Int J Hematol (2014) 99:714–720
DOI 10.1007/s12185-014-1581-3

ORIGINAL ARTICLE

Peripheral blood hematopoietic stem and progenitor cell frequency is unchanged in patients with alpha-1-antitrypsin deficiency

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Received: 22 November 2013 / Revised: 2 April 2014 / Accepted: 3 April 2014 / Published online: 22 April 2014

Abstract Granulocyte-colony-stimulating factor (G-CSF)-induced hematopoietic stem and progenitor cell (HSPC) mobilization is associated with the release of neutrophil-derived proteases. Previously, we have shown that alpha-1-antitrypsin (AAT) inhibits these proteases in mice, resulting in inhibition of HSPC mobilization. Here, we studied the relationship between AAT and HSPC in steady state and cytokine-induced mobilization in humans. Patients with alpha-1-antitrypsin deficiency (AATD) have an 85–90 % decrease of AAT in the peripheral blood (PB). We hypothesized that this leads to increased proteolytic activity in the bone marrow and increased steady-state PB HSPC numbers. Using flow cytometry and semi-solid cell culture, we found no significant difference in PB HSPC in AATD patients ($n = 18$) as compared to controls ($n = 22$). Healthy stem cell donors ($n = 43$) were mobilized with G-CSF for 5 days and the number of $CD45^+/CD34^+$ HSPC were determined in PB. We found that, during mobilization, PB AAT levels increased significantly, positively correlating with PB $CD45^+/CD34^+$ cells ($r = 0.31$, $p = 0.005$). In conclusion, although serum AAT levels and HSPC mobilization in healthy stem cell donors are positively correlated, AAT is not an indispensable protease-inhibitor in the constitutive circulation of HSPC. These findings suggest a model in which both protease-dependent and -independent pathways contribute to HSPC mobilization.

Keywords Stem cells · Alpha-1-antitrypsin deficiency · Hematopoietic stem cell mobilization · Granulocyte-colony-stimulating factor

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Introduction

Granulocyte-colony-stimulating factor (G-CSF) mobilized stem and progenitor cells (HSPC) are routinely used as a source for clinical stem cell transplantation. In recent years, research has started to unravel the mechanisms underlying cytokine-induced HSPC mobilization. G-CSF administration induces expansion and activation of hematopoietic progenitor cells in the bone marrow, which leads to increased numbers of neutrophils [1, 2]. Upon activation, neutrophils release proteases, including neutrophil elastase (NE), cathepsin G (CG) and matrix metalloproteinase-9 (MMP-9) [3–6]. These proteases degrade adhesive interactions between HSPC and their microenvironment, including CXCR4/CXCL12, VLA-4/VCAM-1 and c-KIT/SCF [5, 7, 8]. Levels of CXCL12, a chemokine that attracts HSPC, are down regulated in the BM, which induces a gradient, allowing HSPC to mobilize towards the peripheral blood [8]. Furthermore, administration of G-CSF induces apoptosis of trophic endosteal macrophages (osteomacs) that support osteoblast function [9]. These osteoblasts are an important constituent of the stem cell niche and play a critical role in HSPC maintenance, via soluble and membrane-bound factors such as CXCL12 and SCF [10]. During G-CSF-induced HSPC mobilization, expression of alpha-1-antitrypsin (AAT) is strongly reduced in

osteoblasts, both at the protein and mRNA levels [11, 12]. Alpha-1-antitrypsin is a potent inhibitor of serine proteases, including NE and CG. Decreased levels of AAT are associated with an accumulation of active proteases in the bone marrow compartment, resulting in HSPC mobilization from the bone marrow towards the peripheral blood [12]. Increased levels of AAT, either by induction via low-dose irradiation or via intraperitoneal administration of exogenous AAT, result in a near-complete inhibition of HSPC mobilization in mice [13]. In the peripheral blood of G-CSF-mobilized healthy individuals, proteases such as MMP-9 and NE, are significantly increased as compared to non-mobilized donors [14–16]. Thus far, peripheral blood levels of AAT during G-CSF-induced HSPC mobilization in humans have not been studied.

The importance of the balance between proteases and protease-inhibitors in retention and mobilization of HSPC suggests that modulation of AAT levels could be a novel strategy to induce HSPC mobilization. To test this hypothesis, we studied the presence of HSPC in the peripheral blood of patients with alpha-1-antitrypsin deficiency (AATD). AATD patients have a mutation in the gene responsible for the expression of AAT, SERPINA1, resulting in severely decreased serum levels of AAT as only 10–15 % of AAT is released into the circulation. As a result, the lungs of these patients are exposed to enzymatic damage by proteases such as NE, leading to early-onset emphysema and bronchiectasis [17–19]. Such a constitutively decreased AAT level, as observed in AATD patients, could lead to a protease/protease-inhibitor imbalance with elevated protease activity in the bone marrow. Increased proteolytic activity may degrade adhesive interactions between HSPC and their environment and as a result steady-state peripheral blood HSPC frequencies could be affected in these patients. Therefore, we determined the number of $CD45^+/CD34^+$ cells, as well as the frequency of colony-forming cells in the peripheral blood in a cohort of AATD patients.

In addition, to determine the relationship between serum AAT levels and mobilizing capacity, we investigated HSPC frequencies in the peripheral blood of G-CSF-mobilized healthy donors and correlated this with serum AAT levels.

Materials and methods

AATD patients

During routine follow-up visits to the pulmonology outpatient clinic, blood samples were taken from patients with AATD. All samples were taken between 8 a.m. and 10 a.m. to minimize the possible effect of the circadian rhythm on HSPC mobilization [20]. All patients had a ZZ-phenotype (PI*ZZ) as confirmed by isoelectric focusing. Healthy volunteers and patients with chronic obstructive pulmonary disease (COPD) levels were used as controls. Individuals with a C-reactive protein (CRP) level $\geq$5 μg/ml or non-MM phenotype were excluded from the analysis. Blood samples were analyzed for AAT level, AAT phenotype and HSPC frequency. The levels of two other physiologically important protease-inhibitors, alpha-2-macroglobulin (A2M) and secretory leukocyte proteinase inhibitor (SLPI), were also determined [21].

Human donors undergoing G-CSF-induced stem cell mobilization

Healthy allogeneic peripheral blood stem cell donors were mobilized by injection of recombinant-human-G-CSF (Filgrastim; Amgen, Thousand Oaks, CA), which was given subcutaneously at a dose of 10 μg/kg on days 1–3. On day 4, two doses of 10 μg/kg were given, one in the morning and one in the evening approximately 12 h before the onset of apheresis on day 5. On days 3, 4, and 5, blood samples were taken prior to G-CSF administration to determine white blood cell counts, neutrophil counts and the frequency of $CD34^+$ cells, as described previously [16]. Plasma samples were collected to determine AAT levels. All donors gave informed consent and the local ethical committee approved this protocol.

Determination of peripheral blood HSPC

Peripheral blood HSPC were determined by flow cytometry and by HSPC culture. For flow cytometric analysis, peripheral blood mononuclear cells (PBMC) were co-stained with conjugated monoclonal antibodies against CD45 (PE, clone HI30) and CD34 (FITC, clone 8G12) after red blood cell lysis with NH_4Cl (LUMC Pharmacy, Leiden). Antibodies were obtained from BD PharMingen, San Diego, CA. HSPC were defined as the $FSC^{lo}/SSC^{lo}/CD45^+/CD34^+$ fraction.

To determine the frequency of HSPC by semi-solid cell culture, 2.0×10^5 PBMC, obtained after Ficoll separation of heparinized whole blood, were plated in 3.5-cm dishes according to the manufacturer's recommendations (Methocult® H4434 Classic, Stemcell Technologies, Grenoble, France). After 12–14 days of culture in a fully humidified atmosphere of 37 °C containing 5 % CO_2, the number of colonies was scored using an inverted light microscope. Colonies were scored according to the type of progenitors they contained: granulocytes (CFU-G), monocytes (CFU-M), granulocytes and monocytes (CFU-GM), granulocytes, monocytes and erythroid progenitors (CFU-GEMM) or erythroid progenitors only [CFU-E and burst-forming colony (BFU)-E].

Determination of plasma levels of protease-inhibitors and AAT phenotype

Serum levels of AAT were measured quantitatively by radial immunodiffusion using commercially available plates (NOR Partigen, Siemens, Germany). A2M and SLPI levels were measured using an enzyme-linked immunosorbent assay using a polyclonal rabbit anti-human A2M antibody (DAKO, Glostrup, Denmark) and human anti-SLPI antibody (clone 31, Hycult Biotech, Uden, The Netherlands), respectively.

AAT phenotype was determined by isoelectric focusing; subjects were divided in having a MM-phenotype (normal), ZZ-phenotype (AATD patients) or other phenotype (e.g., MZ or SS).

Statistical analysis

Statistical differences were determined using GraphPadPRISM (GraphPad Software, San Diego, CA). Data are presented as mean values ± standard error of the mean (SEM); p Values of <0.05 were considered statistically significant. Spearman's correlation coefficient (r) was used to describe the strength of association between two ranked variables.

Results

Hematopoietic stem cell frequencies in the peripheral blood of AATD patients and healthy controls are similar

To investigate the relationship between serum levels of peripheral blood AAT and steady-state peripheral blood HSPC frequencies, patients with AATD were compared with healthy volunteers. To exclude the possibility that the results would be biased by the presence of chronic lung disease alone, a second control group consisting of patients with COPD was included. In total, 20 healthy controls, 21 patients with AATD and 12 patients with COPD were studied. In the healthy control group, 6 subjects were excluded from the analysis ($n = 3$ due to a non-MM phenotype; $n = 3$ due to a CRP level ≥5 μg/ml). In the AATD patient group and in the COPD patient group, 3 and 4 subjects, respectively, were excluded because of a CRP level ≥5 μg/ml. Therefore, 18 patients in the AATD group, 14 healthy controls and 8 COPD patients were available for further analysis. Patient characteristics were comparable, with the exception of age, which was higher in the COPD patient group (Table 1). As expected, serum AAT levels in AATD patients were significantly decreased compared to both control groups (0.21 ± 0.04 vs. 1.80 ± 0.32 mg/ml, respectively, $p < 0.0001$). No significant differences were observed in A2M or SLPI levels between healthy controls and AATD patients (Fig. 1a, b).

Table 1 Patient characteristics of AATD patients and control groups

	n	Age (years, mean ± SD)	Age (years, range)	PI phenotype
AATD patients	18	52.3 ± 12.2	22–69	ZZ
Healthy controls	14	39.3 ± 11.6	22–61	MM
COPD patients	8	63.9 ± 6.0	55–71	MM

Subsequently, the absolute number of $CD45^+/CD34^+$ cells was determined by flow cytometry. No significant difference was observed for the AATD patients as compared to both control groups (AATD patients $3.2 \times 10^{-3} \pm 2.0 \times 10^{-3}$/ml, healthy controls $2.2 \times 10^{-3} \pm 0.9 \times 10^{-3}$/ml, COPD patients $2.2 \times 10^{-3} \pm 2.7 \times 10^{-3}$/ml, $p = 0.12$ for comparison between AATD patients and healthy controls, $p = 0.35$ for comparison between AATD patients and COPD patients; Fig. 1c).

Next, CFU-C frequencies were determined by colony assays in semi-solid cultures. No significant difference was observed between the three groups with respect to the peripheral blood frequency of CFU-G/M/GM/GEMM (AATD patients 281.0 ± 178.0 colonies/10^6 PBMC, healthy controls 259.5 ± 75.8 colonies/10^6 PBMC and COPD patients 223.4 ± 96.5 colonies/10^6 PBMC; $p = 0.67$ for comparison between AATD patients and healthy controls, $p = 0.40$ for comparison between AATD patients and COPD patients; Fig. 1d). However, the peripheral blood frequency of BFU-E was significantly increased in the AATD patients group as compared to healthy controls (AATD patients 357.2 ± 167.8 colonies/10^6 PBMC, healthy controls 159.3 ± 67.1 colonies/10^6 PBMC), but was lower than BFU-E frequencies in COPD patients (612.5 ± 464.0 colonies/10^6 PBMC, $p < 0.05$ for comparison between all groups; Fig. 1e). No significant correlation was found between the serum AAT level, the absolute number of $CD45^+/CD34^+$ cells ($r = -0.18$, $p = 0.53$) and the total number of CFU-G/M/GM/GEMM ($r = -0.19$, $p = 0.50$; data not shown).

In conclusion, very low serum levels of AAT are not associated with changes in steady-state HSPC frequencies in the peripheral blood of patients with AATD.

AAT serum levels are increased during G-CSF-induced HSPC mobilization in healthy human donors

To investigate the effect of G-CSF administration on AAT levels in peripheral blood, 43 healthy human HSPC donors (mean age 40.9 years, range 16–65 years; 26 males, 17 females) were treated with G-CSF for 4 consecutive days. On days 3, 4 and 5 serum AAT levels and $CD45^+/CD34^+$

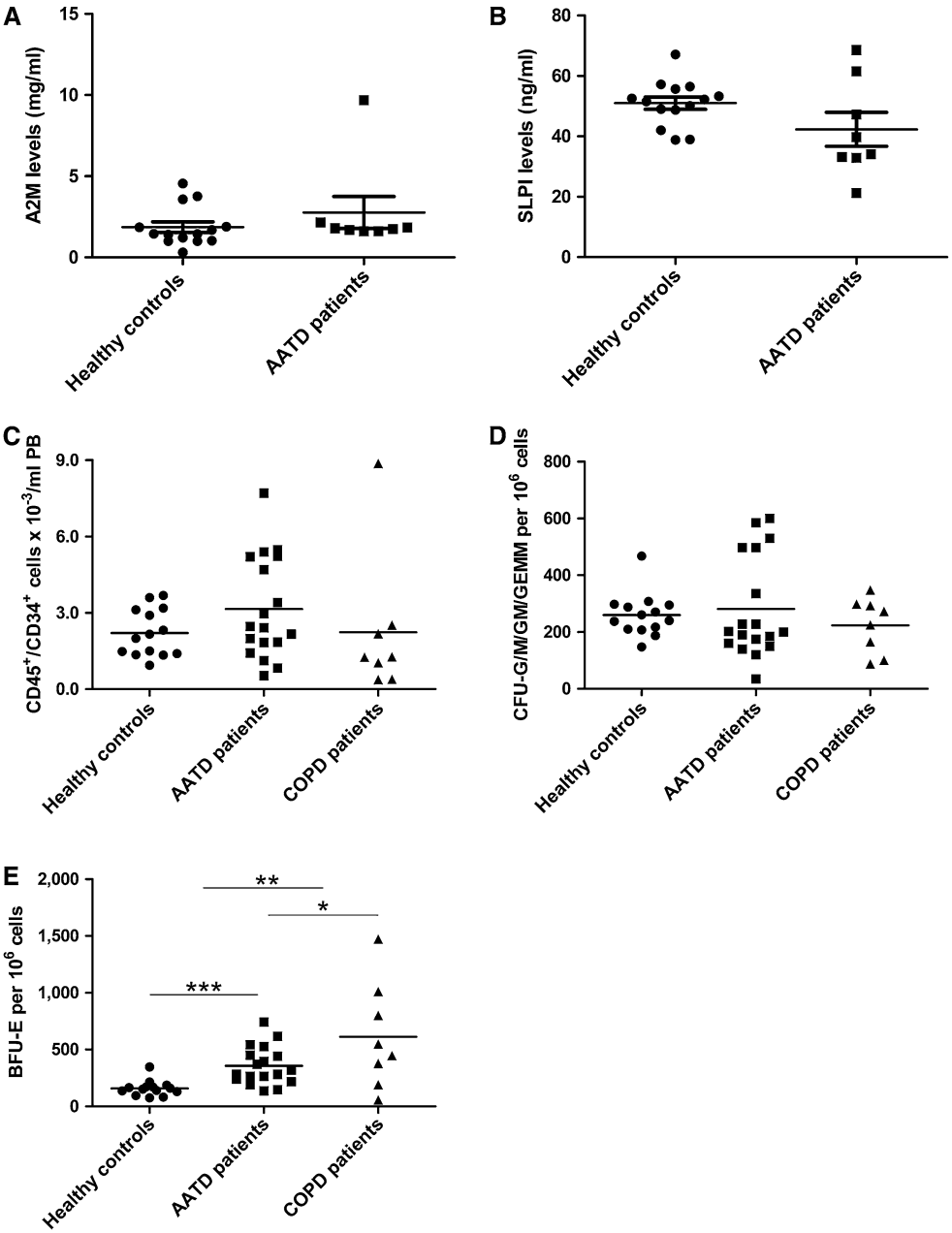

Fig. 1 Hematopoietic stem cell frequencies in the peripheral blood of AATD patients are not significantly different as compared to healthy volunteers and patients with COPD. No significant differences were observed in **a** A2M or **b** SLPI levels in peripheral blood serum obtained from healthy controls and AATD patients. **c** The absolute number of $CD45^+/CD34^+$ cells/ml peripheral blood was determined by FACS analysis. AATD patients are compared with healthy controls and COPD patients ($p \geq 0.10$). The frequency of HSPC in the peripheral blood was obtained by semi-solid cell culture of 2.0×10^5 PBMC. Colonies were manually scored according to the type of progenitors they contained: **d** myeloid (CFU-G/M/GM/GEMM) or **e** erythroid progenitors (BFU-E) (*$p = 0.048$, **$p = 0.016$ and ***$p = 0.0003$)

numbers in peripheral blood were determined. In peripheral blood, $CD45^+/CD34^+$ numbers increased significantly from 14.2×10^3/ml $\pm$ 10.5×10^3/ml on day 3 to 99.9×10^3/ml $\pm$ 56.0×10^3/ml on day 5 ($p < 0.0001$ for day 3 vs. day 5; Fig. 2a). Similarly, AAT levels increased significantly from day 3 to day 5 of G-CSF administration (AAT 1.40 mg/ml $\pm$ 0.20 mg/ml, 1.47 mg/ml $\pm$ 0.22 mg/ml and 1.54 mg/ml $\pm$ 0.25 mg/ml on day 3, 4 and 5, respectively; $p < 0.0001$ for day 3 vs. day 5; Fig. 2b). To examine whether $CD45^+/CD34^+$ numbers and AAT levels were correlated, a Spearman correlation test was applied. A higher number of peripheral blood $CD45^+/CD34^+$ cells was associated with a higher level of serum AAT ($r = 0.31$, $p = 0.005$; Fig. 2c).

Neutrophil numbers and neutrophil elastase levels in the peripheral blood are positively correlated with the extent of HSPC mobilization [16]. Therefore, we investigated the relationship between neutrophil numbers, neutrophil elastase and AAT levels. An increase in neutrophil numbers is positively associated with a concurrent increase in AAT levels ($r = 0.49$, $p < 0.0001$; Fig. 2d). Furthermore, a concomitant increase was observed for white blood cell counts and AAT. No correlation was found for MMP-9 levels and AAT.

In conclusion, G-CSF-induced HSPC mobilization in human donors is associated with a concomitant increase in peripheral blood AAT levels between days 3 and 5 of mobilization.

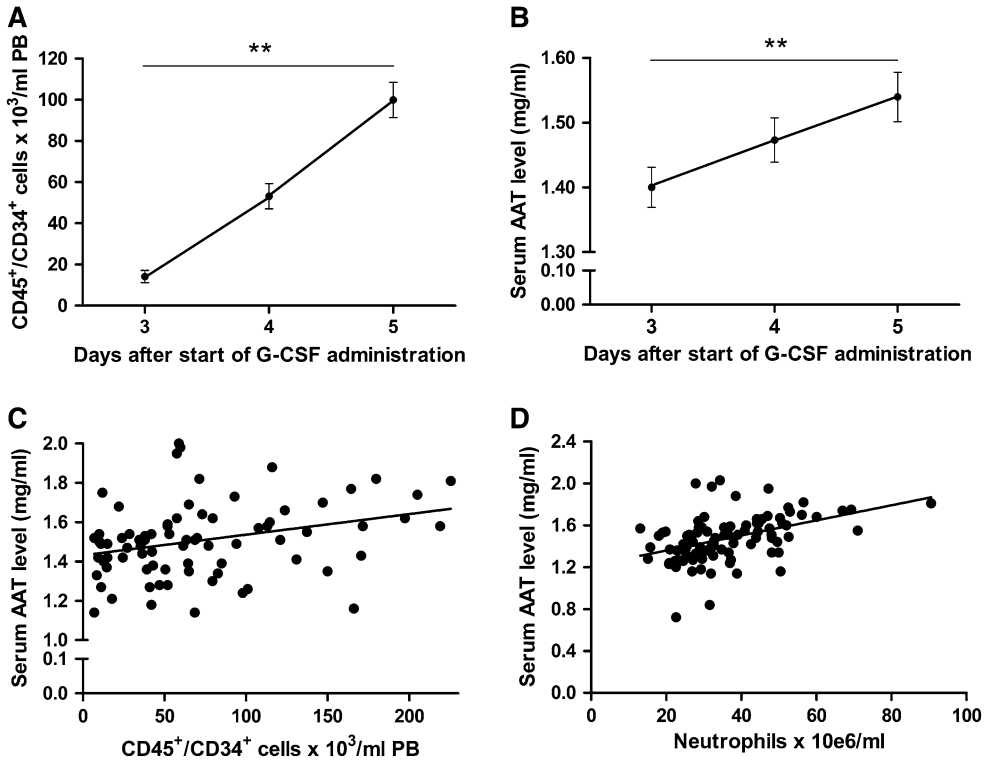

Fig. 2 G-CSF-induced peripheral blood HSPC mobilization in human donors in relation to serum alpha-1-antitrypsin levels. **a** Peripheral blood $CD45^+/CD34^+$ numbers were determined by flow cytometry in the peripheral blood of human peripheral stem cell donors on days 3, 4 and 5 of G-CSF administration. **b** Levels of AAT are measured quantitatively through radial immunodiffusion in the serum of human peripheral stem cell donors on days 3, 4 and 5 of G-CSF administration (**$p < 0.0001$). **c** $CD45^+/CD34^+$ numbers are shown in correlation with serum AAT levels in G-CSF mobilized peripheral stem cell donors ($r = 0.31$, $p = 0.005$). **d** Serum AAT levels and neutrophil numbers/ml of peripheral blood are positively correlated at day 5 of G-CSF-induced HSPC mobilization ($r = 0.49$, $p < 0.0001$)

Discussion

Steady-state HSPC content of peripheral blood in healthy persons is relatively low compared to the HSPC content of bone marrow [22]. Peripheral blood HSPC numbers may increase upon internal signals (inflammation, tissue injury) or external signals (cytokines, drugs). We have previously found that HSPC frequencies in the peripheral blood are significantly reduced in germ-free mice and that these numbers increase upon addition of endotoxin to the drinking water [23]. These results indicate that the "constitutive" presence of HSPC in the peripheral blood is actually, at least in part, an induced event, most likely in response to bacterial products. In addition, the level of mobilization is determined by a balance between proteases and their inhibitors, such as AAT [13]. We therefore hypothesized that HSPC frequencies in patients with a deficiency of AAT are increased. However, our study shows that in these patients the frequency of peripheral blood steady-state HSPC is not significantly different from controls. The higher number of BFU-E in patients with AATD or COPD most likely reflects a physiological response to a chronic hypoxic condition. The lack of differences in peripheral blood steady-state numbers of HSPC could not be explained by a compensatory increase in other protease-inhibitors, as A2M or SLPI levels did not significantly differ between healthy controls and AATD patients.

During G-CSF-induced HSPC mobilization in humans, increased levels of proteases are present in the peripheral blood [16]. In the current report, we show that serum AAT levels increase on days 3–5 of G-CSF-induced HSPC mobilization in healthy donors. Furthermore, serum AAT levels are positively correlated with HSPC mobilization. The increase in serum AAT levels most likely is a response to the high serum levels of proteases that is observed during G-CSF-induced mobilization in healthy human donors [16]. In this previous study, NE levels increased about 40-fold on day 3 of G-CSF-induced HSPC mobilization as compared to levels at the onset of G-CSF administration. Since AAT binds irreversibly to NE as soon as it is released into the peripheral circulation, an increase in serum NE during HSPC mobilization will likely induce a concomitant increase in serum AAT. In mice, we have observed a similar phenomenon, as serum levels of AAT

increase during G-CSF-induced HSPC mobilization (M.P., unpublished data). The increase of AAT levels in the peripheral blood is in contrast with the decrease of AAT levels that is shown in murine bone marrow during G-CSF-induced HSPC mobilization [12]. Similar to what is observed in mice, it can be postulated that in humans AAT levels in the bone marrow are decreased. However, since bone marrow AAT levels were not measured in our study, we were not able to confirm this hypothesis.

During recent years, it became clear that besides the secretion of proteases, other mechanisms also play a role in HSPC mobilization. Mice that are conditionally deficient for one or more proteases including MMP-9, NE and CG mobilize in response to G-CSF [24]. Furthermore, a role has been observed for osteal macrophages and osteoblasts [9, 25, 26].

In conclusion, we show that AAT is unlikely to be an indispensable protease-inhibitor in the constitutive circulation of HSPC, suggesting a complex model in which both protease-dependent and -independent pathways may contribute to HSPC mobilization.

Author contribution E.J.K. and M.P. were responsible for conception and design, collection of data, data analysis and interpretation, manuscript writing; G.A. and R.O., for collection of data, data analysis; W.F., for conception and design, final approval of the manuscript.

Acknowledgments The authors would like to thank Jan Stolk and Joost van den Muijsenberg for their assistance in obtaining patient data and Bram van der Linden for technical assistance.

Conflict of interest The authors report no potential conflicts of interest.

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