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Original Research Article

Matrix metalloproteinase-9: defining a normal reference range

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Abstract: Unstable carotid plaques, characterized by increased levels of macrophages and T lymphocytes, have high emboligenic potential and carry a risk for producing cerebral ischemic events. It has been suggested that plaque instability may be mediated by a family of extracellular proteinases:- the matrix metalloproteinases. These matrix metalloproteinases (MMPs) are required for numerous developmental and disease-related processes and contribute to the development of *de novo* atherosclerotic plaques as well as the rupture of the plaques by degrading the associated extracellular matrix. It is important therefore that the reference range accurately reflects the levels of MMP in the patient. The aim of this study was to calculate a reference range from a normal population. Results from twelve hundred and fifty patients were analysed, and using this normal population the reference range for females was calculated to be 14.3 – 34.6 (M ±2SD) ng/mL with a 95 % CL while the males was 19.8 – 99.5 (M ±2SD) ng/mL with a 95 % CL. Having a correct reference range for the MMP-9 is a potential means of reducing patient anxiety, iatrogenic morbidity and mortality, and cost.
Keywords: Reference range, matrix-metalloproteinases, artherosclerosis

INTRODUCTION

The matrix metalloproteinases (MMPs) are a family of extracellular proteinases required for numerous developmental and disease-related processes. The ability to degrade extracellular proteins is essential for any individual cell to interact properly with its immediate surroundings and for multicellular organisms to develop and function normally. They are central to the regulation of extracellular matrix turnover, having the ability to cleave majority of extracellular matrix (ECM) proteins. The MMPs were described in 1962 by Gross and Lapiere who observed enzymatic activity during tadpole metamorphosis [1]. The family can be broadly subdivided into divisions which include the collagenases, stromelysins, gelatinases, and membrane-type MMPs (MT-MMP), designated by the substrate specificities of the enzyme. This breakdown of extracellular matrix (ECM) proteins is essential for embryonic development, morphogenesis, reproduction and tissue re-sorption and remodelling [2]. And, as new discoveries are made it increases the argument that MMPs are common across both vertebrate, invertebrate and plant species and possibly share a common origin [3-5].

One of the most extensively studied MMPs is MMP-9, also known as 92-kD gelatinase B or type IV collagenase, and it has been shown to be causally involved in the remodelling processes associated with atherogenesis and plaque rupture due its expression in the vulnerable regions of atherosclerotic plaques [6-8]. Further studies have shown that elevated levels of circulating matrix MMP-9 have been found in patients with established tendinosis, a process normally associated with the degenerative changes brought on by aging [9]. Although little is known about the roles of mechanisms responsible for aging in the degeneration of tendons, biophysical investigations have implicated a role for imbalanced homeostatic turnover of the extracellular matrix (ECM) of the tendon due to the release of MMPs [10]. Other studies have also shown that the higher the MMP expression in the tumours, the more aggressive the cancer. Measuring the MMP level in the serum, plasma, or CSF has been shown to be a possible predictor of tumour stage, metastasis, angiogenesis and recurrence [11].

During the primary validation of the MMP-9 assay at Q^2 Solutions (formerly Quest Diagnostics) a reference range had been assigned based on the manufactures recommendation (13-105 ng/mL)

irrespective of gender. 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. The recommended protocol for setting a reference interval is to perform a reference interval study according to standard published procedures [12, 13]. Other analytical factors and pre-analytical factors, however may also need to be considered, along with partitioning on the basis of sex or age, either as part of a reference interval study or when interpreting other studies. The number of significant figures for the reference limits, the possible adoption of common reference intervals and clinical consultation also need consideration after the data has been obtained.

Of course, it is important to define the analyte (measurand) for which the reference interval is being established, and the common reasons for measurement of the analyte, in order to ensure that any intervals are appropriate for those purposes. The analyte description should also include the units to be used and it may be useful to provide conversion factors from other units which may be encountered in the literature. The subjects being tested (the reference population) should be as similar as possible to that for which the test will be applied, with the exception of the presence of disease. The aim of this study was to analyse levels of MMP-9 in a larger asymptomatic population and define an in-house reference range, confirm the validation range and see if the range needed to be gender specific.

MATERIAL AND METHODS

Patients and Sample Collection

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* cancers, known cardiac problems or severe dementia. All samples were also tested for fibrinogen, insulin, free fatty acids and oxidised LDL using standardised methods. Patients with any abnormal analytes were excluded from the statistical analysis.

Plasma for the MMP-9 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 to remove particulate matter.

MMP9 Analysis

The concentrations of MMP-9 were measured in Heparin plasma using a plate assay (Quantikine® Human MMP-9 [total] Assay) supplied by R&D Systems (R&D Systems Europe Ltd., Abingdon, UK). The assay was a quantitative sandwich enzyme immunoassay, with a monoclonal antibody specific for MMP9 pre-coated onto the wells.

Statistical Analysis

The data were analysed using a combination of the Kolmogorov–Smirnov test (K–S test), the Shapiro–Wilk test and a Box-Cox normality plot was used to eliminate any ‘skewness’ and other distributional features that could complicate analysis. Reference ranges were calculated as the mean ± 2 standard deviation (SD) of the subjects tested.

RESULTS

1250 patients were included in the analysis, with an average age of 67.5 (median 69) ranging from 21-80 years of age. There was a male to female ratio of 1.0: 0.8. There were 260 patients excluded because of abnormal results from one or more the other analytes. An initial graph was plotted showing the range of values in the population by gender (Figure 1).

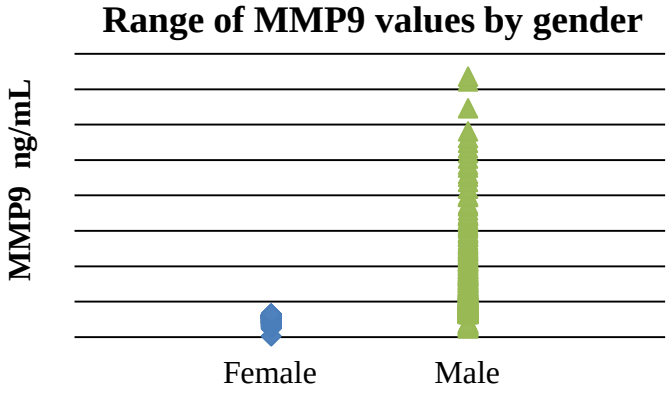

Fig-1: Range of MMP9 values by gender

These excluded patients included 50 patients with abnormal insulin levels (9 with hypoinsulinaemia and 41 showing hyperinsulinaemia) and 210 patients with raised fibrinogen levels (136 males and 74 females). Using this larger population figures the reference range for females was calculated to be 14.3 – 34.6 (M ±2SD) ng/mL with a 95 % CL (Figure 2) while the males was 19.8 – 99.5 (M ±2SD) ng/mL with a 95 % CL (Figure 3).

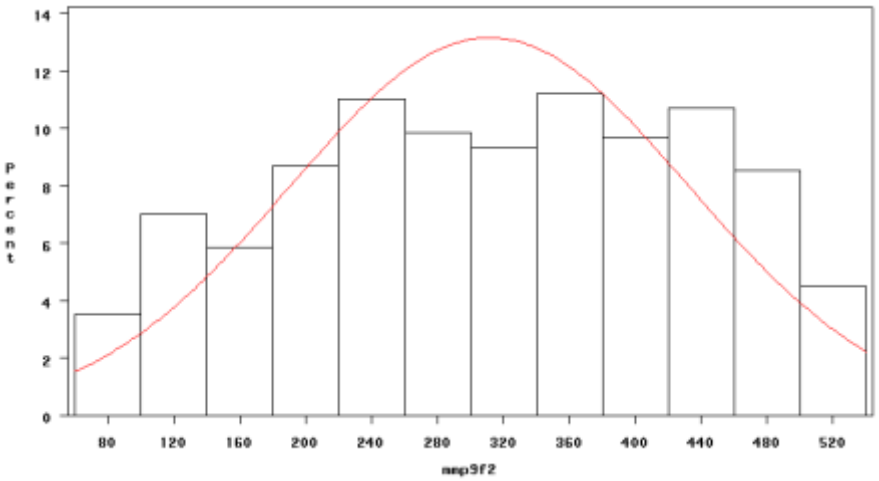

Fig-2: BoxCox Transformed Data–Female (λ=2)

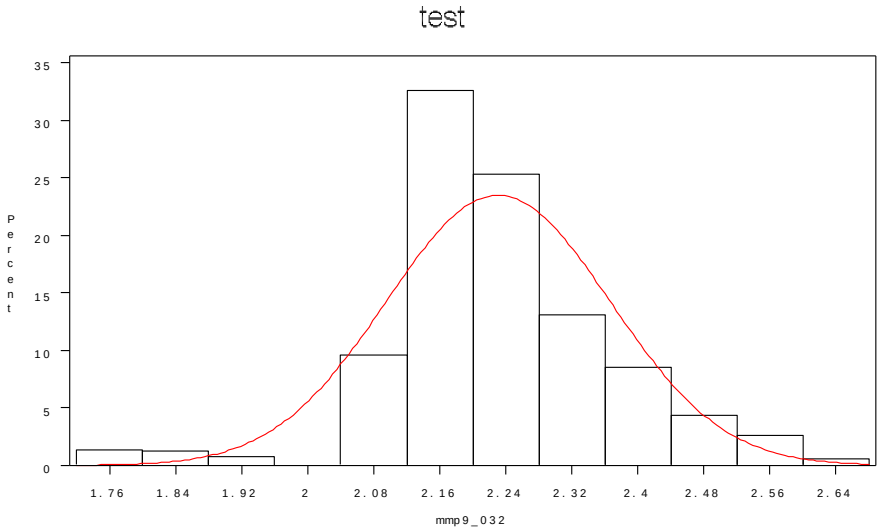

Fig-3: BoxCox Transformed Data–Male (λ=-0.32)

DISCUSSION

This was only a small study but it allowed the laboratory to define its own in-house range for MMP9 in our 'normal' population. During the primary validation a reference range of 13- 105 ng/mL had been used from a limited number of samples which had been based upon the manufacturer recommendations. Of course, there were a number of patients removed from the dataset for having abnormal results in two analytes (Fibrinogen and Insulin) as these have been shown to influence the levels of MMP9 [14,15]. Insulin has been shown to demonstrate a potent anti-inflammatory and subsequently a potential anti-atherogenic effect in human aortic endothelial cells and mononuclear cells at physiologically relevant concentrations [16]. And as MMP9 plays a central regulatory role in angiogenesis and accelerates atherosclerosis any change in insulin concentration could have an effect on the MMP9 [17]. Also, the role of the inflammatory agent fibrinogen (Fg) has been, amongst others, associated with cardiovascular disease, however it may also be elevated in any form of inflammation, as it is an acute-phase protein and it was for this reason these samples were removed from the dataset [18].

Altered levels of MMPs may reflect relevant pathogenic mechanisms of disease conditions and a study by Samnegard [19], found a gender related difference in the pattern of associations between inflammatory markers and MMPs, they speculated that MMP-3 was more important for smooth muscle cell (SMC) migration and proliferation, and might be the cause of plaque erosions that are more common in women, while MMP-9 was more important for macrophage infiltration and activation and might initiate the classic plaque rupture that is more common in men [20].

Certainly, elevated levels of MMP9 have been implicated in the pathogenesis of polycystic ovary syndrome (PCOS) through regulating ovarian tissue remodelling [21]. In addition to degrading the extracellular matrix, MMPs exhibit the ability to cleave insulin-like growth factor binding protein-1 (IGFBP-1), the major regulator of insulin-like growth factor-I (IGF-I) in serum [22]. So, although a large increase in circulating MMP9 could suggest an advanced stage of PCOS, a slight rise might be useful as a pro-indicator, especially if the woman was in the correct age group and showed an increased insulin result [23].

By comparison, studies have shown that in cases of hypertrophic cardiomyopathy (HCM) the circulating levels of MMP9 can be higher in men than women [24]. Hypertrophic cardiomyopathy presents as a diverse clinical spectrum from asymptomatic cases to progressive heart failure and disability in addition to risk for sudden death, often in young people. It can be defined by the presence of unexplained left ventricular hypertrophy, myocyte disarray, and interstitial fibrosis. Collagen turnover is enhanced in HCM, maintaining collagen I synthesis over degradation, with changes in MMP release and activity, mainly high levels of MMP-2 and MMP-9 [25].

The MMPs have been shown to be involved with many conditions, from CAD to arthritis and ulcerative colitis and it is important that clinicians are able to determine if the levels are out of the reference range [26-29]. Although the validation reference range (13-105 ng/mL) is similar to that quoted in other studies, it does not take into account the clear difference in concentrations between genders [30-32]. Our data demonstrate this difference. Thus, we have calculated separate reference ranges for the 2 genders. Use of gender-specific reference ranges will lead to more accurate interpretation of MMP-9 test results.

So in conclusion the validation reference range was correct but it encompassed both male and female results and needed to be split into gender to take into account the wide variation between the sexes.

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