.264)	1.160 (0.878)↑	-2.571	0.604	1.099	100	80
N-Acetyl-D-glucosamine (439,174)	1.528 (0.667)	2.499 (0.361)↑	-0.710	0.402	1.456	60	60
Glycogen and galactose metabolism							
Galactose (439,357)	0.040 (0.005)	0.093 (0.041)↑	-1.228	0.479	1.303	80	80
β -D-Glucopyranuronic acid (441,478)	0.118 (0.047)	0.181 (0.056)↑	-0.622	0.558	1.138	80	60
Pentose phosphate pathway							
Arabitol (94,154)	0.123 (0.09)	0.633 (0.311)↑	-2.361	0.486	1.641	80	80
5-Deoxyribose (270,738)	0.181 (0.015)	0.269 (0.073)↑	-0.570	0.480	1.198	80	80
Erythritol (222,285)	1.116 (0.16)	1.507 (0.336)↑	-0.434	0.175	1.162	80	60
Ribitol (6912)	21.651 (3.863)	16.249 (5.360)↓	0.414	0.354	1.008	60	60
Xylitol (827)	1.053 (0.369)	1.499 (0.439)↑	-0.509	0.505	1.015	80	80
Lipid and associated intermediates							
Cholesterol metabolism							
Cholesteryl acetate (6,427,285)	0.496 (0.089)	0.640 (0.031)↑	-0.368	0.238	1.611	80	80
Mevalonic acid (449)	0.061 (0.005)	0.081 (0.015)↑	-0.411	0.289	1.289	100	80
4 α -Methylzymosterol (22,212,495)	1.643 (0.429)	1.181 (0.421)↓	0.476	0.206	1.076	80	80
Fatty acid metabolism							
Citraconic acid (643,798)	0.410 (0.040)	0.518 (0.062)↑	-0.338	0.541	1.745	100	80
Dodecanedioic acid (12,736)	3.268 (0.332)	4.149 (1.181)↑	-0.345	0.910	0.746	100	60
Ethylmalonic acid (11,756)	0.138 (0.109)	0.266 (0.127)↑	-0.947	0.607	1.009	80	60
Glycerol 1-palmitic acid (14,900)	6.444 (0.618)	7.434 (0.713)↑	-0.206	0.253	1.387	80	60
Hexadecanedioic acid (10,459)	0.316 (0.059)	0.412 (0.094)↑	-0.382	0.433	1.024	100	80
3-Hydroxydodecanedioic acid (16,663,321)	0.145 (0.033)	0.183 (0.030)↑	-0.334	0.456	1.136	100	60
3-Hydroxysebacic acid (3,017,884)	0.693 (0.12)	0.422 (0.235)↓	0.717	0.384	1.157	80	80
Linolenic acid (5,280,934)	0.343 (0.079)	0.203 (0.077)↓	0.761	0.298	1.774	60	60
Malonic acid (867)	0.510 (0.197)	1.054 (0.354)↑	-1.048	0.726	1.535	80	80
Palmitic acid (985)	5.473 (4.541)	21.052 (8.737)↑	-1.944	0.218	1.783	100	100
Sebacic acid (5192)	23.696 (2.081)	28.085 (6.839)↑	-0.245	0.899	0.642	100	40
Tetradecanedioic acid (13,185)	0.978 (0.112)	1.155 (0.277)↑	-0.240	0.903	0.638	80	60

Table 1 (continued)

Metabolite (PubChem ID)	Average concentration (ug/L) (standard deviation)		Fold change (mean)	PCA (power value)	Cohens (effect size: d-value)	XERp classification accuracy %	
	Wild-type mice	PKC δ deficient mice				Wild-type mice	PKC δ deficient mice
Steroid hormone metabolism							
17 α -Hydroxypregnenolone (91,451)	0.282 (0.198)	0.064 (0.026)↓	2.138	0.314	1.103	80	100
Fatty acid amides metabolism							
Dodecanamide (14,256)	2.744 (0.626)	1.997 (0.264)↓	0.459	0.468	1.193	60	100
Oleamide (5,283,387)	1.090 (0.39)	0.565 (0.269)↓	0.948	0.304	1.326	60	60

Increase and decrease in the metabolite markers are indicated by $\uparrow\downarrow$ respectively

PCA power value > 0.9 , a t-test p-value < 0.05 , or an effect size > 1 . The selected metabolite markers are listed according to their metabolic pathway activity Table 1, along with their respective average concentrations and univariate test outcomes. The metabolome disruptions (Table 1) affected several metabolites related to (i). amino acid metabolism and ketogenesis (α -ketoisocaproic acid, α -ketoisovaleric acid, leucinic acid, serine, and β -hydroxybutyric acid), (ii). carbohydrate metabolism (glucose, 2-phosphoglyceric acid, pyruvic acid, mannose, N-acetyl-D-glucosamine, galactose, β -D-glucopyranuronic acid, arabinol, 5-deoxyribose, erythritol, ribitol, and xylitol), (iii). the tricarboxylic acid [TCA] cycle (malic acid), (iv). fatty acid metabolism (citraconic acid, dodecanedioic acid, ethylmalonic acid, glycerol 1-palmitic acid, hexadecanedioic acid, 3-hydroxydodecanedioic acid, 3-hydroxysebacic acid, linolenic acid, malonic acid, palmitic acid, sebacic acid, and tetradecanedioic acid), (v). cholesterol and steroid hormone synthesis (cholesteryl acetate, mevalonic acid, 4 α -methylzymosterol, and 17 α -hydroxypregnenolone), and (vi). fatty acid amides (dodecanamide and oleamide) (Fig. 4). Of the total, 78.4% (29/37) of the markers were elevated; and 48.65% of these were associated with fatty acid metabolism (18/37). Biological functions and metabolic pathways of these metabolites were deduced using databases such as SMPDB (The Small Molecule Pathway Database), Human Metabolome Database (HMDB), Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database, MetaCyc Metabolic Pathway Database, PubChem, and Chempidier.

4 Discussion

The biochemical analysis of tissue, serum, plasma, whole blood, urine, sweat, and salivary samples, holds great promise for the discovery of biomarkers of disease progression and response to therapy (Tounta et al., 2021; Zhang et al., 2015). Whilst some studies have revealed that a PKC δ -deficiency/inactivation results in an increased susceptibility

to other invasive, recurrent, and/or severe infections, other *in vivo* investigations have shown that PKC δ inactivation was associated with: (1) enhanced organ protection, (2) rapid pathogen eradication, and (3) improved sepsis survival (Liverani et al., 2020). In this investigation, several metabolites were significantly altered in the PKC δ knock-out mice serum, which when interpreted in the context of the previously published literature, gives clues to better elucidate the function of PKC δ , and its possible role in many communicable and non-communicable diseases. As seen in Fig. 2, our results show a clear group separation and sample clustering between the PKC δ deficient and wild-type mice. The changes to the metabolites detected in the PKC δ -deficient mice serum (Table 1), included dodecanedioic acid, tetradecanedioic acid, sebacic acid, ethylmalonic acid, leucinic acid, mannose, galactose, 3-hydroxysebacic acid, serine, ribitol, pyruvic acid, cholesteryl acetate, glucose, α -ketoisovaleric acid and malonic acid, alterations of which have been previously implicated in many metabolic deficiencies and diseases. However, before we describe in detail the metabolic changes induced in the PKC δ knockout (Fig. 4), a general observation needs to be mentioned. PKC activation has been previously associated with increased breakdown of glucose, and both elevated ATP and insulin production (Salway, 2017). PKC δ has also been implicated in the functionality of NADPH oxidase 5 (Nitti et al., 2007, 2010; Thamilselvan et al., 2009)—therewithal, NADPH oxidase 5 catalyses the conversion of NADPH to NADP (Fisher & Zhang, 2006). Consequently, PKC δ deactivation, as was the case in this investigation, would be expected to result in a reduced NADH:NAD ratio (supported by a study done by Mayr et al. (2004), and an elevated NADPH:NADP ratio. Since these cofactors are associated with several enzyme reactions, a change in their ratios will result in a change in the metabolic flux of certain enzyme reactions or even more so, entire metabolic pathways. Considering this in the context of a general metabolic overview, one would expect to see inhibition of glycolysis and fatty acid synthesis, and subsequently activation of alternative metabolic pathways,

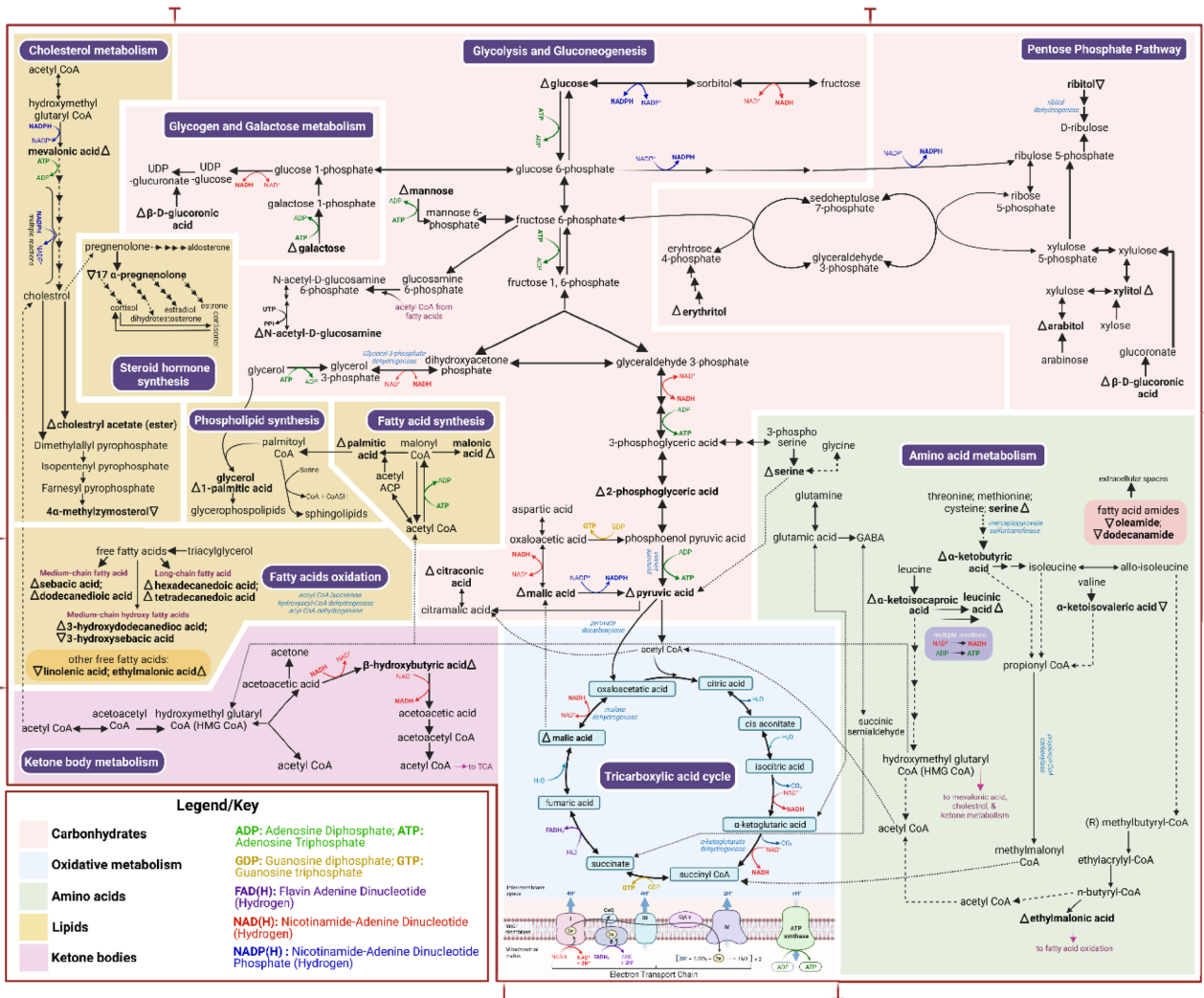


Fig. 4 Figure created with <https://biorender.com/>

to supply the necessary intermediates (acetyl-CoA to the TCA cycle) for energy production, such as via elevated β -oxidation, ketogenesis, and branched chain amino acid metabolism.

Apart from this general change in metabolic flux expected due to the change in the aforementioned ratios, PKC δ has been directly associated with the expression of many specific enzymes. Although the elevated levels of glucose (and additionally mannose and galactose), 2-phosphoglycerate, and pyruvate observed in the PKC δ deficient mice serum in this study supports the aforementioned inhibition of glycolysis, Mayr et al. (2004), additionally indicated that a PKC δ deficiency results in a marked decrease in the enzyme activities of glycerol-3-phosphate dehydrogenase, pyruvate kinase 3, and lactate dehydrogenase, which directly would result in the elevated glucose (and additionally mannose and galactose), 2-phosphoglycerate, and pyruvate seen in our investigation. The increased activity of a malic enzyme,

as previously reported by Mayr et al. (2004), further contributes to the elevated pyruvate and the increased NADPH:NADP ratio, previously mentioned. Furthermore, the accumulation of pyruvate is also an indication of inhibition of the TCA cycle in the PKC δ deficient mice as previously mentioned. This is most likely due to the inhibition of α -ketoglutarate dehydrogenase as previously reported by Mayr et al. (2004), which would result in inhibition of the TCA cycle post α -ketoglutarate, and possibly also result in an accumulation of the latter metabolite (although not detected as a marker in this investigation using the strict stats criteria for marker selection). Elevated levels of another TCA cycle intermediate; malic acid, was however observed and can be ascribed to be derived from the elevated pyruvate, via oxaloacetate, catalysed via the enzymes pyruvate carboxylase and malate dehydrogenase (Garrett & Grisham, 2016), and also via other complementary pathways feeding into the TCA cycle at succinic acid (e.g. oxidative glutamine

metabolism) (Yang et al., 2014). During glucose metabolism impairment (such as during a mitochondrial pyruvate import blockage), cell survival is dependent on glutamate dehydrogenase (Yang et al., 2009), whilst glutamine-derived malate can then be converted into pyruvate via malic enzyme 2 (Salway, 2017; Yang et al., 2014), which was previously also seen to be altered in a PKC δ deficient model (Mayr et al., 2004). Furthermore, single carbon metabolism and sulphur-containing metabolic pathways (serine-cysteine metabolism) can also account for the elevated pyruvic acid in this study, driven by the elevated concentrations of the aforementioned 2 phosphoglyceric acid and serine detected in this investigation (Salway, 2017), and also a source for the elevated concentrations of the ethylmalonic acid detected in the PKC δ deficient mice (Barth et al., 2010).

Out of the 5 sugar alcohols or polyols (erythritol, arabinol, ribitol, xylitol, and 5-deoxyribitol) detected in the serum of the PKC δ deficient mice, only ribitol showed a marked decrease. Although detection of polyhydric alcohols (e.g. polyols) (in the human metabolome), has been mostly attributed to dietary sources (e.g. fruits, vegetables, and nuts), polyols are derived from aldoses and ketoses through NADPH reduction (Gao et al., 2012). PKC deactivation causes elevated NADPH (Bey et al., 2004), with activation of the polyol pathway, which is NADPH dependent (Wolf et al., 2008). Limited information exists on 5-deoxyribitol, however, in rats, it has been produced from the metabolic degradation of 5-deoxyribose 1-phosphate (dR1P) (Ichihara et al., 1985; Sekowska et al., 2019). In this study, the increased polyols were possibly due to the aforementioned general glycolytic system dysfunction, which correlates with the study of Gao et al. (2012) and Sato et al. (1999). The elevated concentrations of galactose corroborate the increased concentrations of the polyols. Elevated polyols with glucose, mannose, and oleamide, along with decreased urinary sugars (fructose and galactose) have been associated with amplified aldose reductase (AR) activity (Semmo et al., 2015). Naturally, AR has a low affinity for glucose (limiting the conversion of glucose to polyols) (Tanenberg & Donofrio, 2008), however, in high glucose levels/hyperglycaemia conditions, the polyol pathway converts glucose into sugar polyols using aldose reductase and is accompanied by NADPH oxidation to NADP⁺ (Laaksonen & Sen, 2000). Polyol pathway stimulation vis-à-vis elevated polyol levels, could promote oxidative stress/hyperosmotic stress in cells and possibly PKC reactivation as well (Jeng-Miller & Baupal, 2018; Lorenzi, 2007), however reactivation of this wouldn't be possible in the PKC δ knock out mice used in this investigation.

Significantly evaluated levels of *N*-acetyl glucosamine were also detected and have previously been associated with induction of the galactose metabolic pathway (Kamthan et al., 2013), and both metabolites are rate-limiting intermediates in the biosynthesis of glycoproteins and proteoglycans

(Dani & Broadie, 2012). *N*-Acetyl-D-glucosamine is also considered important for the formation of articular cartilage glycosaminoglycans, glycoproteins, and proteoglycans (Dunsmore, 2006; Wagermaier & Fratzl, 2012), hence, its use as a dietary supplement in osteoarthritis therapy (Jain et al., 2016; Kubomura et al., 2017). Furthermore, *N*-acetyl-D-glucosamine is a key component of the extracellular matrix of animal cells, influencing cell signalling through the post-translational modification by glycosylation, of a variety of intracellular proteins, including NF κ B, *c-myc*, and p53 (Konopka, 2012).

The fatty acids as a group were the most significantly dysregulated metabolite class in the study and this indicates the strong role of PKC δ in fatty acid metabolism or the upregulation of β -oxidation as previously described (Greene et al., 2014; Schmitz-Peiffer, 2013; Yang et al., 2013). Various enzymes associated with lipid metabolism (e.g. phosphorylated isoforms of acyl-CoA dehydrogenases) were also significantly altered in a proteomics study done by Mayr et al. (2004) in PKC δ -deficient heart tissue, confirming this finding (Fig. 4). Apart from the aforementioned mechanisms, in the bid to downregulate the elevated concentrations of pyruvate in the PKC δ deficient mice, citramalate can be synthesized from both pyruvate and acetyl CoA, in a reaction catalysed by citramalate synthase (Sugimoto et al., 2021). Thereafter, isopropylmalate isomerase (2-methylmalate hydrolase) converts citramalate to citraconic acid, which was also elevated in our study. Lastly, α -linolenic acid [a n-3 polyunsaturated fatty acid (PUFAs)] functions as an energy substrate when carbohydrate reserves are depleted (Lyudinina et al., 2020). Its involvement in the efficient supply of energy in comparison to other n-3 PUFAs, has also been documented (Baker et al., 2016). α -Linolenic acid was reduced in the PKC δ -deficient mice in our study, suggesting the metabolite serves as an energy substrate, agreeing with the report by Tsukamoto and Sugawara (2018).

Additionally, it is also well described, that under conditions of reduced energy production via glycolysis, elevated branched-chain amino acid (BCAA) catabolism exists, and subsequently ketone body formation (Garrett & Grisham, 2016). As previously mentioned, a PKC δ deficiency has been associated with an inhibition of α -ketoglutarate dehydrogenase (Mayr et al., 2004), which would be expected to result in elevated concentrations of α -ketoglutarate, which would further drive BCAA catabolism (Fig. 4). Confirmation of this in our investigation was the elevated concentrations of α -ketoisocaproic acid (from elevated leucine metabolism in particular). Leucine metabolism is also strongly linked to ketogenesis and cholesterol metabolism, and the elevated concentrations of both β -hydroxybutyric acid and mevalonic acid (Fig. 4), indicate that a PKC δ deficiency not only results in increased β -oxidation, and leucine catabolism, but also ketogenesis

and cholesterol synthesis. Of the 3 cholesterol/steroid intermediates (cholesteryl acetate, 4 α -methylzymosterol, and 17 α -hydroxypregnenolone), cholesteryl acetate (a cholesterol ester synthesized by formal acylation of the hydroxyl group of cholesterol by acetic acid) was the only one of the 3 to be elevated. Cholesteryl esters are the key transport and storage form of cholesterol in lipoproteins and several cell types (Kuivenhoven et al., 1998), and hence the elevated cholesteryl acetate levels indicate that knocking out the PKC δ gene, results in elevated cholesterol transport and storage as opposed to it serving as a substrate for hormone production (Fig. 4), and hence the reduced levels of 4 α -methylzymosterol, and 17 α -hydroxypregnenolone in the PKC δ knock-out mice. This is additionally confirmed by Mayr et al. (2004), showing an elevated activity of Apolipoprotein A1 activity in their PKC δ knock-out model, which is responsible for reverse cholesterol transport.

Two fatty acid amides (FAMS) (oleamide and dodecanamide) with hypocholesterolemic, anti-inflammatory, and anticancer activity (Li et al., 2014; Lo et al., 2001), were down-regulated in the PKC δ deficient mice in this investigation. Whilst, limited information exists on dodecanamide, FAMS generally (e.g. oleamide, and anandamide) exhibit vasorelaxant potentials (Hoi & Hiley, 2006; Hopps et al., 2012), and are reportedly a competitive substrate for the brain enzyme; fatty acid amide hydrolase (FAAH) (Cravatt et al., 1996; Lichtman et al., 2002). FAAH catalyses the hydrolytic degradation of these FAM chemical messengers. Therefore, functional impairment of the hydrolase (suspected hyperactivity) caused the down-regulation of the signalling lipids. Waluk et al. (2014), previously showed that downregulation of oleamide in mice ensued with active FAAH. The mechanism of FAMS synthesis in the body is unclear (Farrell et al., 2012; Mueller & Driscoll, 2009). Some investigations show that FAMS can disrupt the intestinal absorption of cholesterol via the inhibition of the enzyme acyl-CoA:cholesterol acyltransferase (Roth et al., 1992; Xu et al., 2004). Besides this, it's also been reported that FAMS are synthesised following a two-step catalytic metabolism of N-fatty acylglycine, by peptidylamidoglycolate lyase (Farrell et al., 2012; Merkler et al., 1999; Wilcox et al., 1999). Considering this, a deficiency (loss of activity) of this lyase would also result in the downregulation of the FAMS. The upregulation of the monoglycerol (MG); glycerol 1-palmitic acid (1-monopalmitin) here, is consistent with the study by Su et al. (2020). This upregulation signifies dysregulation of monoacylglycerol pathway and monoglyceride lipase deficiency (MGL). MGL influences energy metabolism by hydrolysis of MG into fatty acids and glycerol, and degradation of 2-arachidonoyl glycerol (Taschler et al., 2011; Vujic et al., 2016). Hence, glycerol 1-palmitic acid upregulation would

be towards compensating for energy losses, serving as an alternative substrate for β -oxidation. 1-Monopalmitin is a serum biomarker in obesity and diabetes (Hameed et al., 2020; Park et al., 2015; Taschler et al., 2011), and as a potential salivary biomarker of hepatocellular carcinoma (Hershberger et al., 2021).

Lastly, the largely increased levels of serum palmitic acid, accompanied by the elevations in glycerol 1-palmitic acid, are indicative of a metabolic flux towards phospholipid synthesis, in particular, the glycerophospholipids (Carta et al., 2017), in the PKC δ knock-out model. Considering the functionality of these phospholipids is for regulating membrane permeability (Rajasekaran et al., 2011; Yu & Rasenick, 2012), fat absorption from the intestine (Murota, 2020), elevated mitochondrial function (Hishikawa et al., 2014), cholesterol transport and accumulation in the cells (Blanco & Blanco, 2017; Carta et al., 2017; Castro-Gómez et al., 2015), and preventing fat accumulation in the liver (Carta et al., 2017; Wang et al., 2013), supports the aforementioned observations.

5 Conclusion

Metabolic biomarkers associated with various metabolic disorders, infectious diseases, and many other autoimmune diseases, need to be specific enough to predict the presence of their corresponding conditions and also explain the mechanisms associated with the pathophysiology of the disease. In this study, the metabolite markers that were significantly altered in the PKC δ knock-out mice, accurately coincided with the changes to the specific enzymes detected in the proteomics study done by Mayr et al. (2004), proving the accuracy of not only metabolomics as a standalone technology, but also that of proteomics. That said, the use of multiple omics techniques, does have value, for surety, when it comes to better characterizing a perturbation. From the results of this metabolomics investigation, it is clear that total loss of PKC δ results in an inhibition of glycolysis and the TCA cycle, accompanied by upregulation of the pentose phosphate pathway, fatty acids oxidation, cholesterol transport/storage, single carbon and sulfur-containing amino acid synthesis, branched-chain amino acids (BCAA), ketogenesis, phospholipid synthesis, and cell signalling. The measurement of the dysregulated serum metabolites in this study may represent an additional tool for the early detection and screening of PKC δ -deficiencies.

Acknowledgements We thank the University of Cape Town (UCT) Animal Unit staff, Rodney Lucas for technical assistance, Faried Abbas for maintenance of the BSL3 facility, Nazila Ghodsi, Munadia Ansari, and Fadwah Booley for genotyping of mice and maintenance of the laboratory, respectively. AAA appreciates North-West University for post-doctoral fellowship.

Author contributions DTL, SPP, MO, and FB: helped conceive the project, performed experiments, developed methods, provided equipment for experiments, and analyzed results. AAA: performed extensive literature review, curated data, analyzed results, and created metabolic map. MVR: helped conceive the project, curated data, and statistically analyzed results. All authors contributed to the manuscript, provided intellectual input, and approved it for submission.

Funding No funds, grants, or other support was received.

Declarations

Conflict of interest The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Ethical approval All experiments were carried out following South African National Guidelines and University of Cape Town laboratory animal procedures. The Animal Ethics Committee, Faculty of Health Sciences, University of Cape Town, Cape Town, South Africa, approved the protocol (AEC: 012/036).

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