Confirming the Reference Range of the Oxidised LDL Assay for a Normal Population
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Abstract: Reference ranges are the most common decision support tool used for interpretation of numerical pathology reports. As laboratory results may be interpreted by comparison with these ranges, the quality of the reference intervals can play as large a role in result interpretation as the quality of the result itself. One of the key inflammatory markers in the progression of atherosclerotic lesions is the level of circulating oxidised low density lipoprotein (oLDL) using a sandwich type EIA. Having a reference range that is correct for the target population is important. The manufacturer of the assay had calculated a reference range of 26–117 mU/L for the oLDL assay using only 148 ‘normal’ patients. Our data calculated the in-house reference range as 14.4 to 102.7 mU/L with a tolerance interval of 20.5 to 96.8 mU/L for 90% of the population. In this instance there was a slight difference between the manufacturer’s range and the calculated in-house range.

Keywords: atherosclerotic lesions, oxidised low density lipoprotein (oLDL), Assay

INTRODUCTION
Inflammation is an important feature of atherosclerotic lesions and acute coronary syndromes and studies have shown an increase in inflammatory markers including C-reactive protein and interleukins in both stable and acute angina [1-5]. One of the other key markers in the genesis of the inflammatory process has been oxidised low density lipoprotein (oLDL) [6-8]. Oxidative modification of LDL alters its biological properties, resulting in chemotaxis of monocytes or T lymphocytes in addition to the modulation of growth factors and cytokine production from endothelial cells, smooth muscle cells, and macrophages [9]. Goldstein et al. originally reported a process of LDL modification involved in the phenotypic change of macrophages to foam cells in evolving stages of atherosclerosis [10]. Studies have shown that increased circulating oLDL concentrations are related to cardiovascular disease, although not always independently after adjustment for classical lipid markers and can be affected by many biological and lifestyle factors, as well as (generalized) subclinical atherosclerosis [11-13].

During the primary validation of the oLDL assay at Quest Diagnostics in Heston, UK, a reference range had been assigned based on the manufacturer’s recommendation (26-117 mU/L), for heparin plasma. The aim of this study was to analyse levels of oLDL in a larger asymptomatic population to confirm the validation range and see if a new in-house range had to be defined.

MATERIALS AND METHODS
Study Population
1510 samples were collected from patients of north European origin. They were healthy adults evenly distributed by sex and age. Exclusion criteria were any self-reported disease eg diabetes, cancers, known cardiac problems or severe dementia.

Plasma for the oLDL was collected by centrifugation of the blood sample for 15 minutes at 1000 x g within 30 minutes of collection. The plasma samples were then frozen and stored at -20°C before being transported to the laboratory. The samples were kept frozen until testing. Prior to testing the samples were thawed, vortexed to remove layering effects and then centrifuged 15 minutes at 1000 x g. All samples were also tested for MMP-9, fibrinogen, insulin, and free fatty acids using standardised methods.
Oxidised LDL Assay

The concentrations of oLDL were measured in Heparin plasma using a plate assay (Oxidised LDL, supplied by Mercodia AB, Sweden). The assay was a solid phase two-site enzyme immunoassay. Based on the direct sandwich technique in which two monoclonal antibodies are directed against separate antigenic determinants on the oxidised apolipoprotein B molecule.

Statistical Analysis

The data were analysed using a combination of the Kolmogorov–Smirnov test (K–S test) and the Shapiro–Wilk test. A Box-Cox variable transformation technique was used to stabilize variance, to make the data had a more normal distribution and to improve the validity of measures of association such as the Kolmogorov–Smirnov test and the Shapiro–Wilk test [14]. As there were a number of results in excess of the manufacturers’ recommended range, the Iglewicz and Hoaglin robust test for multiple outliers was used to remove extraneous values [15].

Reference ranges were calculated as the mean result± 2 standard deviation (SD) of the subjects tested.

RESULTS

Two hundred and sixty-four subjects were excluded from the final analysis because of abnormal results from one or more the other analytes leaving 1246 subjects in the studied population. These included 50 patients with abnormal insulin levels, 210 patients with raised fibrinogen levels (136 males and 74 females) and 4 patients with raised MMP-9 results. A graph was plotted showing the range of oLDL values which showed that there was very little gender bias(Figure 1). A graph and a histogram plot showing the oLDL distribution were plotted (Figure 2 and Figure 3). The Kilgomorov-Smirnov test gave a p-value of <0.0001 and the Shapiro-Wilk a value of <0.05. As the p-value was very small it suggested that the data did not follow a normal distribution, if 0.01 and 0.05 were being used for the threshold. Because of this a Box-Cox transformation calculation was used to remove any bias in the distribution (Figure 4), and finally to plot a normality graph (Figure 5) this had a λ value of 0.37 and a correlation of 0.998. These gave a mean value of 58.7mU/L for the oLDL assay, with a standard deviation of 22.1 for the population under investigation.
Fig-2: Distribution of oLDL results

Fig-3: Histogram of Original oLDL Data

Fig-4: Histogram of Transformed oLDL Data (Box-Cox Transformation) $\lambda = 0.37$ (Correlation of 0.998)
The manufacturer gave a reference range of 26-117 mU/L for the oLDL assay using only 148 ‘normal’ patients, our data calculated the in-house reference range as 14.4 to 102.7 mU/L with a tolerance interval of 20.5 to 96.8 mU/L for 90% of the population.

DISCUSSION
Recent studies have suggested the plasma oLDL concentrations may change under pre-pathological and post-pathological conditions. It has been shown that oLDL may be transferred between tissues and plasma and does not merely accumulate in the lesions but is equilibrated between the tissues and circulation with the liver being the major organ for the clearance of oLDL from circulation [16]. The biological effects oLDL have been shown to contribute to initiation and progression of the atherosclerotic process, and there is an association between cardiovascular disease and oxidation of LDL [17, 18]. Because oLDL is thought to play a key role in the genesis of the inflammatory process in atherosclerotic lesions, the question arises whether an increase in the blood levels of oLDL could be involved. Certainly, oLDL generated in the vessel wall may diffuse into the circulation and LDL in the circulation may in part be oxidatively modified. Also LDL extracted from atherosclerotic lesions in part oxidatively modified, and second, immune-histochemical investigations show that atherosclerotic lesions react with antibodies generated against oLDL antibodies. Although either is possible, evidence favours the former as the mechanism by which circulating oLDL is generated [19-21].

Given the obvious clinical atherogenic role of oLDL, studies have been directed at detecting oLDL levels in the circulation, with expectations that oLDL levels would be a biochemical marker for atherosclerosis [22, 23]. The limitation of the present study is there were no results for the relationship between circulating oLDL and other potential risk factors, such as Lp(a) and homocysteine, although studies have shown the additive value to lipid measurement for cardiovascular risk prediction as inconsistent. But having a reference range that is correct for the target population is important. In this instance there was a slight difference between the manufacturer’s range and the calculated in-house range.

REFERENCES
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