Determination of a reference value for plasma myeloperoxidase in healthy Chinese population

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Abstract: Plasma myeloperoxidase (MPO) has been proved to be an important marker of future cardiovascular disease risk. In the present study, we tried to establish a reference range for plasma MPO in apparently healthy Chinese population. The level of plasma MPO in healthy Chinese individuals was evaluated by a quantitative enzyme-linked immunosorbent assay (ELISA) kit. We enrolled 820 (422 males, 398 females) healthy individuals in our study and divided them into subgroups according to their gender and age. The necessity of setting reference intervals of MPO for subgroups was determined according to the CLSI standard. Our results show that the level of plasma MPO was elevated along with the increase of age in healthy Chinese. No statistical differences in the level of plasma MPO was found between males and females ($P>0.05$). There was no need to establish reference intervals for each subgroup respectively. The reference value of plasma MPO in apparently healthy Chinese people is 90.53ng/ml, which may be of great importance in clinical practice in the future.
[MA Chun-mei, WU Jian-rong, YE Dao-bin, DENG Xiao-wei, WEI-wei, GUO Yue-ling. **Determination of a reference value for plasma myeloperoxidase in healthy Chinese population.** *Life Sci J* 2015;12(1):89-93]. (ISSN:1097-8135). http://www.lifesciencesite.com. 13

Key words: plasma MPO, healthy Chinese population, reference value

Abbreviations

MPO: myeloperoxidase
ELISA: enzyme-linked immunosorbent assay
CLSI: Clinical and Laboratory Standards Institute
NCCLs: the National Committee for Clinical Laboratory Standards
CFDA: China Food and Drug Administration
TMB: Tetramethylbenzidine
OD: optical density
NO: nitric oxide
HOCl: hypochlorous acid
LDL: low-density lipoprotein
HDL: high-density lipoprotein
CHD: coronary heart disease

1. Introduction

Myeloperoxidase (MPO), a member of the peroxidase superfamily, is predominantly found in neutrophils, monocytes and tissue macrophages and stored in the azurophilic granule. The molecular mass of MPO is 144 kDa [1]. It contains 2 subunits, each composed of a heavy chain with 60kDa and a light chain with 15kDa. MPO is both a biomarker of systemic inflammation and a medium of oxidative stress, which can promote the formation of oxidizing substances. The releasing of oxygen free radical and inflammatory mediators can further cause endothelial dysfunction. At the same time, through its effect on the oxidation of LDL and HDL and some other mechanisms, MPO plays a critical role in the formation, development, damage and even rupture of atherosclerotic plaques and the process of thrombosis [2]. Brennan et al. reported that plasma myeloperoxidase levels predicted the risk of cardiac events in patients in whom unstable angina was suspected, independent of CRP and other biologic markers. The level of plasma myeloperoxidase in patients with acute chest pain can independently predicts the early risk of myocardial infarction, as well as the risk of major adverse cardiac events in the ensuing 30-day and 6-month periods, highlighting its potential usefulness for risk stratification among patients who present with chest pain [3]. In this study, we measured the MPO levels in 820 (422 males, 398 females) healthy individuals and tried to determine the reference range of plasma MPO levels in healthy Chinese population in order to provide some valuable information for future applications.

2. Materials and Methods

2.1 Study population

This study collected individuals who attended the medical examination centers of the General Hospital of Chinese People's Armed Army for their annual health check-up from April to September, 2013. The inclusion criteria include: 1) no history of heart diseases, physicians could rule out coronary artery disease after a comprehensive assessment during the current health check-up; or 2) coronary artery disease was excluded after receiving CTA or CAG. The exclusion criteria include: 1) history of heart disease, hypertension, dyslipidemia and glucose metabolism abnormal; 2) ECG or X-ray abnormal during the current check-up; and 3) white blood cell count abnormal, hepatic or renal dysfunction during the current examination. 422 male, age from 17 to 97 years, and 398 female, age from 14 to 91 years, were included at last.

2.2 Samples preparation

From each subject, a 3ml venous blood was collected by sterile percutaneous vein puncture and drawn into a heparin tube. The samples were stored at room temperature and centrifuged within 4 hours after collection to separate plasma. Centrifugation was carried out at 3000rpm for 10 min in a refrigerated centrifuge.

Plasma samples were stored at room temperature (20-26℃) if they could be analyzed within 8 hours. If the analysis could not be finished within 8 hours, plasma samples was stored at 2-8℃ for no more than 7 days. For longer storage of the samples, they were put in a refrigerator at -20℃ (or lower). The plasma can be frozen and thawed for two times at the most.

2.3 Materials

A. Instruments: Microplate reader for ELISA, TECAN SUNRISE®, Austria.

B. Reagents: Quantitative detection kit for MPO (ELISA), Peking Unionluck Biologic Technology Co., LTD.

2.4 Mechanism of myeloperoxidase detection

The standard samples and the plasma samples were put into the microplate coated with MPO antibodies. After that, MPO antibodies labeled with horseradish peroxidase were added into the microplate. Antibody-antigen-antibody complexes, which just like sandwiches, were formed afterwards. The horseradish peroxidase on the complexes can convert the substrate 3,3′,5,5′-Tetramethylbenzidine (TMB) into a soluble blue (~650nm) material, which further turns into a super-oxidized soluble yellow (450nm) product while the reaction is blocked by strong acid. The shade of the color is positively proportional to MPO concentration of the samples. Thus, we can determine the level of MPO by detecting the optical density (OD) at 450nm wavelength using a microplate reader.

2.5 Precision test

Two standard MPO solutions of different concentration (80ng/ml and 300ng/ml, respectively) were tested repeatedly for 10 times by the quantitative ELISA kit. The mean ($\overline{X}$), standard deviation (SD) and coefficient of variation (CV) were calculated in order to determine the precision of the kit.

2.6 Accuracy test

Another two standard samples of MPO solution of different concentration (400ng/ml and 100ng/ml, respectively) were added to hormones-free serum samples. Recovery rate was then calculated according to the formula below (the ratio between the MPO solution and serum was kept not more than 1:9).

Calculate formula 1:

$$R = \frac{C \times (V_0 + V) - C_0 \times V_0}{V \times C_S} \times 100\%$$

Note: R-recovery rate;

V-volume of sample A (the standard MPO solution);

V_0-volume of sample B(human serum);

C-detected concentration of the serum after adding sample B;

C_0-concentration of sample B(human serum);

C_s-concentration of sample A.

2.7 Subgroup analysis

According to the CLSI standard *How to Define and Determine Reference Intervals in the Clinical Laboratory; Approved Guideline- Second Edition (NCCLS document C28-A2)* and the standard of CFDA *Series of guiding principles for the assessment of the analytical properties of in vitro diagnostic reagents,* we divided the study population into several subgroups according to their gender and age and we further determined whether there were any statistically significant differences between the means of subgroups.

2.8 Outlier treatment:

According to NCCLS, Dixon outlier range statistic was used to find out outliers. The Dixon test is as follows: let R = the range of the values (maximum - minimum) and let D = the absolute difference between the most extreme (large or small) value and the next most value (large or small). If the ratio D/R exceeds 1/3, then the extreme value is considered an outlier and deleted. If there are two or three outliers on the same side of the sample, the least extreme outlier is tested. If the D/R test rejects the least extreme outlier, then the more extreme outliers are rejected as well.

2.9 Subgroup reference intervals:

The necessity of setting reference intervals of plasma MPO levels for each subgroup was determined according to the CLSI standard. The statistical significance of the difference between means of subgroups was tested by the standard normal deviate

test,

$$Z = \frac{\overline{X}_1 - \overline{X}_2}{\sqrt{\frac{S_1^2}{n_1} + \frac{S_2^2}{n_2}}}$$

Calculate formula 3:

$\overline{X}_1$ and $\overline{X}_2$ are the observed means of the two subgroups, S_1^2 and S_2^2 are the observed variances, and n_1 and n_2 are the number of reference values in each subgroup, respectively.

The calculated statistic z should be compared with a "critical" value Z^*.

$$Z^* = 3 \times \sqrt{\frac{(n_1 + n_2)}{240}}$$

Calculate formula 4:

In addition, the larger standard deviation, for example, S_2, should be checked to see whether it exceeds $1.5S_1$. If the calculated Z value exceeds Z*, or if the larger standard deviation exceeds 1.5 times the smaller, regardless of the Z value, then separate reference intervals should be calculated for each subgroup. If these conditions do not hold, then a single reference interval for the combined group of reference subjects should be calculated for general use.

2.10 Statistical analysis:

Statistical analysis was performed with the SPSS software (version 19.0; SPSS Inc., Chicago, Illinois). Differences between two groups were analyzed using the t test. Comparison between more than 2 groups was made based on one-way ANOVA analysis. A p value < 0.05 was considered statistically significant.

Result

Precision test

For the low concentration sample (80ng/ml), the mean ($\overline{X}$) of the 10 results was 77.43ng/ml, with the SD = 2.21ng/ml, the CV = 2.85%.

Meanwhile, for the high concentration sample (300ng/ml), $\overline{X}$ = 246.87ng/ml, SD = 12.77ng/ml, CV = 5.17%.

Accuracy test

According to our test result, the concentration of the background human serum was 14.92ng/ml. When a low concentration standard MPO solution (100ng/ml) was added, the detected concentration of the mixture was 110.91ng/ml. Therefore the recovery rate in this condition was 95.99%. While another standard MPO solution with a higher concentration (400ng/ml) was added, the concentration of the mixture was 357.92ng/ml. Thus, in accordance with the formula introduced above, the recovery rate was 85.75% in this condition.

The reference intervals of MPO concentration in Chinese healthy population

The results of MPO concentration in 820 healthy Chinese subjects were described in Table 1 and Figure 1.

Table 1. Frequency distribution of MPO concentration in healthy Chinesse population

Concentration(ng/ml)	Frequency (n)	Cumulative frequency
0-10	33	4.00%
10-20	208	29.40%
20-30	172	50.40%
30-40	129	66.10%
40-50	82	76.10%
50-60	54	82.70%
60-70	47	88.40%
70-80	29	92.00%
80-90	23	94.80%
90-100	15	96.60%
100-120	11	97.90%
120-150	8	98.90%
>150	9	100.00%
Total	820	

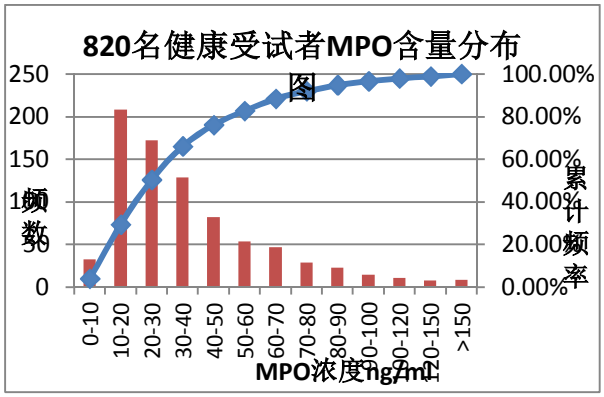

Figure 1: The MPO concentrations of healthy people

The distribution of MPO concentration in healthy Chinese population. According to the 95% confidence limit, the reference value of plasma MPO in healthy Chinese population should be 90.53ng/ml.

The MPO concentrations of healthy male and female Chinese were 36.99±26.08ng/ml and 39.03±29.73ng/ml, respectively. With the age growing, the MPO levels in healthy population were progressively increased. We further compared the MPO concentration of different age groups, in male and female population separately, and found that the differences among different age groups were statistically significant (F=9.939 VS F=4.185, P<0.01). While comparing the MPO level between healthy male and female population, there was no significant differences between them (t=1.047, P>0.05). In the population less than 60 years old, the MPO level was higher in female than in male. However, in people over 60 years old, this relationship is reversed and the MPO concentration in male became slightly higher than in female.

Table 2. MPO concentration of different age groups in male and female

	Age(years)	N	Mean±SD (ng/ml)	Median [5th, 95th percentile] (ng/ml)
Female	<30	69	29.15±21.16	20.97[8.08, 85.40]
	30~40	70	31.39±28.13	21.67[5.00, 93.05]
	40~50	75	40.45±31.77	26.28[11.25, 108.76]
	50~60	93	44.63±32.15	36.15[11.66, 107.95]
	60~70	47	44.71±32.04	36.75[18.45, 94.95]
	>70	44	45.68±27.35	41.2[15.77, 87.58]
	Total	398	39.03±29.73	29.85[19.9, 96.88]
Male	<30	73	23.36±13.24	18.99[11.66, 47.94]
	30~40	77	29.78±19.72	20.97[10.44, 70.67]
	40~50	75	37.10±24.24	31.60[10.31, 84.57]
	50~60	99	42.91±29.14	32.74[12.13, 92.51]
	60~70	49	45.34±32.62	32.6[15.47, 119.03]
	>70	49	48.16±29.37	40.37[20.35, 88.77]
	Total	422	36.99±26.08	29.36[11.67, 87.22]

The MPO concentration of healthy male and female Chinese population of different age groups was show in Table 2 and Figure 2. Calculated according to the formula 3 and formula 4, we got the results of Z and Z^* were 1.25 and 5.55. At the same time, the standard deviation of the female group (29.73) did not exceed 1.5 times the male group (26.08). According to the document C28-A2, there was no need to calculate separate reference intervals for each subgroup.

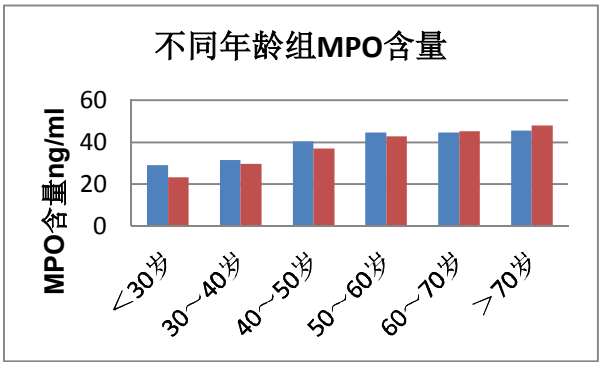

Figure 2: The plasma MPO levels in different age groups

MPO concentration of different age groups in male and female. A) As the age growing, the MPO levels in healthy population were increased progressively. The differences among age groups were statistically significant (F=9.939 VS F=4.185, P<0.01). B) The MPO level in male and female population was similar (t=1.047, P>0.05). In the population less than 60 years old, the MPO level was higher in female than in male. However, in people over 60 years old, this relationship is reversed and the MPO concentration in male became slightly higher than in female.

The plasma MPO levels of healthy Chinese population in different age groups are shown in Table 3. As the age grows, the MPO levels in healthy individuals were progressively increased and the differences between age groups were statistically significant (F=13.022, P<0.001). However, according to the NCCLS document C28-A2, there is also no need to set separate reference intervals for each subgroup. In accordance with the 95% confidence limit, the reference value of plasma MPO in healthy Chinese population should be 90.53ng/ml.

Table 3. The plasma MPO concentration in 820 healthy Chinese individuals

Age(years)	N	Mean±SD (ng/ml)	Median [5th, 95th percentile] (ng/ml)
<30	142	25.65±16.66	19.41[10.62, 66.10]
30~40	147	30.54±24.02	21.59[5.66, 77.67]
40~50	150	38.77±28.23	27.48[10.31, 92.78]
50~60	192	43.74±30.56	33.69[12.10, 94.80]
60~70	96	45.03±32.17	36.45[18.26, 119.03]
>70	93	47.31±26.99	40.37[16.18, 87.58]
Total	820	37.98±27.92	29.68[10.68, 90.53]

Discussion

Myeloperoxidase (MPO) is a member of the peroxidase superfamily. It is primarily hosted in human polymorphonuclear neutrophils (PMN) and release from these cells into the circulation [4]. MPO has been proved to be linked to events that participate in the initiation and progression of plaque formation including lipid peroxidation, generation of atherogenic lipoproteins and dysfunctional HDL, and catalytic consumption of nitric oxide (NO) [5]. For example, MPO-derived hypochlorous acid (HOCl) can interfere with intracellular signaling events and oxidize lipoproteins, such as LDL and HDL [6]. MPO-mediated depletion of endothelial derived nitric oxide impairs endothelium-dependent vasodilatation and causes vascular dysfunction which is an early risk factor of atherosclerosis, and nitrotyrosine (NO2Tyr) formation by MPO sequestered into the vessel wall may affect matrix protein structure and function [7]. Reactive nitrogen species generated by MPO can convert LDL into a form (NO2-LDL) that is avidly taken up and degraded by macrophages, leading to massive cholesterol deposition and foam cell formation in the vessel walls, and take part in essential steps in atherosclerotic lesion development. Myeloperoxidase can further selectively modify apolipoprotein A-I and result in the generating of dysfunctional high-density lipoprotein (HDL) whose cholesterol transport ability is impaired [8]. The abnormal cholesterol and HDL ratio easily lead to plaque instability, even rupture. MPO level in the circulation is already proved to be an independent marker of plaque stability. MPO levels are

higher in patients with coronary heart disease (CHD) and can predict future cardiovascular events in these patients and patients with chest pain [9]. Therefore, MPO can be used as a new independent risk factor for CHD: it can predict the development and outcome of CHD.

Our study showed that there were differences in the plasma MPO concentration in different gender and age of healthy Chinese population. However, the differences did not meet the requirements of setting particular reference intervals. According to the method required by CLSI document C28-A2 [10], we have established a reference value (90.53ng/ml) for plasma MPO concentration in healthy Chinese individuals, which might provide a scientific basis for the further application of MPO in the clinical practice.

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1/7/2015