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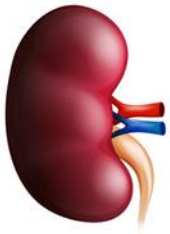

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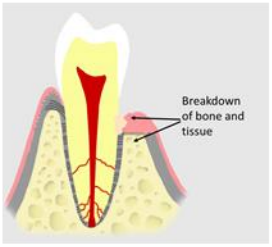

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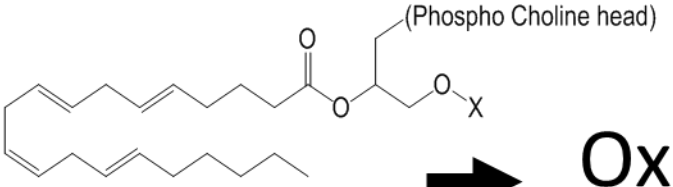
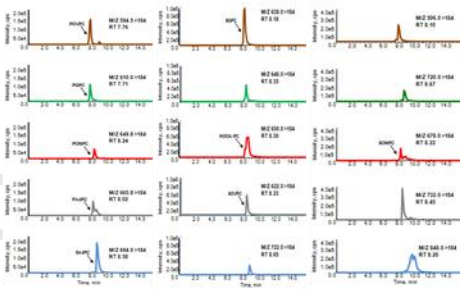


PCs

OxPCs



Mass Spectrometry

Distribution of plasma oxidised phosphocholines in chronic kidney disease and periodontitis as a co-morbidity

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Abstract

Individuals with chronic kidney disease (CKD) and periodontitis as a co-morbidity have a higher mortality rate than individuals with CKD and no periodontitis. The inflammatory burden associated with both diseases contributes to an increased risk of cardiovascular and all-cause mortality. We previously demonstrated that periodontitis is associated with increasing circulating markers of inflammation and oxidative stress. We propose that inflammatory oxidised phosphocholines may contribute to the increased risk of cardiovascular disease in patients with CKD. However, the analysis of oxidised phospholipids has been limited by a lack of authentic standards for absolute quantification. Here, we have developed a comprehensive quantification liquid chromatography-mass spectrometry-based multiple reaction monitoring method for oxidised phospholipids (including some without available authentic species) that enables us to simultaneously measure twelve oxidised phosphocholine species with high levels of sensitivity and specificity. The standard curves for commercial standards 1-palmitoyl-2-glutaryl-*sn*-glycero-3-phosphocholine (PGPC); 1-palmitoyl-2-(9'-oxo-nonanoyl)-*sn*-glycero-3-phosphocholine (PONPC), 1-palmitoyl-2-azelaoyl-sn-glycero-3-phosphocholine (PAzPC) and 1-palmitoyl-2-(5'-oxo-valeroyl)-*sn*-glycero-3-phosphocholine (POVPC), were linear with a correlation coefficient greater than 0.99 for all analytes. The method is reproducible, with intra- and inter-day precision <15%, and accuracy within ±5% of nominal values for all analytes. This method has been successfully applied to investigate oxidised phosphocholine in plasma from CKD patients with and without chronic periodontitis and the data that was obtained has been compared to plasma from healthy controls. Comparative analysis demonstrates altered chain fragmented phosphocholine profiles in the plasma samples of patients with CKD and periodontitis as a co-morbidity compared to healthy controls.

Key words: CKD, periodontitis, oxidised phospholipids, MRM-LC/MS, oxidative stress

Abbreviations

CV	Coefficient of variation
CKD	Chronic kidney disease
dDMPC	1, 2-dimyristoyl-*sn*-glycerol-3-phosphocholine-1,1,2,2-d4-N,N,N-trimethyl-d9
MRM	Multiple reaction monitoring
OxPC	Oxidized phosphocholine
PAPC	1-palmitoyl-2-arachidonyl-sn-glycero-3-phosphorylcholine
PAzPC	1-palmitoyl-2-azelaoyl-sn-glycero-3-phosphocholine
PGPC	1-palmitoyl-2-glutaryl-*sn*-glycero-3-phosphocholine
PL	Phospholipid
PLPC	1-palmitoyl-2-linoleoyl-sn-glycero-3-phosphocholine
PONPC	1-palmitoyl-2-(9'-oxo-nonanoyl)-*sn*-glycero-3-phosphocholine
POVPC	1-palmitoyl-2-(5'-oxo-valeroyl)-*sn*-glycero-3-phosphocholine
PPD	Probing pocket depth
QC	Quality control
ROS	Reactive oxygen species
SAPC	1-stearoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine
SAzPC	1-stearoyl-2-azelaoyl-sn-glycero-3-phosphocholine
SD	Standard deviation
SE	Standard error
SGPC	1-stearoyl-2-glutaryl-*sn*-glycero-3-phosphocholine
SLPC	1-stearoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine
SONPC	1-stearoyl -2-(9'-oxo-nonanoyl)-*sn*-glycero-3-phosphocholine
SOVPC	1-stearoyl-2-(5'-oxo-valeroyl)-*sn*-glycero-3-phosphocholine
TLR	Toll like receptors

1. Introduction

Chronic kidney disease (CKD) affects up to 16% of the population worldwide, increases in prevalence with age and is associated with high morbidity and mortality compared to individuals without CKD [1, 2]. Traditional risk factors for CKD such as age, gender, ethnicity, family history, smoking habits, and socioeconomic status are also associated with diabetes, hypertension, and lipid disorder comorbidities; individually and collectively these contribute to a higher cardiovascular disease morbidity and mortality [3]. Non-traditional risk factors are also associated with poorer CKD outcomes, including inflammation, which is strongly associated with an increased risk of progression to end-stage renal failure and mortality [1]. Identifying and targeting novel, modifiable risk factors that contribute to systemic inflammation in CKD and are causal for poorer clinical outcomes represents an effective strategy for reducing morbidity and mortality in those affected [4].

Periodontitis is the most common chronic inflammatory disease of humans, affecting about 40-50% of the global population and in its most severe form is the sixth most common human disease (11.2%)[5]. The global burden of periodontitis increased from 1990 to 2010 by 57.3% [6]. Indeed, oral diseases, including periodontitis were the leading causes of non-fatal health loss in males and females, determined by years lived with disability, in The Global Burden of Diseases, Injuries, and Risk Factors Study 2017 (GBD 2017). Periodontitis leads to significant elevations in both acute-phase reactants (CRP, IL-6) [5, 7] and oxidative stress biomarkers in plasma [8]. Our previous work demonstrated that CKD patients who are at high risk of progression to end-stage renal disease [9], had a significantly greater prevalence (88%) and severity of periodontitis compared to a local, community dwelling control population (55%) [10]. Using survival analysis carried out in the Third National Health and Nutrition Examination Survey (NHANES III) of the USA and linked mortality data, we demonstrated a strong association between periodontitis and increased mortality in individuals with CKD [10, 11]. Given the existing oxidative stress burden in periodontitis [8], others have analysed the plasma oxidation status of patients with CKD and found that the lipid peroxidation product F2-isoprostane was elevated [12].

The unsaturated fatty acid chains present in sn-1 or sn-2 position of phospholipids (PLs) can undergo either enzymatic (e.g. by lipoxygenases) or non-enzymatic oxidation (by reactive oxygen species; ROS such as the hydroxyl radical, superoxide anion, peroxynitrite, hypochlorite anion and peroxide) to yield oxidised phospholipids (oxPLs) and after release by phospholipases, to form isoprostane species. Oxidative modifications include oxidation of the unsaturated fatty acid chains, intra- and intermolecular arrangements, cyclisation and fragmentation [13].These full-chain oxidised PLs along with chain fragmented PLs may initiate and modulate inflammatory reactions and have been implicated in the pathogenesis of age-related diseases [14, 15]. OxPLs act as lipid mediators of cellular and immune signaling via Toll like receptors (TLR) and are potential biomarkers of disease pathogenesis [14, 16]. Phosphocholine (PC) species with sn-2 palmitoyl or stearoyl moieties comprising; 1-

palmitoyl-2-arachidonyl-sn-glycero-3-phosphorylcholine (PAPC), 1-palmitoyl-2-linoleoyl-sn-glycero-3-phosphocholine (PLPC) 1-stearoyl-2-arachidonoyl-sn-glycero-3-phosphocholine (SAPC) and 1-stearoyl-2-linoleoylphosphatidylcholine (SLPC) were shown to generate several discrete oxidised phospholipids under different conditions [14, 17]. The oxidised phosphocholines (oxPCs) generated have immunogenic activity and act as ligands for scavenger receptors [17]. Oxidised metabolites are rapidly removed in plasma by cellular uptake and detoxified through catabolic activity in the liver [13].

Our understanding of oxPCs, including their concentration in biological fluids, cells and tissues, is emerging with the aid of quantitative lipidomics. It is important to establish reliable and simplified mass spectrometry methods to analyse oxPCs [18]. However, the major challenge in quantitative oxidative lipidomics is the availability of authentic and deuterated standards for the lipids of interest [13, 19]. To mitigate this limitation, we have prepared additional oxidised products of PAPC, PLPC, SAPC and SLPC using Fenton reaction chemistry to expand the panel of oxPCs.

In this paper we describe a mass spectrometry based multiple reaction monitoring (MRM) method that enables measurement of an extensive panel of oxPCs to investigate the plasma oxPC profiles of patients with CKD and CKD with co-morbid periodontitis compared to healthy controls. Using this method, we investigated the hypothesis that CKD and CKD co-morbid with periodontitis have altered oxPC profiles in plasma compared to healthy controls.

2. Materials and methods

2.1 Chemicals

Authentic lipid standards comprising: 1-palmitoyl-2-(5'-oxo-valeroyl)-sn-glycero-3-phosphocholine (POVPC); 1-palmitoyl-2-(9'-oxo-nonanoyl)-*sn*-glycero-3-phosphocholine (PONPC); - 1-palmitoyl-2-glutaryl-*sn*-glycero-3-phosphocholine (PGPC); 1-palmitoyl-2-azelaoyl-sn-glycero-3-phosphocholine (PAzPC), 1-stearoyl-2-arachidonoyl-sn-glycero-3-phosphocholine (SAPC) and 1-stearoyl-2-linoleoylphosphatidylcholine (SLPC) and one deuterated standard; 1, 2-dimyristoyl-*sn*-glycero-3-phosphocholine 1, 1, 2, 2-d_4-N, N, N-trimethyl-d_9 (dDMPC), were purchased from Avanti Polar Lipids (Alabaster, USA). Solvents for lipid extraction and LC-MS of HPLC grade were purchased from Fisher Scientific, Loughborough, UK. All other chemicals were purchased form Sigma Aldrich (Dorset, UK), unless otherwise stated.

2.2 *In vitro* oxidation of SAPC and SLPC and extraction of oxidised lipids

SAPC and SLPC were oxidised with $FeCl_2$ and H_2O_2 (Fenton reaction) according to the method described by Reis *et al*. [20]. Briefly, 100µg of phospholipids were oxidised with 50mM H_2O_2 / 5mM $FeCl_2$ in 1ml solution. The mixture was allowed to react at 37°C in the dark with sonication and occasional vortexing. Oxidation was monitored by electrospray

ionisation mass spectrometry at 24hrs and 48hrs. Products of lipid peroxidation resulted from cleavage of oxygen radicals producing short chain aldehydes, hydroxyaldehydes and dicarboxylic acids. Phospholipid oxidation products were extracted using the modified methyl tert butyl ether (MTBE) method with MTBE/methanol/water (10:3:2.5, v/v/v) containing 50µg/ml of BHT as we previously described [21].

2.3 Plasma samples

Plasma samples were from a randomly selected group of patients with CKD, and who were periodontally healthy (n=13) recruited to the Renal Impairment in Secondary Care (RIISC) study (Ethical approval for this cohort was covered by West Midlands South Birmingham NRES 10/H1207/6) [10]. The RIISC study is an ongoing, prospective cohort study investigating novel risk factors in the progression of CKD. Further plasma samples were collected from patients with CKD and with periodontitis as a comorbidity (n=20), patients with periodontitis without any self-reported illness (n=17), and without periodontitis or CKD (n=20) in the "INSPIRED TRIAL" (Influence of Successful Periodontal Intervention on Renal and Vascular Systems in patients with Chronic Kidney Disease-A Pilot Interventional Randomised Controlled Trial (INSPIRED). Ethical approval was by the National Research Ethics Service, West Midlands - The Black Country, ref 15/WM/0006) [22]. The INSPIRED trial is an ongoing pilot randomised control trial investigating the effect of periodontal treatment on the cardio-renal health of patients with CKD. The patient demographics are shown in **table 1**. Blood samples were collected in the EDTA tubes and plasma was separated by centrifugation for 10 min at 3000×g at 4 °C within 2 hours of withdrawal and frozen at -80^{o}C until further analysis.

Inclusion criteria for all participants were: patients aged ≥18 years; able to provide consent to participate in the trial. Additional inclusion criteria for patients with CKD, for patients with CKD and periodontitis, and for systemically healthy patients with periodontitis are detailed as supplementary material.

Exclusion criteria for all participants were: patients not meeting the inclusion criteria; or unable to provide informed consent. Additional exclusion criteria for patients with CKD, for systemically healthy patients with periodontitis, and for systemically healthy patients without periodontitis are detailed as supplementary material.

For the purposes of this study, periodontitis is defined as those with a cumulative probing depth ≥30 mm. This is the sum of the deepest probing pocket per tooth, excluding probing depths <5 mm. This represents generalised moderate-severe periodontitis (periodontal health=1).

2.4 MTBE lipid extraction

Lipids were extracted from 10µl of individual plasma samples spiked with 200ng of dDMPC internal standard by MTBE method as previously reported [21]. The dried lipid extracts were reconstituted in 200µl methanol immediately prior to injection. Lipid extracts (10µl in 100% methanol) were injected for separation and analysis by LC-MS/MS.

2.5 Phospholipid quantification

For the purpose of phospholipid assay, lipids were extracted from 10 µl of plasma by the Folch protocol [23]. Phospholipid content of lipid extracts was quantified by spectrophotometry measurement of inorganic phosphorous (λ=797 nm) using a micromethod adapted from Rouser et al. [24], as described before [25].

2.6 Liquid chromatography-mass spectrometry (LC-MS/MS) analysis of lipids

Our previously described MRM-MS method [21] was adapted using the triple quadrupole mass spectrometer (QTRAP 5500, AB Sciex UK Ltd. Warrington) equipped with a standard-ESI source, operated in a positive ion mode with an ionisation voltage of 5kV, entrance potential of 10 V, and ion source temperature of 400 °C, collision gas nitrogen 20V and ion source gas 25V. For optimisation of ESI and MS parameters, standard mixtures containing 1ng/µl of each oxPC species was infused directly to the ESI source through an integrated syringe pump (Harvard apparatus) with a flow rate of 20µl/min. Lipid extracts were separated on a Luna Omega C18 column (internal diameter 2.1mm, column length 50mm, particle size 3µm, Phenomenex, Macclesfield, Cheshire, UK) with column guard installed. Mobile phases consisted of (A) 10 mM ammonium formate in methanol: water: formic acid (20:80:0.1, v/v/v) and (B) 2 mM ammonium formate in 2-propanol: methanol: formic acid (90:10:0.1, v/v/v) at 60 °C. Flow rate was maintained at 200 µl/min with the gradient as follows: 30% B from 0 to 1 min, 30–100% B from 1 to 6 min, 100% B 6–13min, 100–30% B from 13 to 14 min, 30% B 14–24 min. Analytical samples (10µl) were injected by the autosampler at 10^{o}C with a constant flow of 200µl/min. Measurement and data analyses were performed in triplicate using Analyst software (version 1.6.2). Peak area of ions (m/z value) generating the PC-specific fragment at m/z 184, ~~Peak area of the PC-specific fragment ion (*m/z* 184)~~ which corresponds to the cleaved phosphocholine polar head was used for the quantification of oxPCs. The peak areas were normalised to the deuterated internal standard, dDMPC for each sample. Analyte concentrations in each sample were calculated using standard curves against PGPC (10-2000pg/µL), PONPC (10-1000pg/µL), PAzPC (10-1000pg/µL) and POVPC (10-500pg/µL).

2.7 Determination of linear dynamic range, limit of detection/quantification of OxPCs

The method developed was for relative quantification, however, we prepared an external calibration using authentic standards (POVPC, PGPC, PONPC and PAzPC) to determine their linear range, lower limit of detection (LOD) and lower limit of quantification (LOQ). Calibration curves were produced by injecting 6 authentic solutions between 10-2000pg/µl

(10, 50, 100, 500, 1000, 2000pg/µl). The concentration ranges selected for the calibration curves were based on preliminary data on the dynamic ranges. LOD and LOQ were calculated using the blank determination method (n=20) from the International Conference on Harmonisation (ICH) guidelines as described previously [26]. LOD and LOQ are expressed as the analyte concentration corresponding to the sample blank value plus three and ten standard deviations, respectively.

2.8 Evaluation of method reproducibility with intra-day and inter-day assays

Quality control (QC) plasma samples (n=3) were analysed to evaluate the performance of the MS response over time, namely sensitivity of the method and reproducibility. Intra-day reproducibility was obtained from six analyses run consecutively while inter-day reproducibility was obtained from ten analyses run on different days over 1 month.

2.9 Estimation of precision, accuracy, recovery and matrix effect for MRM method

The method recommended by Matuszewski *et al*. [27] was adapted for recovery and matrix effect analysis. The recovery percentages were estimated by comparing the peak areas of four concentrations (100-1000pg/ml) of POVPC, PONPC, PGPC and PAzPC standards injected in methanol to the same phosphocholine standards spiked and extracted from plasma. The accuracy of the assay was determined by six replicates of QC samples at four concentrations during a single analytical run as described by Partani et al.,[28].

2.10 Statistical analysis

Multivariable regression models were constructed with measures of oxidative stress as dependent variables and clinical health parameters (healthy/ periodontitis only/ CKD only/ CKD and periodontitis), age ~~and gender~~ sex (male/female), smoking status (current smoker, ex-smoker, never smoker) and diabetes status (yes/no) as independent variables. All analyses were carried out using Stata/IC version 15.1 (StataCorp LLC). Significance was accepted as $p<0.05$.

3. Results

3.1 Optimisation of LC-MS/MS identification of OxPC panel

Four commercially available oxidised phospholipid standards, two native lipid standards oxidised through Fenton chemistry and one deuterated standard were employed to develop this targeted LC-MS/MS method. First, each commercially available standard was individually injected to identify optimal fragments based on their abundance for MRM analysis. The precursor ion scans were performed between 100 *m/z* to 1000 *m/z* mass range with ESI-MS in a positive ion mode. The ~~At least three diagnostic~~ product ion, 184 *m/z* was selected for each analyte, and collision energy, depolarisation potential and exit potential was optimised for each transition pair (**Table 2**). The product ion spectra of $[MH]^+$ ions for POVPC, PGPC, PONPC and PAzPC standards showed an abundant product ion at *m/z* 184, which corresponds to the polar head group of phosphocholines ($[H_2PO_4(CH_2)_2N(CH_3)_3]^+$).

Next, MRM parameters were optimised to detect commercially available non-oxidised lipids; PAPC, PLPC, SAPC and SLPC (**Figure 1**). To overcome the lack of standards for the identification of oxidised phospholipids, we generated a panel of oxidation products using PAPC, PLPC, SAPC and SLPC through the Fenton reaction between H_2O_2 and $FeCl_2$ as previously described [20]. A range of oxidised phosphocholine species originating from PAPC (*m/z* 782.7), PLPC (*m/z* 758.7), SAPC (*m/z* 810.6) and SLPC (*m/z* 786.6) was monitored in a precursor ion scan for the phospholipid head group, *m/z* 184. Aligning with previous studies [14], chain-shortened, oxidised forms of SAPC were identified as 1-steroyl-2-(5'-oxo-valeroyl)-sn-glycero-3-phosphocholine; SOVPC (*m/z* 622), 1-steroyl-2-glutaryl-sn-glycero-3-phosphocholine; SGPC (*m/z* 638). Chain-fragmented oxidised SLPC ions were identified as 1-steroyl-2-(9'-oxo-nonanoyl)-sn-glycero-3-phosphocholine; SONPC (*m/z* 678), 1-steroyl-2-azelaoyl-sn-glycero-3-phosphocholine; SAzPC (*m/z* 694). Oxidised ions of PAPC were identified as 1-palmitoyl-2-(5-hydroxy-8-oxo-6-octenoyl)-sn-glycero-3-phosphocholine); HOOA-PC (*m/z* 648) and 1-palmitoyl-2-(5-keto-8-oxo-6-octenoic acid)-sn-glycero-3-phosphocholine; KOOA-PC (*m/z* 648). Chain-fragmented oxidised PLPC ions were identified as 1-palmitoyl-2-(4-keto-dodec-3-enadioyl)-sn-glycero-3-phosphocholine; KDdiA-PPC (*m/z* 720), and 1-palmitoyl-2-(9-hydroxy-11-carboxy-undec-6-enoyl)-sn-glycero-3-phosphocholine; HDdiA-PC (*m/z* 722) (**Supplementary Figure 1 and Supplementary table 1**). All oxidized lipids were separated from non-oxidized lipids by reverse-phase column chromatography (**Figure 2**).

3.2 Linear dynamic range, Intra-day and inter-day analyses of MRM-MS method for OxPCs

To estimate the sensitivity of the method, we estimated the linearity, LOD and LOQ of the four commercially available OxPCs. The LOD and LOQ of the standard solutions were in the range 0.25 - 16pg and 0.5 – 37pg respectively with all correlation coefficients greater than 0.99 **(Supplementary table 2).** Intra-day analyses were six consecutive analyses on the same day of QC plasma sample while the inter-day analyses were ten non-consecutive analyses over one month of the QC sample. The QC results show that the LC method is precise and reproducible with an intra-day assay %CV of 4-8 and the inter-day %CV of 6-14. (**Supplementary table 3**)

3.3 Percentage recovery, matrix effect and accuracy of oxPCs standards

Supplementary table 4 shows the average and the CV of oxPC recovery with and without matrix; percentages are in the range 73-91% and 90-99% respectively with CVs <15%. The accuracy was also 97-102%. The precision and accuracy values were satisfactory. Precision is required to be within ±15% and accuracy between 85- 115% [24].

3.4 Analysis of plasma phospholipids and oxidised phosphocholines in CKD patients with or without periodontitis.

Plasma phospholipids were analysed for phospholipid as described. Total phospholipid concentration in plasma was not different between healthy and disease groups (**Figure 3**). The patient demographics are described in Table 1. The distribution of patients and healthy controls were not different for weight but significant changes were observed for body mass index (BMI), age and sex distribution between groups. The percentage of smokers were not significantly different between groups but there was a significant difference observed for ex-smokers between healthy and disease groups. The co-morbidity of diabetes was also high in patients with CKD. To account for these differences, multivariable regression models were constructed with measures of oxidative stress as dependent variables and clinical health parameters (healthy/ periodontitis only/ CKD only/ CKD and periodontitis), BMI, age, sex, smoking status and diabetes as independent variables. Significance was accepted as $p<0.05$.

Individual estimates of specific OxPC showed significant differences (**Figure 4 and Table 3**) between healthy and disease groups. Compared to healthy subjects, patients with periodontitis, CKD and with both diseases had significantly higher levels of HDdiA-PC ($P<0.005$; Figure 4L). SAzPC levels were significantly higher only in CKD group compared to healthy subjects ($P=0.001$; Figure 4H). In periodontitis; POVPC ($P=0.016$; Figure 4A), PONPC ($P=0.029$; Figure 4C) and SOVPC ($P=0.043$; Figure 4E) were significantly higher compared to

the healthy group and KOOA-PC was significantly higher in patients with both diseases than in healthy controls ($P<0.001$; Figure 4J).

Conversely, some oxPCs were found be significantly lower in CKD plasmas; SGPC ($P=0.026$; Figure 4F), and in the presence of both diseases; PAzPC ($P=0.047$; Figure 4D).

4. Discussion

This study has focused on developing a method to simultaneously quantify, with high sensitivity, a panel of 12 oxPCs in biological fluids and applying the method to plasma samples from patients with CKD and chronic periodontitis as a comorbidity for the first time. We optimised chromatography conditions and included in-house oxidised lipid standards to cover a panel of oxPCs. With the use of the Lunar Omega polar C18 column (Phenomenex, UK), this method achieved high selectivity for polar oxidised phosphocholine lipids without diminishing important non-polar interactions. By combining column characteristics with the solvent system, the current method decreased the retention time of the analytes from 44 minutes to 24 minutes compared to our previously published method [21].

We and others have shown the presence of systemic inflammatory burden in periodontitis, induced by periodontal bacteraemia activating peripheral blood neutrophils to release ROS (6). Thus, a measure of periodontitis that captures this infectious-inflammatory exposure is required. The measure used, cumulative probing depth, can be readily calculated from routinely collected periodontal measurements and approximates the extent of the periodontal wound. It can be used to differentiate measures of previous disease experience, such as recession and clinical attachment loss, which may not influence the patient's current systemic health. The current, commonly used case definitions of periodontitis may not be able to achieve this, as has been reported by other researchers [29, 30]. Cumulative probing depth also accounts for tooth loss in a way that other measures, such as mean probing pocket depth (PPD), do not.

Dyslipidaemia and disturbances in lipid metabolism are reported previously in patients with CKD who are pre-dialysis and who are receiving long-term renal replacement therapy with haemodialysis [31, 32]. Many other studies have investigated the lipid parameters including total cholesterol, high density lipoprotein cholesterol (HDL-C), Low density cholesterol (LDL-C), and triglycerides in CKD. These studies indicated that dyslipidemia can increase the risk of atherosclerotic cardiovascular diseases in patients with CKD [32, 33]. We have previously demonstrated that dyslipidaemia is associated with oxidative stress in diabetes patients with periodontitis, relative to people with diabetes alone [34]. We have also shown the damaging effects of oxidised lipids (from oxLDL) including oxidised cholesterol (27-hydroxycholesterol), using an *in vitro* neuronal cell culture system [35] and on endothelial cells [36].

Plasma lipoproteins carry hydrophobic and water insoluble lipids to be delivered to tissue and cells. Phospholipids residing at the surface layer of lipoproteins constituting ~20–25% of the particle by weight [37] are primary targets of oxidative damage with formation of oxidised phospholipids. Primary oxidation products generated from the most abundant molecular species of PCs (PAPC, SLPC, PLPC and SAPC) are present in LDL [38, 39]. Reis et al., previously compared the molecular lipidomic profile of LDL in patients with non-diabetic, advanced renal disease to that of age-matched controls [40]. The study indicated significantly lower levels ~~concentration~~s of PCs in LDL particles. A study conducted by Yang et al., described changes to the urinary phospholipid profile in CKD patients [41]. Collectively, this work suggested an important link between phospholipid profiles and CKD.

Based on these measures, we sought to investigate whether increased oxidative stress may have contributed to differences in the circulating profile of phosphocholines in patients with CKD with or without periodontitis. Elevated peripheral oxidative stress has been reported in periodontitis, arising from peripheral blood neutrophil activation by periodontal bacteraemia, including extracellular release of reactive oxygen species [42]. While the damage to macromolecules, including phospholipids are inevitable, so far none of the studies have investigated oxidised phospholipid profiles in periodontitis. To our knowledge, this is the first time fragmented oxPCs have been analysed in the plasma of patients with CKD with periodontitis as a comorbid inflammatory disease. This paper has focused on the oxidative modification to PAPC, PLPC, SAPC and SLPC classes of lipids.

HDdiA-PC levels were significantly increased in all patient groups tested and the keto acid analogue, KOOA-PC was significantly higher in the presence of periodontitis or comorbid with CKD. These oxPCs share a common structural moiety possessing sn-2 esterified γ-hydroxy (or oxo)-α,β unsaturated carbonyl-containing fatty acids. This suggests that they represent limited oxidation before chain fragmentation to form shorter fatty acid moieties, which may relate to the concentration or nature of radical species involved in oxidation. Eugene et al., described the generation of this family of truncated PCs using unilamellar vesicles in the presence of the myeloperoxidase (MPO)-H_2O_2-NO_2^- system [43]. MPO is most abundantly expressed in neutrophil granules and released either into the phagosome or the extracellular space where it catalyses the conversion of H_2O_2 and chloride in to hypochlorous acid. Therefore, it is possible that neutrophil hyperactivity in chronic periodontitis [42, 44] has a key role in generating this family of PCs.

PCs with an acyl chain at the sn-2 position are known to have high affinity for the macrophage scavenger receptor, CD36 [45]. Chain fragmented oxPCs, such as POVPC, were less effective in binding to the CD36 receptor. Moreover, altering the sn-2 esterified group by repositioning of the γ –hydroxy moiety by one methylene group or completely losing γ –hydroxyl moiety significantly reduced CD36 binding ability. The work by Eugene et al. highlighted the highly conserved nature of the critical structural elements required for oxidised phospholipids to serve as ligands for CD36 and uptake by macrophages [46]. It is

established that LDL loaded macrophages can lead to exacerbation of inflammation and involvement in cardiovascular disease pathologies via foam cell formation. The altered oxPC profile observed here may contribute to increased risk for CVD, as observed in patients comorbid for CKD and periodontitis, due to altered clearance by CD36 and binding to pro-inflammatory TLRs [16].

The biological activity of circulating oxPCs is extensive and has been reviewed previously [13, 17]. Using an in vitro approach, Gargalovic et al., have shown that oxPCs at non-toxic concentrations (50 μg/ml) can regulate >1000 genes in endothelial cells [47]. OxPAPC disrupts endothelial barrier properties and activates both pro- and anti-inflammatory pathways [48]. OxPAPC altered endothelial transcriptome analysis revealed the complexity of various regulatory pathways [48]. Using a systems level network approach, Hitzel et al., described oxPC regulated amino acid metabolism in endothelial cells [49]. They further demonstrated that oxPAPC induces a gene network regulating serine-glycine metabolism with the mitochondrial methylenetetrahydrofolate dehydrogenase/cyclohydrolase (MTHFD2), which is active in atherosclerotic plaque material with implications ~~in~~ for cardiovascular disease.

Taken together, this work highlights the importance of accurate measurement of oxPCs within biological fluids and possible implications of disease comorbidities on phosphocholine profile.

5. Conclusion

We have developed a quantitative oxPC lipidomic method for application in plasma analysis. This sensitive, accurate and improved method is able to detect differences between healthy people and patients with oxidative stress related diseases. The signature of OxPC found in our study of CKD, with or without periodontal comorbidity, discriminated between the two conditions. This study has potential to help understand any role of oxPCs in the complications of CKD and indicates their potential use as biomarkers for diagnosis, prognosis and treatment.

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Declarations of interest: none

7. References

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618 **Figures**

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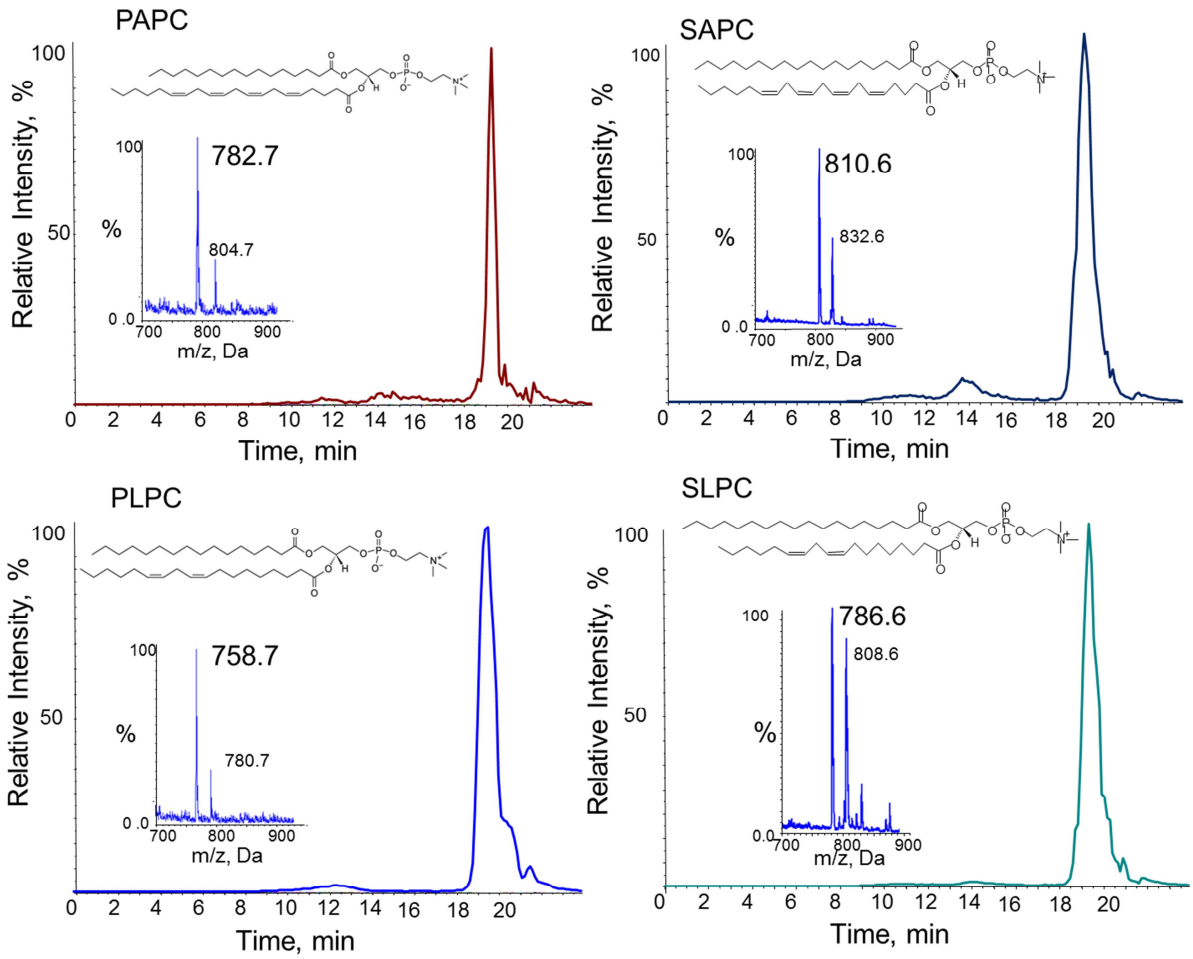

620

621 **Figure 1: Chromatographic separation of native PAPC, SAPC, PLPC and SLPC standards.**
622 Native PAPC, SAPC, PLPC and SLPC in a positive ion mode revealed single chromatographic
623 peaks that corresponded to the protonated molecule at *m/z* 782.7, *m/z* 810.6, *m/z* 758.7
624 and *m/z* 786.6 respectively; with their respective spectra in the inserts. Ions observed in LC-
625 MS spectra at *m/z* 804.7, *m/z* 832.6, *m/z*780.7 and *m/z* 808.6 corresponded to sodiated
626 adducts ($[MNa]^+$) of each analyte.

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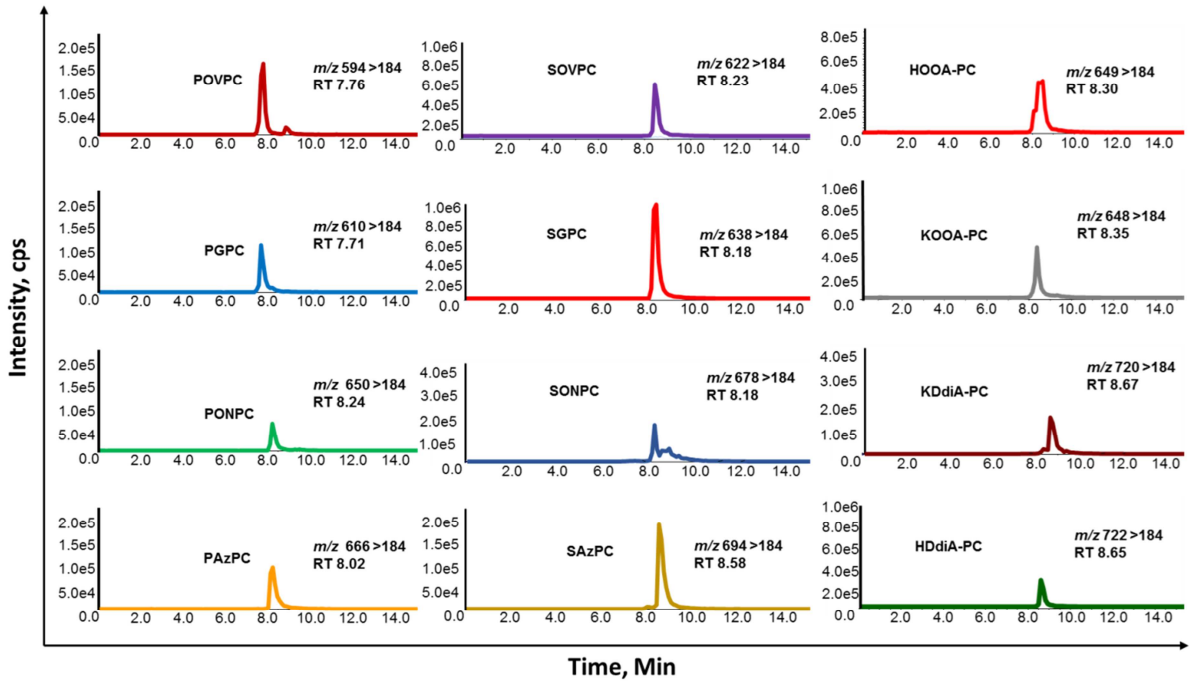
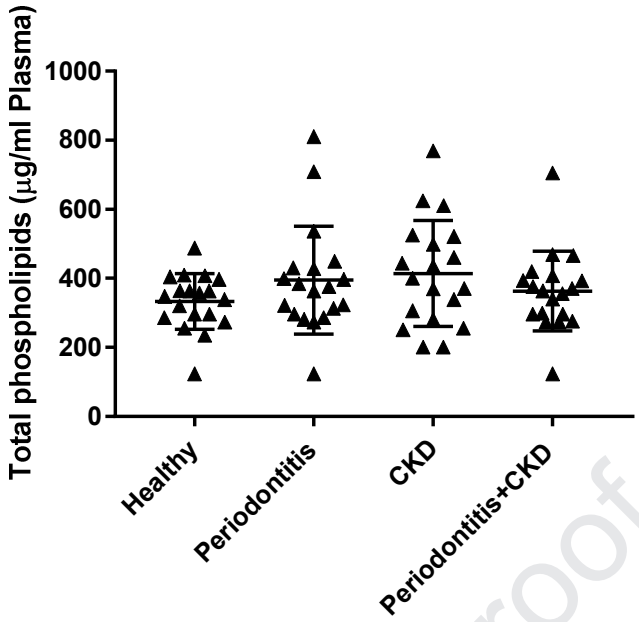


Figure 2: Extracted ion chromatograms (XIC) of individual molecular species of four commercial synthetic standards and eight chain fragmented oxPCs. MRM method developed for 12 oxPCs, which consist of four commercially available standards (POVPC, PONPC, PGPC, PAzPC) and eight iron oxidised products of SAPC and SLPC lipids; namely SOVPC (1-stearoyl-2-(5'-oxo-valeroyl)-*sn*-glycero-3-phosphocholine), SGPC (1-stearoyl-2-glutaryl-*sn*-glycero-3-phosphocholine), SONPC (1-stearoyl -2-(9'-oxo-nonanoyl)-*sn*-glycero-3-phosphocholine) SAzPC (1-stearoyl-2-azelaoyl-sn-glycero-3-phosphocholine), HOOA-PC (1-palmitoyl-2-(5-hydroxy-8-oxo-6-octenoyl)-sn-glycero-3-phosphocholine), KOOA-PC (1-palmitoyl-2-(5-hydroxy-8-oxo-6-octenoic acid)-sn-glycero-3-phosphocholine), KDdiA-PPC (1-palmitoyl-2-(4-keto-dodec-3-enadioyl)-sn-glycero-3-phosphocholine, and HDdiA-PC (1-palmitoyl-2-(9-hydroxy-11-carboxy-undec-6-enoyl)-sn-glycero-3-phosphocholine)



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645 **Figure 3: Total phospholipid content in disease groups.**

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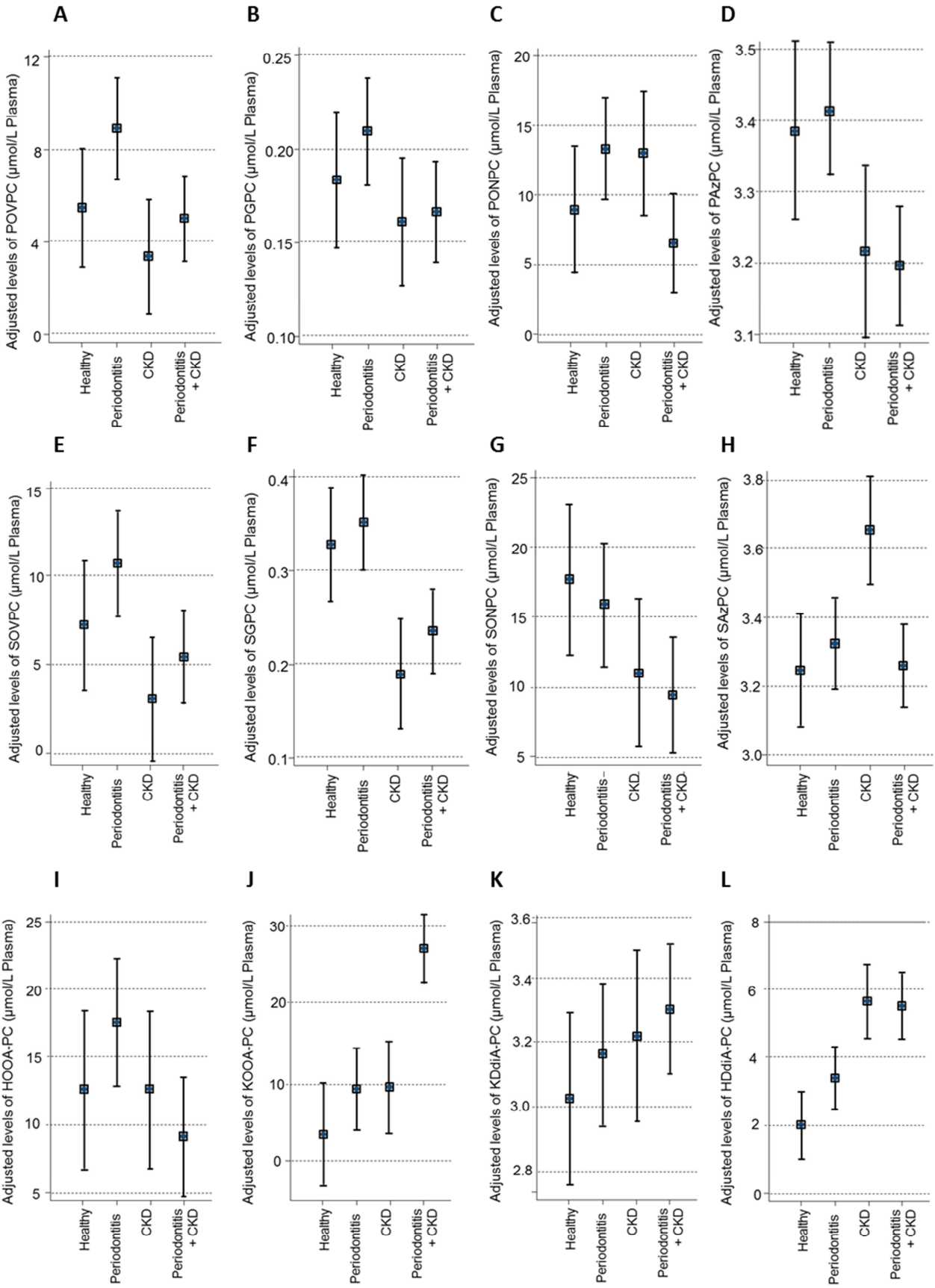
A
Adjusted levels of POVPC (µmol/L Plasma)
12
8
4
0
B
Adjusted levels of PGPC (µmol/L Plasma)
0.25
0.20
0.15
0.10
C
Adjusted levels of PONPC (µmol/L Plasma)
20
15
10
5
0
D
Adjusted levels of PAzPC (µmol/L Plasma)
3.5
3.4
3.3
3.2
3.1
E
Adjusted levels of SOVPC (µmol/L Plasma)
15
10
5
0
F
Adjusted levels of SGPC (µmol/L Plasma)
0.4
0.3
0.2
0.1
G
Adjusted levels of SONPC (µmol/L Plasma)
25
20
15
10
5
H
Adjusted levels of SAzPC (µmol/L Plasma)
3.8
3.6
3.4
3.2
3.0
I
Adjusted levels of HOOA-PC (µmol/L Plasma)
25
20
15
10
5
J
Adjusted levels of KOOA-PC (µmol/L Plasma)
30
20
10
0
K
Adjusted levels of KDdiA-PC (µmol/L Plasma)
3.6
3.4
3.2
3.0
2.8
L
Adjusted levels of HDdiA-PC (µmol/L Plasma)
8
6
4
2
0
Healthy
Periodontitis
CKD
Periodontitis + CKD

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Figure 4. OxPC concentrations (µmol/L plasma ± SEM) measured in plasma from healthy control, patients with periodontitis, patients with CKD and patients with CKD comorbid with periodontitis.

Tables

Table 1: Demographics of patients and healthy control individuals. Data presented is mean (SD) unless otherwise stated

	Healthy control (n=20)	CKD (n=13)	Periodontitis (n=17)	CKD and periodontitis (n=20)	P<0.05
Weight (Kg)	74 (17)	78 (19)	79 (15)	87 (23)	No
BMI (Kg/m^2)	25 (4)	29 (6)	27 (4)	31 (8)	Yes
Age (years)	37 (9)	74 (13)	48 (5)	62 (9)	Yes
Gender (% female)	60	15	59	30	Yes
Periodontal Health	0	0	1	1	
Current smokers (%)	5	10	12	10	No
Ex-smokers (%)	10	65	59	30	Yes
Diabetic (%)	0	40	0	45	Yes

Table 2: Selected multiple MRM parameters (Q1/Q3 transition pair, declustering potential (DP), collision energy (CE), exit quadrupole potential (CXP), retention times (min) used in the analysis

Analyte	MRM transitions	Dwell Time (ms)	DP (V)	CE (V)	CXP (V)	Retention time (min)
POVPC	594.5/184.0	100	96	33	18	7.76

PGPC	610.0/184.0	100	10	37	26	7.71
PONPC	650.0/184.0	100	10	39	14	8.24
PAzPC	666.0/184.0	100	10	37	16	8.02
SOVPC	622.0/184.0	100	26	45	18	8.23
SGPC	638.0/184.0	100	16	35	10	8.18
SONPC	678.0/184.0	100	16	43	18	8.97
SAzPC	694.0/184.0	100	21	37	24	8.58
HOOA-PC	649.0/184.0	100	31	41	10	8.30
KOOA-PC	648.0/184.0	100	26	41	24	8.35
KDdiA-PC	720.0/184.0	100	6.0	45	16	8.67
HDdiA-PC	722.0/184.0	100	21	37	28	8.65
DMPC (d13)	691.0/481.0	100	96	38	12	9.53

Table 3: Multivariable regression analysis of oxPCs between disease groups. Groups were analysed with measures of oxidative stress as dependent variables and clinical health parameters (healthy/ periodontitis only/ CKD only/ CKD and periodontitis), age and sex as independent variables. Significance was accepted as $p<0.05$.

	POVPC	SOVPC	HOOA-PC	PONPC	SONPC	KOOA-PC	PGPC	SGPC	HDdiA-PC	PAzPC	SAzpc	KDdiA-PC
Perio vs Healthy	0.016	0.043	0.083	0.029	0.526	0.052	0.096	0.331	0.005	0.848	0.238	0.372
CKD vs Healthy	0.653	0.376	0.577	0.104	0.195	0.162	0.909	0.026	0.000	0.112	0.001	0.266
CKD+Perio vs Healthy	0.760	0.788	0.946	0.864	0.061	0.000	0.969	0.065	0.000	0.047	0.595	0.051
CKD vs Perio	0.010	0.006	0.445	0.851	0.281	0.932	0.109	0.001	0.003	0.034	0.002	0.529
CKD+Perio vs Perio	0.049	0.025	0.091	0.027	0.106	0.000	0.095	0.003	0.009	0.013	0.623	0.152
CKD+Perio vs CKD	0.240	0.307	0.335	0.006	0.618	0.000	0.895	0.250	0.604	0.889	0.000	0.402

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Highlights:

- We describe a targeted mass spectrometry method to simultaneously quantify an expanded panel of 12 oxidised phosphocholines in plasma.
- This method reproducibly and sensitively quantified oxidised phosphocholines in the individuals with chronic kidney disease and with periodontitis as a co-morbidity.
- Patients with chronic kidney disease and periodontitis as a co-morbidity, have altered oxidised phosphocholine profiles in plasma compared to healthy controls.