Oxidised phospholipids as biomarkers in human disease

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Summary

Oxidised phospholipids (OxPLs) are generated from (poly)unsaturated diacyl- and alk(en)ylacyl glycerophospholipids under conditions of oxidative stress. OxPLs exert a wide variety of biological effects on diverse cell types in vitro and in vivo and are thought to play a role in the development of several chronic diseases including atherosclerosis, a classical lipid-associated and inflammatory disorder. OxPLs are recognised as culprit molecular components responsible for the pathophysiological actions of oxidised low-density lipoproteins. There is growing interest in the potential use of OxPLs as biomarkers of human pathologies. Here we offer a brief overview of current detection methods and knowledge on relationships between levels of circulating OxPLs and disease progression, with particular emphasis on cardiovascular disease.

Key words: Oxidised phospholipids; biomarkers; human pathologies; cardiovascular disease

Structure and generation of oxidised phospholipids

Oxidative stress is a hallmark of many pathological states. Among various types of biomolecules lipids are particularly susceptible to oxidation due to the presence of unsaturated double bonds from which hydrogen can be easily abstracted by oxidants. Phospholipids (PLs) are a major class of polar lipids that are abundantly present within cell membranes and the outer shell of lipoprotein particles. Glycerophospholipids, which are the most abundant subclass of PLs, contain a glycerol backbone, two fatty acid residues and a polar head group (fig. 1). The majority of glycerophospholipids in mammalian tissues contain phosphatidylcholine as a head group, while phosphoethanolamine or phosphatidylserine represent less abundant classes, which, however, are enriched in some tissues such as brain [1]. Polyunsaturated fatty acids in the sn-2 position of the glycerol moiety of PLs are the major target for oxidation. Oxidative attack on polyunsaturated fatty acids results in generation of multiple fragmented or non-fragmented end products with various combinations of functional oxy groups. These products can exert variable effects on cells by modulating activity of intracellular signal transduction and gene expression mechanisms, forming covalent or non-covalent complexes with proteins, inducing cellular stress and apoptosis and further stimulating ROS production [2]. Moreover, newly formed oxidation epitopes on lipid mo-
Molecules are targets for adaptive and innate immune responses which significantly contribute to pathological conditions characterised by chronic inflammation [3].

Methods for detection of circulating oxidised phospholipids

Quantification of oxidised phospholipids (OxPLs) in biological samples is difficult due both to their low concentrations as compared to non-oxidised precursors and the very wide range of structurally different oxidation products. The most sensitive and powerful method for OxPL analysis is mass spectrometry [4, 5]. Development of soft ionisation procedures such as electrospray ionisation mass spectrometry (ESI-MS) or atmospheric pressure chemical ionisation mass spectrometry (APCI-MS) enabled sensitive and efficient metabolic profiling of lipids present in a variety of biological samples (tissues and fluids) including atherosclerotic plaque, brain, plasma and cerebrospinal fluid. However, large-scale mass spectrometry analysis of clinical samples remains a challenge due to the complexity and high costs of the technique. The bulk of existing clinical data on OxPL levels in human disease has been obtained using immunological methods.

Two monoclonal antibodies (mAb) against OxPLs (DLH3 and E06) have been used in clinical studies. DLH3 was generated by immunising mice with a homogenate of human atheroma [6]. A limitation of DLH3-based ELISA is that low density lipoprotein (LDL) fractions have to be isolated from plasma, which makes large-scale screening impractical. In contrast, mAb E06 raised from B-cell clones of apoE-deficient mice [7, 8] is exploited for a sandwich ELISA in which LDL particles are captured directly from plasma by another mAb (MB47) recognising apolipoprotein B-100 (apoB-100) and oxidation-generated epitopes are detected with biotinylated E06 followed by chemiluminescence-based detection; this sandwich ELISA has been used for the majority of clinical investigations on circulating OxPLs. Both DLH3 and E06 antibodies recognise the oxidised, but not native, phosphatidylcholine moiety on PLs, although the exact structures of their respective target epitopes are not characterised. Apart from OxPL present on oxidised LDL (OxLDL), E06 can bind to apoptotic cells which express oxidised phosphatidylcholine-containing epitopes on their surface due to oxidative stress [9] and to phosphocholine present in the capsular polysaccharide of many bacteria (e.g., Streptococcus pneumonia) [10]. There are also other commercially available OxLDL-recognising monoclonal antibodies. One example is ML25 which binds to malondialdehyde-modified LDL (MDA-LDL) and is often used in combination with anti-apoB-100 antibody to measure MDA-LDL in serum [11]. However, the epitopes of these antibodies contain covalent adducts of apoB-100 with small molecules generated by non-enzymatic peroxidation of all sorts of esterified and non-esterified fatty acids (e.g., MDA or 4-hydroxynonenal). Therefore these mAbs are not specific for OxPLs and will not be discussed in this review.

Oxidised phospholipids as biomarkers in cardiovascular disease

Proatherogenic effects of OxPLs

Oxidative stress and oxidation of lipids are held to be decisive events in progression of atherosclerosis and its clinical complications. Initial studies on involvement of oxidised lipids in atherogenesis demonstrated that avid OxLDL uptake mediated mostly by macrophage scavenger receptors SR-A and CD36 promoted lipid accumulation in macrophages and formation of foam cells in atherosclerotic plaques [12, 13]. Today it is recognised that OxLDL and its major active factor OxPLs can elicit multiple proatherogenic effects by acting on several different cell types in blood and the vascular wall [14] (fig. 2). The role of OxPLs in atherosclerosis is supported by studies demonstrating the presence of OxPLs in atherosclerotic vessels of hypercholesterolaemic animal models [15] and in human lesions. Various OxPL species have been documented at different stages of atherosclerosis in different areas of plaques including oxidatively fragmented phospholipid species containing saturated and unsaturated truncated residues, phospholipid-esterified isoprostanes, phospholipid hydroperoxides and others [2]. Amounts of OxPLs increase proportionally with plaque burden and are specifically associated with unstable and ruptured advanced plaques [16]. OxPLs significantly contribute to inflammation in diseased vessels both by inducing expression of proinflammatory cytokines and adhesion molecules on vascular endothelial cells and promoting monocyte adhesion, and by acting directly on leukocytes [17]. Other activities of OxPLs relevant to initiation, progression and development of complications of atherosclerosis include stimulation of ROS production, attenuation of endothelial-dependent vasorelaxation, induction of phenotypic modulation and migration of smooth muscle cells, enhancement of thrombogenic activity of endothelial cells, activation of platelets, induction of smooth muscle cell apoptosis and stimulation of vessel calcification. Activation of intraplaque angiogenesis and increased production of metalloproteinases by OxPLs contributes to destabilisation of coronary plaques, predisposing them to rupture and causing thrombosis and acute

![Figure 2](https://example.com/figure2.png)

The role of OxPLs in the pathophysiology of atherosclerosis.
coronary syndromes. OxPLs can modulate functions of dendritic cells and T-lymphocytes and thus may influence the outcome of adaptive immune reactions. Under certain conditions OxPLs may also stimulate tissue-protective processes via upregulation of stress response genes, attenuation of inflammation and maintenance of the endothelial barrier function [2].

Clinical studies on biomarker value of circulating OxPLs

Since pathological effects of OxLDL and OxPLs have been investigated most extensively in the context of atherosclerosis, the majority of studies aimed at the evaluation of circulating OxPL levels as biomarkers have also been performed in the field of cardiovascular disease. A series of studies utilising E06-based ELISA have demonstrated a clear correlation between OxPL content per particle of apoB (OxPL/apoB ratio) and the presence of coronary and peripheral artery disease. A strong and graded association with the extent of coronary artery disease (CAD) defined as stenosis of more than 50 percent of the luminal diameter was demonstrated in a study involving 504 subjects, the correlation being strongest for patients aged 60 years or younger [18]. The highest quartile of OxPL/apoB was associated with an odds ratio for CAD of 3.12 (P < 0.001) compared with subjects in the lowest quartile. Interestingly, in the entire cohort OxPL/apoB predicted CAD independently of all other clinical markers except for Lp(a), a subclass of lipoproteins characterised by the presence of a unique apolipoprotein apo(a). OxPL/apoB showed strong correlation with Lp(a) levels suggesting that the majority of oxidative epitopes detected by E06 are located on Lp(a) particles, the main function of which is supposed to be sequestration of toxic proinflammatory OxPLs. Recent mass spectroscopy analysis revealed that OxPL are both present in the lipid phase of Lp(a) and are covalently bound to apo(a) [19]. Interestingly, this relationship between OxPL and Lp(a) was dependent on the size of apo(a) isoforms. Apo(a) proteins vary in size due to a variable number of the so-called kringle IV type 2 repeats in the apo(a) gene. Correlation of OxPL was weakest with the largest apo(a) isoforms and strongest with the small isoforms containing lowest number of kringle IV type 2 repeats [20]. Among patients aged 60 years or younger the predictive value of OxPL/apoB was independent even of Lp(a), perhaps reflecting additional proinflammatory Lp(a)-independent risks of OxPL elevation. Studies performed using DLH3 antibody confirmed association of higher OxPL levels with coronary [21] and carotid [22] atherosclerosis.

The prospective Bruneck study performed with a 5 year interval demonstrated high predictive value of OxPL/apoB measured by E06 antibody also for the presence, extent and development of carotid and femoral atherosclerosis [23]. In the 10 year [24] and 15 year [25] follow-up analyses OxPL/apoB and Lp(a) predicted future cardiovascular events (cardiovascular death, myocardial infarction, stroke and transient ischaemic attack) beyond the information provided by the Framingham Risk Score, and allowed reclassification of a significant proportion of patients into higher or lower risk categories after traditional risk assessment. The EPIC-Norfolk study involving 763 cases and 1,397 controls demonstrated that the highest tertiles of OxPL/apoB and Lp(a) were associated with a higher risk of CAD-related events and provided better cumulative predictive value when added to traditional cardiovascular risk factors [26]. However, in patients with previous myocardial infarction no correlation was found between E06-detected OxPL/apoB and recurrent cardiovascular events (cardiovascular death, nonfatal reinfarction or stroke, percutaneous coronary intervention, coronary artery bypass grafting and hospitalisation due to angina pectoris) [27]. Importantly, the Dallas Heart Study demonstrated significant race/ethnicity-related differences in oxidative markers, with the level of OxPL/apoB and its correlation with Lp(a) being highest in black subjects as compared with whites and Hispanics [28]. These data suggest that proinflammatory OxPLs present on Lp(a) represent a genetic predisposition to increased oxidative stress. Positive association of OxPL/apoB with peripheral artery disease has been confirmed in a recent study which included two parallel nested case-control studies within the Health Professionals Follow-up Study and the Nurses’ Health Study [29].

Temporary increases in plasma OxPL detected by E06 or DLH3 antibodies have been observed in acute coronary syndromes such as myocardial infarction [22, 30–33]. Studies performed using DLH3 antibody demonstrated strong accumulation of OxPLs in ruptured lipid cores of culprit coronary and carotid atherosclerotic plaques [22, 31, 32]. Plasma levels of DLH3–OxPL increased in acute cerebral infarction [34], stayed persistently elevated during the early phase after the stroke in association with enlargement of the ischaemic area in patients with cortical lesions [35], and reflected reduction of oxidative brain damage in patients with cortical infarcts treated by free radical scavenger edaravone [36]. Interestingly, a mass spectrometry approach identified a distinct plasma pool of OxPL that is covalently bound to plasminogen. OxPL/plasminogen levels did not correlate with Lp(a) and were acutely increased over the first month in patients following acute myocardial infarction [37].

Percutaneous coronary intervention (PCI) is a standard diagnostic and therapeutic method for management of CAD. A major complication of PCI is vascular restenosis, a gradual re-narrowing of the treated segment that occurs between 3 to 12 months after the intervention. Several studies attempted to establish the potential value of OxPLs as a predictor of restenosis. Results on changes in OxPL levels obtained using E06 and DLH3 antibodies are not strictly consistent: while both approaches demonstrated elevation of OxPL after the percutaneous intervention and stenting [38–40], E06 reactivity in plasma was not associated with the restenosis risk either in balloon- or stent-treated groups [40], while OxPL levels detected by DLH3 were a strong independent predictor of in-stent restenosis at six month follow-up in acute myocardial infarction patients [41]. Interestingly, oxidation epitopes recognised by E06 could be coimmunoprecipitated with Lp(a) from plasma samples at every time point after PCI except for those collected immediately after the intervention, suggesting that OxPLs are released briefly and then reassociate with Lp(a) [38]. A complex study utilising immunoassays and mass spectrometry confirmed the presence of multiple oxidised lipid
species including phosphatidylcholine-containing OxPL in lipid extracts from obstructive plaques and in OxPL released into the circulation during percutaneous coronary and peripheral arterial interventions [42]. Accumulation of fragmented phosphatidylcholine during the reperfusion period was also detected by high performance liquid chromatography after cardiopulmonary bypass [43]. Fast changes in OxPL concentrations after acute events or revascularisation procedures might reflect oxidative stress caused by tissue injury, increased production of ROS during ischaemia/reperfusion or release of lipid components from ruptured atherosclerotic plaques into the circulation. Several studies have addressed the value of OxPL measurements in monitoring efficiency of medicamentous treatments and life style changes aimed at management of cardiovascular disease. Unexpectedly, most data obtained using E06 antibody revealed elevations in OxPL levels in response to cholesterol-lowering agents. In the MIRACL (Myocardial Ischaemia Reduction with Aggressive Cholesterol Lowering) Trial atorvastatin treatment decreased total OxPL on all apoB particles in patients with acute coronary syndromes but increased OxPL/apoB, Lp(a) levels and Lp(a)-associated OxPL [44, 45]. The VISION (Value of oxidant lipid lowering effect by Statin InterventON in hypercholesterolaemia) study compared oxidation biomarker values in two groups of hypercholesterolaemic patients treated either with pitavastatin or atorvastatin. No difference between the groups was observed with respect to OxPL/apoB; however, within each group OxPL/apoB significantly increased upon treatment as compared to baseline [46]. Similar results were obtained in the REVERSAL (Reversal of Atherosclerosis with Aggressive Lipid Lowering) Trial for atorvastatin and pravastatin [47] and in two other studies [48, 49]. Patients on a low-fat, high-carbohydrate diet exhibited elevated levels of OxPLs and an accompanying shift in plasma lipoprotein profile (decrease in LDL particle size and increase of Lp(a)) [50]. As a further example of inverse correlations between OxPL levels and disease progression, increases in OxPL/apoB and Lp(a) were found to strongly correlate with improved vascular function and to predict a lack of progression of coronary artery calcification [51]. Some attempt at explaining these inverse correlative data was made in an experimental study which analysed dietary-induced atherosclerosis progression and regression in cynomolgus monkeys and New Zealand White rabbits. Hypercholesterolaemia was associated with a decrease in plasma OxPL/apoB, whereas during reversal to normocholesterolaemia OxPL/apoB increased and was accompanied with the disappearance of OxPLs from atherosclerotic plaque lesions [52]. Taken together, these data might suggest that statins and lipid lowering diets promote formation of Lp(a) lipoproteins resulting in mobilisation of OxPL from the vessel wall, transfer to Lp(a) particles and improved clearance of OxPLs from the vascular system. However, in contrast to these studies, OxPL levels detected by DLH3 mAb decreased in patients with hypocholesterolaemia after treatment with fluvastatin and pravastatin [53], thus showing a tendency similar to MDA-LDL levels which were reduced by pitavastatin and atorvastatin [46].

Endothelial dysfunction is an early event in the pathogenesis of atherosclerosis which is characterised by decreased vasodilator function, increased coagulation activity and enhanced proinflammatory properties of vascular endothelial cells. Inflammation and oxidative stress which results in reduced availability of the major vasodilator factor NO are potent triggers of endothelial dysfunction. Improvement of endothelial function after lipid lowering therapy in patients with coronary atherosclerosis assessed by quantitative angiography strongly correlated with OxPL levels. The number of E06 epitopes per LDL particle was related to the severity of endothelial dysfunction and was the single most powerful independent risk factor in the post-therapy study suggesting that OxPL may contribute to abnormal coronary vasomotion in atherosclerosis [54].

Oxidised lipids in diabetes and metabolic syndrome

The metabolic syndrome, a constellation of symptoms including obesity, dyslipidaemia, hypertension and insulin resistance, is a risk factor for both CAD and diabetes [55]. Some components of metabolic syndrome are traditional risk factors for these pathologies, but they only partially account for the increased incidence of CAD and diabetes in persons with metabolic syndrome. Among emerging common non-traditional risk factors are low-grade inflammation and oxidative stress. In population studies elevated levels of proinflammatory biomarkers CRP and IL-6 were found to predict the development of type 2 diabetes [56]. Increased oxidative stress in adipose tissue contributes to the metabolic syndrome and is associated with type 2 diabetes [57]. The role for oxidised lipids in metabolic syndrome was suggested by the findings that obesity-associated dyslipidaemia and hyperglycaemia in humans are associated with increased LDL oxidation and that dyslipidaemia and insulin resistance in obese LDL receptor-deficient mice were associated with increased oxidative stress and impaired antioxidant defence [58]. LDL from patients with non-insulin-dependent diabetes mellitus were more susceptible to oxidative modification due to a reduced vitamin E/ lipid peroxide ratio in blood, a factor that may represent a possible link between the increased incidence of vascular disease and diabetes mellitus [59]. High-density lipoproteins isolated from type 2 diabetic patients exhibited a decreased capacity for clearance of OxPLs, which may increase the risk for cardiovascular disease [60]. Further, levels of OxLDL and advanced glycation end products–modified LDL in circulating immune complexes were strongly associated with carotid intima thickening in patients with type 1 diabetes [61].

DLH3 mAb-based ELISA demonstrated higher OxPL in patients with unstable angina pectoris that also had diabetes mellitus as compared to non-diabetic subjects [31] and in subjects with diabetic nephropathy [62]. Altogether, these data suggest that OxPLs may reflect and/or contribute to progression of metabolic disorders and the linkage to a constellation of their complications and related pathologies.
Oxidized phospholipids in renal dysfunction

Disturbances in lipid metabolism are a characteristic feature of chronic renal disease. Renal insufficiency is accompanied by shifts in plasma lipid profiles, and high triglyceride and cholesterol plasma levels are independent risk factors for renal disease progression. Experimental data suggest that oxidative stress may be among possible mechanisms linking hyperlipidaemia with the renal damage [63]. Increased cellular accumulation of lipids and oxidised fatty acids were detected in the glomerulosclerotic lesions [64]. Patients with uraemia, which is a major contributor to oxidative stress, exhibit increased susceptibility of LDL for oxidation [65]. The haemodialysis procedure per se may also promote LDL oxidation due to activation of neutrophils or bacterial contamination [66].

Several studies analysed levels of OxPLs in blood of patients with renal disease and reported variable results. DLLH mAb-based ELISA demonstrated more than eight-fold increase in LDL oxidation in patients receiving haemodialysis [6]. Low levels of lysocephatidylcholine determined by an enzymatic assay were reported to be associated with increased risk of cardiovascular disease in Korean haemodialysis patients [67]. In end-stage renal failure patients undergoing haemodialysis OxPL/apoB levels measured by E06 mAb dropped immediately following the procedure, while other markers of LDL oxidation such as autoantibody titers to copper-oxidised LDL and malondialdehyde-LDL significantly increased. E06-based detection also did not reveal any association between OxPL/apoB levels and cardiovascular disease in chronic haemodialysis patients [68]. Further large-scale prospective studies are required to estimate predictive value of OxPL levels as a biomarker of clinical outcome in renal disease.

Oxidised phospholipids in neurological disorders

Brain and nervous tissue are very susceptible to oxidation due to their high lipid content and intense consumption of oxygen. Oxidative stress and lipid peroxidation have been related to progression of many neurological disorders such as schizophrenia, bipolar disorder and neurodegenerative diseases. Involvement of OxPLs in onset and progression of Alzheimer’s and Parkinson diseases has been proposed [69, 70]. Multiple sclerosis (MS) is a disabling neurodegenerative disease characterised by the presence of demyelinated plaques and axonal degeneration. Autoimmune attack on the myelin sheath in the brain and the spinal cord is supposed to be the major cause for the disease; however, the identity of the antigens remains elusive. Since lipids comprise >70% of the myelin sheath, lipids have been considered capable of inducing autoimmune reactions in MS [71]. Oxidized 1-palmitoyl-2-(5'-oxo)valeryl-sn-glycero-3-phosphatidylcholine, detected by E06 antibody, was found to be present in high amounts in brain tissue of MS patients but almost absent in control samples [72].

Another neuropathological condition involving OxPLs is that of neurobehavioral problems in children treated for acute lymphoblastic leukaemia. Chemotherapy with methotrexate causes an injury of the central nervous system leading to neurocognitive deficiencies, anxiety and depression. Children with high-risk acute lymphoblastic leukaemia receiving the most intensive methotrexate treatment displayed the highest levels of oxidised phosphatidylcholine in the cerebrospinal fluid suggesting that this OxPL species may be a marker of therapy-induced central nervous system injury [73]. OxPL levels also predicted behavioural changes such as executive dysfunction [74], aggression at the end of therapy and postconsolidation adaptability [75]. These studies would justify development of OxPL-based biomarker methods for predicting the degree of neuropathological symptoms caused by chemotherapy, and also suggest that use of antioxidants may limit toxic effects of the treatment.

Other pathologies characterised by accumulation of oxidised phospholipids

Lung injury
PLs are a major component of pulmonary surfactant and easily undergo oxidation under pathological conditions characterised by oxidative stress, such as viral or bacterial infections. Accumulation of OxPLs has been reported in animal models of lung injury as well as in humans infected with SARS, Anthrax or H5N1 [76]. Experimental evidence suggests that OxPLs have dual pro- and anti-inflammatory functions in the lung. On the one hand OxPLs stimulate production of proinflammatory cytokines and TLR4 signalling in alveolar macrophages [76] thus contributing to lung tissue injury, and they increase endothelial permeability by inducing cellular cytoskeleton reorganisation [77, 78]. On the other hand, under certain conditions OxPLs may inhibit LPS-induced inflammation in animal models [79, 80] and also protect endothelial barrier function, the differential effects being dependent on concentration and structural characteristics of OxPL species [77, 78]. Quantitative assessment of levels of tissue and systemic OxPLs in pulmonary injury has not been performed.

Leprosy
Accumulation of fatty acids and PLs is a characteristic feature of the lepromatous (disseminated, L-lep) form of human leprosy. Lipid accumulation is related to changes in the expression profile of genes involved in lipid metabolism in the host, such that the newly synthesised lipids in lepromus lesions derive from human tissue and not from the mycobacteria. Functionally, OxPLs produced mainly by macrophages promoted survival of the pathogen by interfering with innate and specific immune responses such as CD11b-mediated presentation of antigens to T-cells, TLR2/1 activity and IL-12 secretion [81]. Interestingly, the accumulation of OxPLs in leprosy lesions was very similar to atherosclerosis, suggesting common innate immunity-controlled mechanisms in progression of infectious and metabolic diseases.
Cancer

Biliary strictures may develop due to a number of pathologies, among them cholangiocarcinoma and pancreatic cancer. Correct diagnosis of the biliary stricture aetiology remains a challenge since existing diagnostic methods such as bile duct brushing do not allow differentiation between malignant and benign origin of the strictures. Lipidomic profiling using liquid chromatography-ESI-MS technique demonstrated elevation of two OxPL species, 1-palmitoyl-2-[(9-oxonanoyl)-sn-glycero-3-phosphatidylcholine and 1-palmitoyl-2-succinoyl-sn-glycero-3-phosphatidylcholine, in bile samples of patients with cholangiocarcinoma, distinguishing these cases from strictures of other origins with 100% sensitivity and 83.3% specificity. This approach may enhance the accuracy of endoscopic tests during diagnosis of indeterminate biliary strictures [82].

Conclusion

Emerging data suggest that OxPLs significantly contribute to progression of many pathological conditions and might serve as biomarkers to predict the risk of the diseases, monitor disease progression and check therapeutic intervention efficacy. Broadening the range of monoclonal antibodies to enable detection of various types of OxPLs as well as better characterisation of their target oxidation-specific epitopes would help to overcome the limitations of current OxPL detection methods. Mass spectrometry analysis of the spectrum and structure of OxPL species differentially expressed in health and disease has great potential for rapid progress in the field and will allow identification of both novel biomarkers and molecular mechanisms underlying pathological effects of OxPLs.

References


Figure 1
Figure 2
The role of OxPLs in the pathophysiology of atherosclerosis.