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# Trypsin, elastase, plasmin and MMP-9 activity in the serum during the human ageing process

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## Abstract

**Objective:** the aim of this work was to define the influence of the ageing process on the activity of proteolytic enzymes, such as trypsin, elastase, plasmin and active MMP-9 concentration, as well as the inhibitor α 1-antitrypsin. Moreover, we assessed associations between enzyme activity and selected clinical and biochemical parameters.
**Methods:** healthy normotensive volunteers ($n = 60$, 30 women) aged 20–82 years were split into subgroups: young (aged 20–22), middle-aged (49–52) and elderly (77–82). Serum enzyme activity was assessed using fluorometric methods.
**Results:** overall, active MMP-9 concentration and trypsin activity decreased with age, and α1-antitrypsin concentration and plasmin activity increased. Activity of elastase increased with age when compared to the young age group. An inverse correlation was identified between MMP-9 concentration and BMI and a direct correlation found between BMI and elastase, plasmin activity and α1-antitrypsin concentration. In the middle-aged group, glucose correlated directly with trypsin activity and inversely with MMP-9 concentration. Trypsin activity and MMP-9 concentration correlated inversely with cholesterol concentration and plasmin and elastase activity, and the α1-antitrypsin concentration correlated with cholesterol concentration in the overall group.
**Conclusions:** the results confirm the influence of the ageing process on the activity of serum proteolytic enzymes. The activity of individual proteolytic enzymes in the serum changes with age.

Keywords: *ageing trypsin, plasmin, elastase, metalloproteinases,*

## Introduction

In humans, ageing is a physiological process of a gradually advancing accumulation of characteristic structural changes in cells, tissues and organs. The changes cause functional disorders and contribute to the disability of adaptation skills, an increased susceptibility to illnesses and a growing probability of death [1]. Ageing is characterised by an increase in the amount of cross-links in proteins and the development of aggregates resistant to the activity of proteolytic enzymes, which leads to the accumulation [2]. A change in the susceptibility of proteins to proteolysis and, above all, damage to proteolytic systems responsible for the elimination of these proteins, are seen as the causes of this occurrence [3]. A few separate proteolytic systems take part in the intra-cellular degradation of proteins. They differ in sub-cellular localisation and substrate specificity.

Plasmin, trypsin and elastase belong to the serine protease group. Serine proteases are proteolytic enzymes that contain a highly reactive serine residue. Plasmin intensifies the degradation of the extra-cellular matrix proteins directly and indirectly by activating the matrix metalloproteinases (MMPs) MMP-3, MMP-9, MMP-1 and MMP-2 [4, 5]. The accumulation of the products of plasmin breakdown and extra-cellular vessel wall matrix degradation plays an important role in the pathogenesis of blood vessel ageing [6]. Moreover, an increase in concentrations of plasmin–α2-antiplasmin complexes has been observed during the process of ageing and in people with acute coronary syndrome [7].

Trypsin is synthesised by the exocrine pancreas in the form of a proenzyme, and in serum, it is associated with the protease inhibitors α1-antitrypsin and α2-macroglobulin. The trypsin–α2-macroglobulin complex is not degraded by other proteases and at the same time retains its enzymatic activity [8]. Trypsin hydrolyses both proteins present in food and extra-cellular matrix proteins by indirectly activating latent forms of many MMPs, including MMP-9, MMP-8 and MMP-1 [9, 10].

Elastase works in inter-cellular spaces and a micro-environment and is secreted by smooth muscle cells, fibroblasts, endothelium cells and white blood cells [11]. The

substrates for granulocytic elastase are both extra-cellular matrix proteins such as elastin, fibronectin, laminin, types III, IV, VI collagen and proteoglycans, and also plasma proteins, including immunoglobulins, coagulation factors, α2-antiplasmin and bacterial proteins [12]. This enzyme digests the elastic fibres present in the arterial vessel walls, which results in an increased concentration of elastin degradation products in the serum. The elastic fibres are degraded during ageing and atherogenesis [13, 14].

The α1-antitrypsin, with a molecular mass of 51,000 kD, is an important inhibitor of serine proteases in the serum. It is synthesised by hepatocytes and macrophages as well as by epithelial cells in the bronchial tubes and is responsible for 90% of antitrypsin serum activity; however, its main function is to halt the elastase freed from the granulation of neutrophils [15].

MMPs are endopeptidases involved in many physiological and pathological processes, including the pathogenesis of various malignancies [16]. Moreover, MMP-9 catalyses post-translational activation of cytokines such as IL-1β, TNF-α, TGF-β and chemokines [17, 18].

The aim of this work was to assess the activity of proteolytic enzymes during human ageing and simultaneously to attempt to define the role of proteolytic enzymes in the development of the ageing process.

## Materials and methods

The study involved 60 healthy participants (30 women) aged 20–82 years. Participants had no history of chronic inflammatory diseases, chronic glomerular kidney inflammation, systemic connective tissue diseases, joint diseases, neurological diseases, neoplastic diseases, acute coronary syndromes or diabetes, and gave their written consent to take part in the study. History of tobacco smoking, alcohol consumption and arterial blood pressure was assessed. The study was conducted with approval by the Bioethical Commission at the Medical University of Warsaw (no. KB/248/2003). Study volunteers were divided into age subgroups: young (average age: 21.3 years; age range: 20–22), middle-aged (average age: 50.8; age range: 49–52) and elderly (average age: 79.7; age range: 77–82). Each subgroup had 10 women and 10 men. The study material was fasting venous blood from the cubital vein. For assessment of serum MMP-9, trypsin, elastase activity and α1-antitrypsin concentration, 30 min after the blood draw serum was removed after a 10-min centrifugation at $1,000 \times g$ in 4°C. Blood for measuring plasmin activity was drawn in heparin vials and centrifuged for 10 min at $1,000 \times g$ in 4°C. Serum and heparinised plasma samples were kept in −70°C until analysis. Blood was also collected for morphology and basic biochemical studies, such as ionogram, alkali reserve, transaminase and bilirubin levels, creatinine concentration, total cholesterol concentration, proteinogram, C reactive protein (CRP), concentration of fasting glucose and lipase concentration. BMI was assessed and arterial blood pressure measured.

### Analytical methods

The activity of trypsin was studied using the fluorometric method, applying the Z–Arg–AMC HCl substrate (Bachem, Biochemica GmbH Heidelberg, Germany). The samples were incubated in an activating buffer (50 mM TES/0.4 mM Z–Arg–AMC HCl, pH 8.0) for 60 min at room temperature and then measured on a spectrofluorometer (Perkin-Elmer LS-50B, USA) at an activation light wavelength $\lambda = 355$ nm and emitted light wavelength $\lambda = 460$ nm. A dilution of the AMC mother solution (20 mM in 5% $CH_3COOH$) in TES buffer in the range of 0.312–10 μM constituted the standard curve. Enzymatic activity is given as IU/l [19].

Elastase activity was also assessed using the fluorometric method with an Ac–Ala–Ala–Pro–Ala–AMC substrate (Bachem, Biochemica GmbH Heidelberg, Germany) [19], as was plasmin activity using the Boc–Val–Leu–Lys–AMC AcOH substrate (Bachem, Biochemica GmbH Heidelberg, Germany) [20].

The concentration of the active form of serum MMP-9 was measured by fluorometry with the Fluorokine E Active MMP-9 reagent set (R&D Systems, Wiesbaden, Germany). A fluorogenic substrate was used at 1 mM in DMSO, and recombined human proenzyme MMP-9 was the standard. *Para*-aminophenyl mercury acetate was the activator of the MMP-9 proenzyme. After a 20-h incubation at 37°C, the intensity of fluorescence was read on the spectrofluorometer (Perkin-Elmer LS-50B, Seer Green, UK) at the activation light wavelength $\lambda = 340$ nm and emission light wavelength $\lambda = 405$ nm. The concentration of active enzyme is given as μg/l.

### Statistical methods

Statistical calculations were made using SPSS version 12.0 PL, made available by the Warsaw University Department of Psychology. To perform the statistical assessment of the age groups of men and women, a student's *t*-test was applied for separate samples. In cases of three or more comparable groups, a simple single-factor variation analysis was used to assess the relevance of the differences, using the Smallest Relevant Difference test. The assessment of the intensity of the connection between the variables was performed using the Tau-Kendall correlation coefficient. The results were considered relevant at $P<0.05$.

## Results

Table 1 shows basic clinical and laboratory parameters. All results of biochemical and haematology studies fell within the reference value range. Clinical and biochemical data were compared in the studied subgroups, and *P*-values are given in the table, as well.

Table 2 shows proteolytic enzyme activity. The data were compared in the studied subgroups, and *P*-values are given in the table. There was overall dependence between proteolytic enzyme activity and the selected factors (aged 20–82).

Table 1. Basic clinical and laboratory parameters

| $n = 60$ | Subgroups (age) | | | $P$ | | |
|---|---|---|---|---|---|---|
| Age (years) average (range) | Young 21.3 (20–22) | Middle-aged 50.8 (49–52) | Elderly 79.7 (77–82) | Young versus middle | Middle-aged versus elderly | Young versus elderly |
| Haemoglobin (mmol/l) | 8.82 ± 0.76 | 8.78 ± 0.62 | 8.85 ± 0.48 | NS | NS | NS |
| Haematocrit (l/l) | 0.42 ± 0.03 | 0.40 ± 0.03 | 0.42 ± 0.03 | NS | NS | NS |
| Leukocytes (G/l) | 6.82 ± 1.46 | 6.3 ± 1.5 | 6.59 ± 1.45 | NS | NS | NS |
| Platelets (G/l) | 238.1 ± 41.05 | 228.6 ± 42.7 | 220.4 ± 40.27 | NS | NS | NS |
| Erythrocytes (T/l) | 4.82 ± 0.58 | 4.6 ± 0.29 | 4.66 ± 0.29 | NS | NS | NS |
| Na (mmol/l) | 142.2 ± 1.77 | 141.8 ± 2.37 | 142.7 ± 1.34 | NS | NS | NS |
| K (mmol/l) | 4.4 ± 0.24 | 4.4 ± 0.29 | 4.6 ± 0.35 | NS | NS | NS |
| Alkali reserve (mmol/l) | 26.5 ± 2.3 | 26.7 ± 1.7 | 28.0 ± 1.23 | NS | 0.01 | 0.01 |
| Creatinine (μmol/l) | 75.14 ± 15.9 | 69.84 ± 10.6 | 82.21 ± 8.8 | NS | 0.001 | NS |
| Glucose (mmol/l) | 4.95 ± 0.48 | 4.92 ± 0.38 | 5.29 ± 0.27 | NS | 0.001 | 0.01 |
| ASPAT (IU/l) | 27.2 ± 6.01 | 25.5 ± 8.58 | 24.25 ± 5.3 | NS | NS | NS |
| ALAT (IU/l) | 26.6 ± 7.87 | 27.3 ± 8.46 | 22.05 ± 7.01 | NS | 0.04 | NS |
| Bilirubin (μmol/l) | 11.8 ± 4.7 | 12.5 ± 4.6 | 13.3 ± 3.8 | NS | NS | NS |
| ALP (IU/l) | 71.9 ± 16.0 | 184.1 ± 40.7 | 193.4 ± 51.30 | 0.001 | NS | 0.001 |
| Lipase (IU/l) | 219.8 ± 32.5 | 45.2 ± 21.1 | 31.4 ± 15.3 | 0.001 | 0.02 | 0.001 |
| CRP (mg/l) | 1.14 ± 0.31 | 2.31 ± 1.04 | 1.76 ± 0.82 | 0.001 | NS | 0.01 |
| Ca (mmol/l) | 2.46 ± 0.08 | 2.39 ± 0.08 | 2.46 ± 0.10 | 0.01 | 0.02 | NS |
| Cholesterol (mmol/l) | 4.50 ± 0.51 | 5.96 ± 0.86 | 5.72 ± 0.70 | 0.001 | NS | 0.001 |
| Albumin (g/l) | 47.9 ± 3.5 | 48.3 ± 3.3 | 47.5 ± 1.7 | NS | NS | NS |
| Total proteins (g/l) | 78.5 ± 2.2 | 76.1 ± 3.6 | 78.6 ± 1.4 | 0.02 | 0.01 | NS |
| Globulins (g/l) | 10.9 ± 2.0 | 10.3 ± 2.0 | 11.1 ± 1.3 | NS | NS | NS |
| BMI ($kg/m^2$) | 22.55 ± 1.32 | 25.41 ± 1.32 | 25.56 ± 1.33 | 0.001 | NS | 0.001 |
| Systolic arterial pressure (mmHg) | 110 ± 10.1 | 125.7 ± 5.2 | 127.5 ± 5.7 | 0.001 | NS | 0.001 |
| Diastolic arterial pressure (mmHg) | 68.5 ± 6.9 | 76.7 ± 4.4 | 75.5 ± 4.6 | 0.001 | NS | 0.001 |

Values as mean ± SD; NS, not significant; ASPAT, asparagine aminotransferase; ALAT, alanine aminotransferase; ALP, alkaline phosphatase; CRP, C reactive protein; BMI, body mass index.

Table 2. Proteolytic enzyme activities and serum concentration of MMP-9 and of α1-antitrypsin

| $n = 60$ | Subgroups (age) | | | $P$ | | |
|---|---|---|---|---|---|---|
| Age (years) average (range) | Young 21.3 (20–22) | Middle-aged 50.8 (49–52) | Elderly 79.7 (77–82) | Young versus middle-aged | Middle-aged versus elderly | Young versus elderly |
| Trypsin (IU/l) | 0.376 ± 0.084 | 0.291 ± 0.107 | 0.201 ± 0.09 | NS | 0.01 | 0.001 |
| Elastase (IU/l) | 0.086 ± 0.01 | 0.125 ± 0.01 | 0.102 ± 0.01 | 0.001 | 0.001 | 0.001 |
| Plasmin (IU/l) | 0.183 ± 0.009 | (−) | 0.261 ± 0.014 | (−) | (−) | 0.001 |
| MMP-9 (μg/l) | 198.0 ± 58.0 | 137.0 ± 14.0 | 127.0 ± 13.0 | 0.001 | 0.03 | 0.001 |
| α1-antitrypsin (g/l) | 1.397 ± 0.064 | 1.701 ± 0.054 | 1.895 ± 0.046 | 0.001 | 0.001 | 0.001 |

Values as mean ±SD; MMP, matrix metalloproteinase; (−),test not performed; NS, not significant.

Figure 1A–D shows the significant statistical correlations among MMP-9 concentration, elastase activity, serum α1-antitrypsin concentration, plasmin activity and BMI in the study group. A statistically important correlation was identified among elastase activity, the MMP-9 concentration, serum α1-antitrypsin concentration, plasmin and systolic and diastolic blood pressure (Appendix 1A–D, 2A–C, see the journal's website http://www.ageing.oxfordjournals.org). No important correlation was noted between blood pressure and serum trypsin activity. A statistically important dependence was identified between glucose concentration and MMP-9 concentration and serum α1-antitrypsin concentration (Appendix 3A–B, see the journal's website http://www.ageing.oxfordjournals.org). No significant association was identified between serum glucose concentration and activities of elastase, plasmin or trypsin. Significant statistical correlations were identified between the activity of elastase, trypsin, plasmin, MMP-9 concentration, and α1-antitrypsin and the serum total cholesterol (Appendix 4A–E, see the journal's website http://www.ageing.oxfordjournals.org).

## Discussion

The exact mechanisms leading to the ageing of an organism remains unknown, but there is increasing evidence of the role of proteolytic enzymes in the pathology of diseases that accompany age, such as atheromatosis, cataracts, degenerative joint disease, neoplasms and cardiovascular

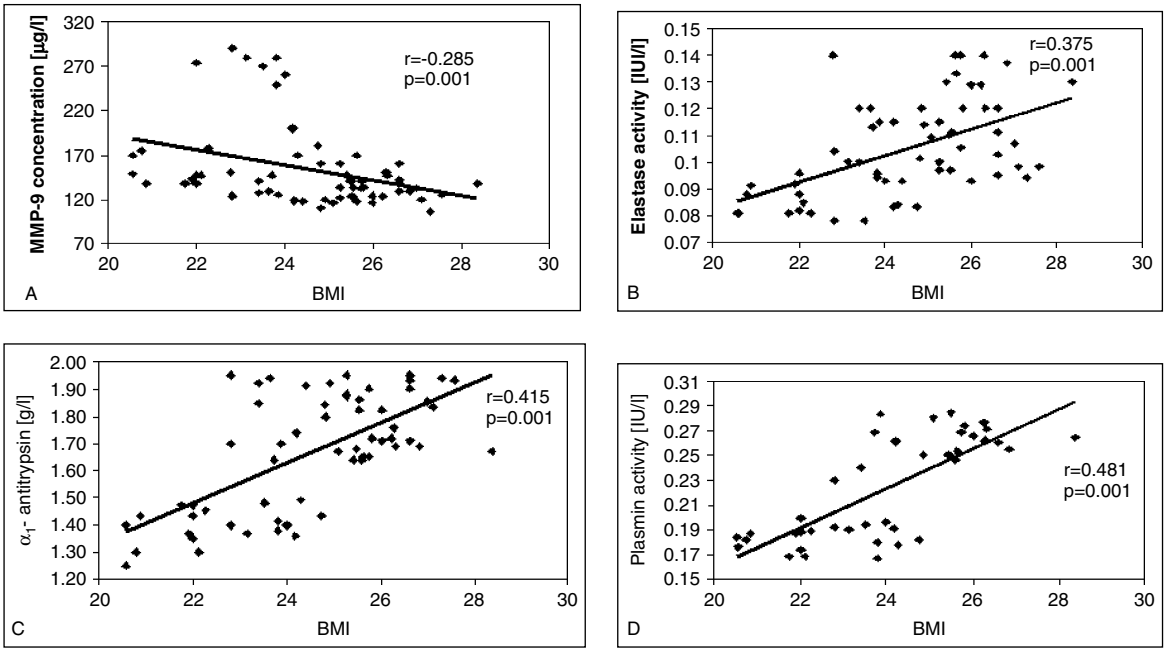


Figure 1. Concentration of MMP-9 (A) and α1-antitrypsin (C) and activity of elastase (B) in the serum and activity of plasmin (D) in the plasma in relation to BMI.

system diseases. The aim of this work was to define the influence of the process of ageing on the proteolytic enzyme activity in the serum. Many authors have found a role, for example, for elastase in processes associated with ageing, including its role in atherogenesis and atheromatotic changes [21], aneurysms [22], and ischaemic heart disease [23].

On the basis of the recent reports in the literature [22, 23] and on the current results, it can be inferred that a decrease in serum elastase activity (when compared between the middle-aged and elderly group) and increase in serine protease inhibitor concentration may serve as a marker of arterial vessel wall inflammatory change. Such changes are characteristic of atheromatosis, a chronic process that develops with age. Available data lead to the hypothesis that intensive secretion of elastase in vessel walls undergoing atheromatosis may lead to its loss and to decreased serum activity. Our current results suggest that an increase in α1-antitrypsin concentration with age halts elastase activity in the serum.

The middle-aged group exhibited a statistically significant higher trypsin activity than the elderly group. A study assessing the age-dependent activity of trypsin in brain structures found that activity decreases with age in the cortex, cerebellum, striatum and pale globe of mice and rats in old age compared to young mice and rats. Changes in the activity of this enzyme may play a role in the pathogenesis of Parkinson's disease [24]. The decrease in trypsin activity in the serum with age may arise from an increase in α1-antitrypsin activity, the serine protease inhibitor.

Plasmin is the main enzyme involved in the fibrinolysis process. In cardiovascular diseases, D-dimer concentrations are diagnostically quite relevant. D-dimer concentration increases with age; higher concentrations in middle-aged men are associated with increased risk of ischaemic heart disease, and the age increase is also statistically relevant for people with atheromatosis. High D-dimer levels have also been observed in atheromatosis of coronary, cerebral and peripheral arteries [25]. The current results and previous findings suggest that the increase in plasma plasmin activity reflects increased activation of blood clotting and fibrinolysis and may serve as an atheromatosis development marker and marker for cardiovascular system diseases, both characteristic illnesses of old age.

We also identified a statistically relevant inverse relationship between both systolic and diastolic pressure and serum MMP-9 concentration. With increase in arterial pressure, the MMP-9 concentration decreased. However, the relationship of elastase, plasmin activity and the α1-antitrypsin concentration with systolic and diastolic pressure was positive. No correlation was noted between arterial pressure and trypsin activity.

Other studies have demonstrated a relationship between elastase and MMP-9 activity and atheromatosis (see [26, 27]). These enzymes take an active part in the remodelling of vessel walls and play an important role in the pathogenesis of cardiovascular system diseases. On the basis of previous reports and current findings, it can be inferred that proteolytic enzymes are actively involved in the remodelling of vessel walls, causing stiffness in them and contributing to the development of hypertension.

A directly proportional dependence was found between BMI and the activity of elastase and α1-antitrypsin, but no relationship between BMI and trypsin activity was found.

One large study of 1,400 patients with cardiovascular disease demonstrated that the activity of elastase in the serum was directly proportional to the BMI and glucose and inverse proportional to triglycerides, while the concentration of α1-antitrypsin was inversely correlated with total cholesterol concentration. The authors concluded that the remodelling of vessel walls depends not only on the degradation of elastin but also on lipid metabolism [28].

In addition, an inverse relationship between glucose concentration and MMP-9 concentration was identified, as was a directly proportional dependence between α1-antitrypsin concentration and glucose concentration. No relationship between glucose concentration and the activity of the other enzymes was found. Other studies have found links between ageing, decreased metalloproteinase and proteolytic enzyme activity, and pathogenesis of renal fibrosis. One study found that the decreased activity of proteolytic enzymes is conducive to protein accumulation of renal glomeruli of the extra-cellular matrix, which may be one of the causes of renal failure [29, 30]. Another group showed *in vitro* that trypsin decreases protein degradation, corrects cell proliferation, and increases protein synthesis and its content in the cell. On the basis of their results, these authors hypothesised that trypsin decreases glycolysation of proteins on cell surface. Trypsin decreases the advanced glycation end products–receptor for advanced glycation end products interaction due to cleavage of the external domain of receptor for advanced glycation end products [31, 32]. These findings suggest that the decrease in the activity of proteolytic enzymes with age may influence the pathogenesis of kidney diseases.

The current results confirm the influence of the ageing process on the activity of proteolytic enzymes in the serum. With age, the activity of individual proteolytic enzymes in the serum changes.

## Key points

- Concentration of active MMP-9 and activity of trypsin decreased with age.
- Plasmin activity and concentration of α1-antitrypsin increased during ageing process.
- Significant correlation was identified between activity of the proteolytic enzymes and clinical and biochemical parameters.

## Conflicts of interests

No conflicts of interest

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## Supplementary data

Supplementary data for this article are available online at http://ageing.oxfordjournals.org.

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