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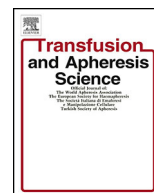
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Does smoking, age or gender affect the protein phenotype of extracellular vesicles in plasma?



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ABSTRACT

Extracellular vesicles (EVs) are involved in several diseases, which have formed the basis for the potential use of EV analyses in a clinical setting. The protein phenotype of EVs can provide information on the functionality of the vesicles and may be used for identification of disease-related biomarkers.

With this extensive study of 161 healthy individuals it was elucidated that certain markers of plasma EVs are influenced by demographic variations such as gender, age and smoking status. When the purpose is to use EVs as a diagnostic tool, it should be emphasized how important it is to choose the correct demographic group when comparing marker levels of plasma EVs.

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1. Introduction

Extracellular vesicles (EVs) are a heterogeneous population of membrane-enclosed vesicles. EVs are recognized as important players in cell-to-cell communication and are described to be involved in numerous biological and pathological processes. The *in vivo* role of EVs still remains

elusive, but their presence in several body fluids such as urine [1], saliva [2], breast milk [3] and plasma [4] suggest an important role.

The fact that EVs are involved in the development and progression of several diseases has formed the basis for the potential use of EV analyses in a clinical setting [5]. The protein profiles and phenotype of EVs can provide information on the functionality of the vesicles and may be used for identification of disease-related biomarkers.

The potential of EVs as clinical and noninvasive biomarkers has already been demonstrated in studies of various diseases, such as Alzheimer's [6], systemic lupus

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erythematosus (SLE) [7] and numerous cancer-types: prostate [8], colorectal [9], ovarian [10] and non-small cell lung cancer [11].

In order to use EVs in a clinical setting, it is of great importance to know whether the protein phenotype of EVs in a healthy cohort are dependent on demographic parameters such as gender, age or smoking status.

Age is one of the most important risk factors for human malignancies. At the subcellular level, regular human aging has been linked to increased genomic instability [12], global epigenetic changes [13], and altered expression of genes involved in cell division and extracellular matrix remodeling [14]. While aging is unavoidable, the aging mechanism is still unclear because of its complexity [15]. Smoking causes premature death and is considered an environmental aging accelerator. The leading causes of death by smoking are cancer, cardiovascular disease, and pulmonary disease, which are also known as aging-related diseases [16].

Both occurrence and development of various diseases have been seen to vary between the genders, as seen for lung cancers [17,18], neuro diseases [19], cardiovascular diseases [20,21], and autoimmune diseases [22] among others. The higher endogenous estrogen levels in women also seem to have an influence on the levels of cholesterol, fatty acids and other lipids. EVs display lipids such as eicosanoids, fatty acids and cholesterol, organized in a bilayer membrane and is involved in EV-mediated lipid trafficking [23]. Investigation of differences in protein profiles of plasma EVs between genders has not previously been reported but it is important in order to use the EVs as a diagnostic- or screening tool for diseases.

The Extracellular Vesicle Array (EV Array) is a sandwich-ELISA-based method capturing EVs using an antibody-panel targeting selected membrane- or membrane associated proteins [24]. The EV Array constitutes a fast, automated, economical and highly sensitive method for exploration of plasma-EVs. In this study, we collected plasma from 161 apparently healthy men and women over 40 years of age and extensively investigated the protein phenotypes of the plasma EVs using a panel of 37 markers of interest. The obtained data were analyzed according to the demographic parameters of gender, age and smoking status.

2. Material and methods

2.1. Plasma samples

Venous peripheral blood was obtained from 161 healthy individuals at Aalborg University Hospital (North Region, Denmark) using EDTA tubes (Vacuette®, Greiner Bio One, DE). The collection tubes were centrifuged at $1.800 \times g$ for 6 min at 20 °C and the plasma was collected, mixed to homogenize, aliquoted and stored at -40 °C until further analysis.

2.2. Antibodies for production of the EV Array

A total of 37 anti-human antibodies were used and all are listed in the following with the corresponding clone, if

available: CD146 (P1H12), Flotillin-1, HB-EGF (4G10), HER3 (2F9), HER4 (H4.77.16), Hsp90 (IGF1), N-Cadherin (8C11), p53 (pAb240), TAG72 (O.N.561), and TSG101 (5B7) from Abcam (MA, USA); SFTPD (VIF11) from Acris Antibodies GmbH (DE); EGFR and EGFRvIII from Antibodies-online.com (DE); CD63 (MEM-259) from Biolegend (CA, USA); HER2 (29D8) from Cell Signalling (MA, USA); CD9 and CD81 from LifeSpan BioSciences (WA, USA); SPA (6F10) from Novus Biologicals (CO, USA); CD13 (498001), CD14 (50040), CD142 (323514), CD151 (210127), CD206, CEA (487609), MUC1 (604804), TNF RI, TNF RII and Tspan8 (458811) from R&D Systems (MN, USA); AREG (S-13), EpCam (O.N.277), Mucin16 (X306), NY-ESO-1 (E978) and PLAP (8B6) from Santa Cruz Biotechnologies (TX, USA); CD171 from Sigma-Aldrich (MO, USA); c-MET (016) and PD-L1 from Sino Biological Inc. (China); and CD163 (Mac2-158) from Trillium Diagnostics, LLC (ME, USA).

2.3. EV Array analysis

Microarray slides were produced using a Spotbot® Extreme Protein Edition Microarray Printer assembled with a 946MP4 pin (ArrayIt Corporation, CA, USA) and epoxy-coated slides (SCHOTT Nexterion, DE) as described by Jørgensen et al. [25]. The antibodies were diluted with PBS, added 5% glycerol and printed in triplicates at 75–200 µg/mL together with positive (100 µg/mL biotinylated human IgG containing 5% glycerol) and negative (PBS containing 5% glycerol) controls.

The EV Array analysis was performed as described by Jørgensen et al. [24]. The microarray slides were initially blocked (50 mM ethanolamine, 100 mM Tris, 0.1% SDS, pH 9.0) prior to incubation with 10 µL plasma sample diluted (1:10) in wash-buffer (0.05% Tween®20 in PBS). The incubation was performed in Multi-Well Hybridization Cassettes (ArrayIt Corporation) at RT for two hours followed by overnight incubation at 4 °C. Following a short wash, the slides were incubated with a cocktail of biotinylated detection antibodies (anti-human-CD9, -CD63 and -CD81, LifeSpan BioSciences, WA, USA) diluted 1:1500 in wash-buffer for two hours at RT. After a wash, a subsequent 30-minute incubation step with Cy5-labelled streptavidin (Life Technologies) diluted 1:1500 in wash-buffer was carried out for detection. Prior to scanning, the slides were washed first in wash-buffer, then in deionized water and finally dried using a Microarray High-Speed Centrifuge (ArrayIt Corporation).

Scanning and spot detection were performed using an Innoscan 710AL scanner (Innosys, Carbonne, FR) as previously described by Jørgensen et al. [24].

2.4. Data and statistical analysis

Graphs and statistics were carried out using GraphPad Prism (version 6.04, GraphPad Software, Inc., San Diego, CA, USA) and Excel (version 2013, Microsoft, Redmond, WA, USA). Heat maps and Hierarchical cluster analysis were produced using Genesis (version 1.7.6, IGB TU Graz, Graz, Austria). Unpaired non-parametric t-test for

differences between groups was applied and differences were considered statistically significant, when $p < 0.05$ (*). Unless otherwise specified, the data are presented as mean SD.

3. Results

The EV Array analysis was performed on 161 plasma samples from healthy individuals. The cohort represented both males and females categorized as either smoker or non-smoker (never smoked). It was selected to include individuals with ages ranging from 40 to 70 years, and the demographic distribution is shown in Table 1.

Thirty-seven different antibodies were printed in triplicates and used for the analysis of EVs in 10 μ L of unpurified plasma. The protein profiles of the EVs (defined as positive for CD9, CD63 and/or CD81) revealed a high variety of expression among the individuals (Fig. 1), which was also seen in previous studies of healthy individuals [24,25]. The hierarchical cluster analysis also showed co-variance among certain markers, e.g. the tetraspanins CD9, CD81 and CD151, whereas the levels of another tetraspanin, CD63, showed co-variance with other markers. The cluster analysis of the individuals revealed several co-varying groups, but none of them seems to correlate to a specific demographic type (Fig. 1, left side).

The log₂ transformed signal values were used for a comprehensive statistical analysis and included more than 6,000 data points obtained from approximately 18,000 microarray spots from where 1147 comparisons (t-tests) were generated (summarized in Table 2).

In general, no differences in the plasma EV profiles were observed according to gender when all individuals are included (Table 2, Row 1). When looking at smoking status in general the levels of plasma EVs expressing CD9 and CD151 were found to be increased (Fig. 2); however, this was not seen for CD81 (Table 2) even though these tetraspanins were found to cluster (Fig. 1). When comparing the influence of smoking in general (all individuals) only EpCAM (Epithelial cell adhesion molecule) was found to be decreased in smokers.

Although no significant differences were observed in EVs according to gender, differences occurred when allocating

the gender cohorts with regard to their smoking status (Fig. 3). It was found that EVs from male smokers carried a higher level of CD171 (L1CAM), PD-L1 (Programmed death-ligand 1) and TSG101 (Tumor susceptibility gene 101) than the group of female smokers. The tendency of increased levels in smoking males was also seen when comparing to the group of non-smoking males. For females the EV level of TSG101 was seen to be significantly decreased in the group of smokers.

As described, the influence of smoking on the EV profiles were found to be different between the genders. The level of several proteins was found to be significantly decreased in female smokers, whereas this was not seen for male smokers (Table 2). The reduction was most pronounced for AREG (amphiregulin), MUC1 (CA15-3), CD146 (MUC18), the alanine aminopeptidase CD13 and TSG101 (Fig. 4).

To test whether the level of plasma EVs and their proteins changes during aging the cohort was divided into three groups (ages 40–49, 50–59 and 60–69). The EV level of the estrogen receptor AREG was generally found to decrease with aging (Fig. 5). This tendency was seen for both genders, but was only found to be statistical significant for males. The group of males also showed a reduction in the EV level of MUC1 during aging, which was not seen for females.

4. Discussion

Circulating EVs are a promising biomarker source in various diseases. So far, only a limited number of studies have examined the diagnostic potential of EVs [11,26]. For all types of diagnostics it is important to determine the diversity of the reference group(s). These reference individuals could be either carrying a benign type of the disease or other related diseases. For the purpose of screening for diseases, a healthy cohort will be the reference group, and hence it is of great importance to know the diversity of such a healthy cohort.

Currently, no proteins are known to be constitutively sorted into vesicles independently of the subcellular origin and the activation status of the producing cell. In this study we used a cocktail of antibodies against CD9, CD63 and CD81 to detect the captured EVs. These markers are considered general exosomal markers [27–29], which are the vesicle type of primary interest in this study. Also, the EV Array catches mainly vesicles in the exosomal size-range [24]; hence it is relevant to use detection antibodies aiming for these vesicles.

The protein profiles of 37 different markers obtained from the EV Array from the 161 healthy individuals tested showed a high degree of variation, both with regard to amount of markers/antigens, but also the number of different antigens present (Fig. 1). The considerable heterogeneity in the expression levels of individual markers in healthy individuals has previously been presented [24,25]. The hierarchical cluster analysis showed co-variance among certain markers, e.g. CD9, CD81 and CD151. The co-variance of these tetraspanins was also found in lung disease patients and patients with non-small cell lung

Table 1

Demographic characteristics of the 161 included individuals according to gender, smoking status and age.

	Female	Male
All individuals	71	90
Non-smokers	48	62
Age 40–49	19	25
Age 50–59	17	25
Age 60–69	12	12
Smokers	23	28
Age 40–49	10	8
Age 50–59	7	13
Age 60–69	6	7

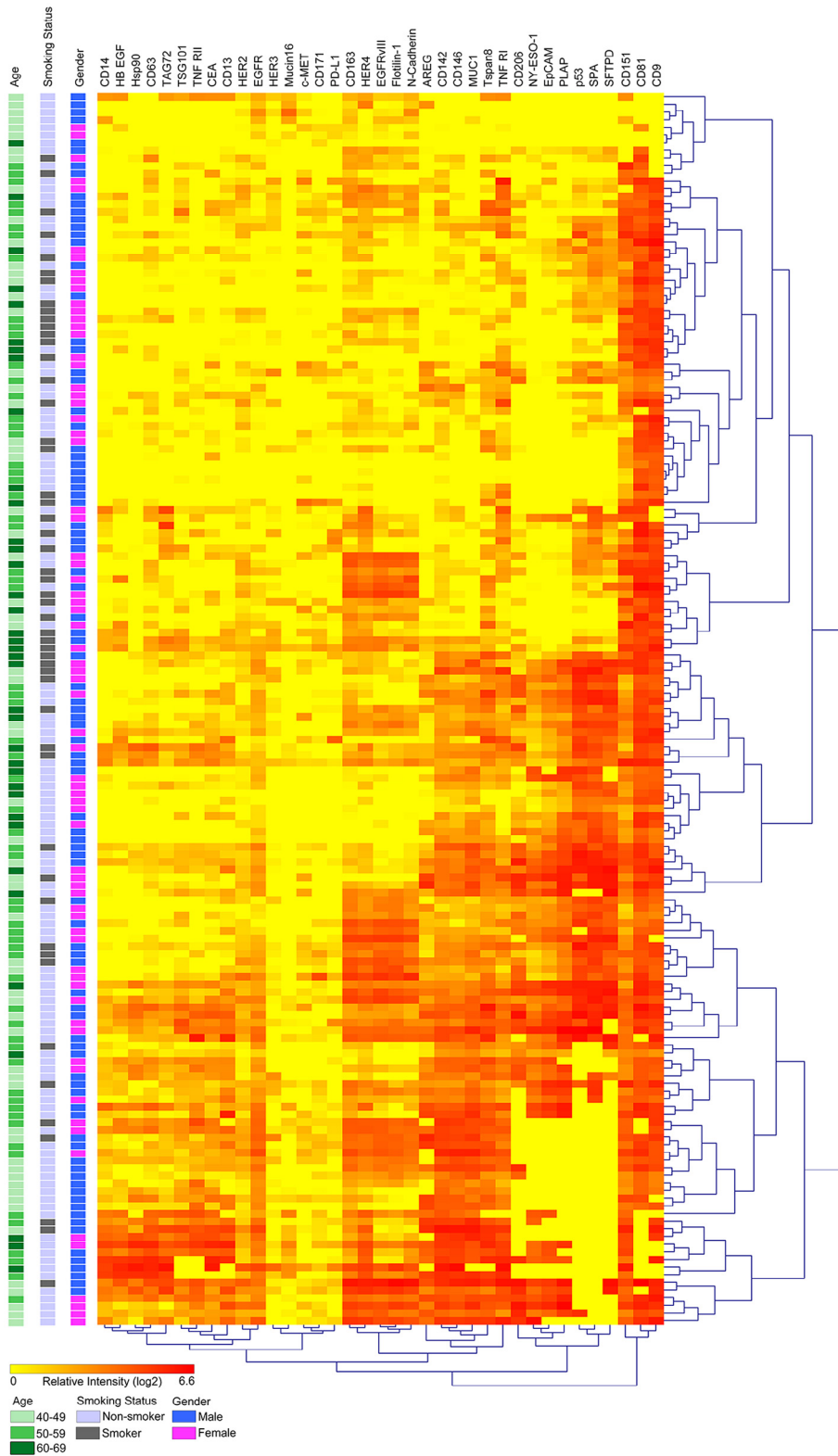


Fig. 1. Hierarchically clustered heatmap summarizing the EV Array data from all individuals. The color coded bar illustrates the relative intensities of each analyte. To the left are selected demographic data shown. The first column indicates the aging group, second column the smoking status and the third column the gender.

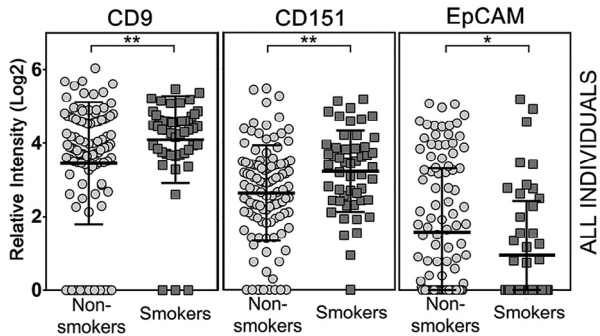


Fig. 2. Comparison of non-smokers and smokers (both males and females) and their vesicular expression of CD9, CD151 and EpCAM. Mean \pm SD is shown and significant differences are marked with asterisks, $p < 0.05$ (*), $p < 0.01$ (**).

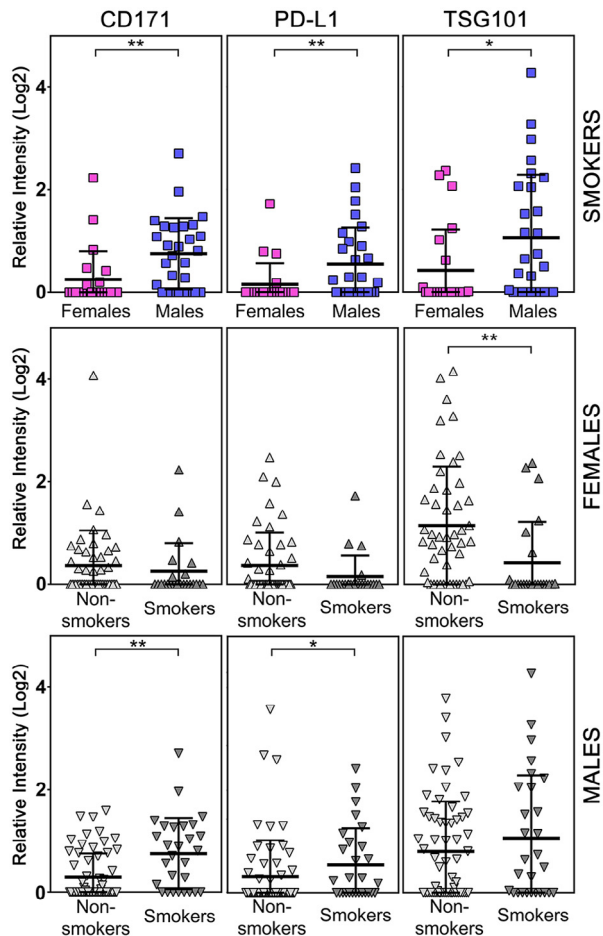


Fig. 3. Levels of the markers CD171, PD-L1 and TSG101 are shown for the smoking cohort with respect to gender and for the gender cohorts with respect to the smoking status. Mean \pm SD is shown and significant differences are marked with asterisks, $p < 0.05$ (*), $p < 0.01$ (**).

cancer (NSCLC) [11]. The expression of CD63 was considerably lower than CD9 and CD81, and the expression was seen to co-variate with another group of markers including TNF RII and Hsp90. Once more, this implies questioning the use of CD63 as a standard EV marker which is supported by earlier results obtained with the EV Array [11,24,25] and other technologies [30–33].

Clustering the EV Array data hierarchical, as illustrated in Fig. 1, no obvious grouping with regards to the demographical variables of gender, age or smoking status was seen. Co-variation was seen in a group of markers containing AREG, CD142, CD146, MUC1, Tspan8 and TNF RI. The statistical comparisons in this group of markers showed significant differences with regards to the age of the cohorts (Table 2). Including both males and females, the plasma level of AREG on EVs was found to be decreased with aging. The decrease was found not to be significant for females, although a decreasing tendency is observed. AREG is an estrogen-responsive gene, and its expression has been found to be increased in a younger cohort (≤ 45 years) of breast cancer patients when compared to an elder cohort (≥ 70 years) [34]. The same tendency is seen in this study with the amount of AREG carried on EVs, although the decrease during aging was found to be more pronounced for males, which was also observed for MUC1 and Tspan8 (Fig. 5, Table 2). Regarding females, their smoking status seemed to affect the amount of AREG on EVs more than their aging, as female smokers were found to have a decreased level (Fig. 4).

In this study the smoking status was found to effect the level of CD146 (MUC18) carried on EVs (Fig. 4). Female smokers were found to have a decreased amount of CD146 on their vesicles compared to non-smoking females, whereas no significant differences were found in the cohort of males. CD146 is a transmembrane glycoprotein belonging to the immunoglobulin superfamily, which is constitutively expressed in the human endothelium [35]. CD146 is involved in the control of cell cohesion and the expression has been observed on endothelial and smooth muscle cells in the airway wall [36]. A soluble form of CD146 has been identified by ELISA in plasma of healthy subjects and the concentration is modulated in inflammatory diseases [37]. Furthermore, its expression was observed to be up-regulated in alveolar macrophages in diseased/inflammatory lungs of patients with chronic obstructive pulmonary disease (COPD) and asthma [38]. Comparing these findings of soluble CD146 with the level of CD146 carried by EVs in plasma it could be speculated that, during the process of smoking-caused inflammation in the lungs, the cells seem to change the protein profile of the EVs. This change in the EV profile could be due to the need for the cells to increase the secretory amount of soluble CD146. Future studies will need to elucidate why this response to smoking is only observed in females.

Additionally, CD146 expression has been shown to be closely associated with advanced stage malignant melanoma, prostate, lung and ovarian cancers [39]. For lung cancer patients (NSCLC) no significant differences were seen in the levels of soluble CD146 according to gender, age and smoking status [35], whereas in patients with adenocarcinoma of the lungs, CD146 expression was more frequently

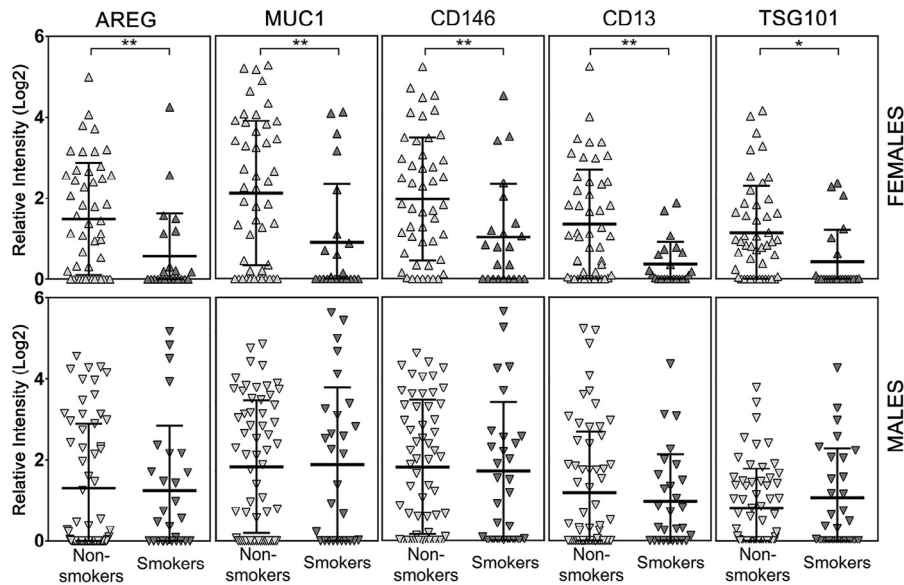


Fig. 4. The plasma levels of EVs carrying AREG, MUC1, CD146, CD13 and TSG101 in the gender cohorts divided in non-smokers and smokers. Mean \pm SD is shown and significant differences are marked with asterisks, $p < 0.05$ (*), $p < 0.01$ (**).

detected in males than females [40]. Accordingly, some gender variation of the CD146 expression are seen in cancerous patients, but the relations to the level of plasma EVs carrying CD146 still remains to be clarified.

Until now no studies with regard to protein profiles of plasma EVs and the influence of smoking have been found. Qazi and coworkers purified exosomes from bronchoalveolar lavage fluid (BALF) and found major differences between the exosomes from patients with sarcoidosis and healthy individuals [41]. Patients showed increased numbers of exosomes in their lungs compared with healthy individuals and had higher levels of MHCI and II, some tetraspanins and hsp70. Taken together, the data from Qazi and coworkers suggests that exosomes may contribute to the inflammatory state of sarcoidosis [41]. They found no significant differences in total exosomal protein ($\mu\text{g}/\text{ml}$) between non-smokers and smokers of sarcoidosis patients. However, significantly higher BALF exosome contents were measured in patients compared to healthy individuals. Whether the exosomes or other EVs can pass from the BALF to plasma remains unclear; however, since the levels and contents of exosomes in the BALF are affected by smoking and lung inflammation, it is expected that the plasma levels are affected as well.

BALF exosomes from smoking and non-smoking sarcoidosis patients showed no significant differences in the expression of HLA-DR, HLA-ABC, CD54, CD63, CD81, CD86, MUC1, and CD9. But in relation to the non-inflammatory, healthy individuals, they found that HLA-DR, HLA-ABC, CD9, CD63, CD81 and CD54 were significantly increased in the patients [41]. Sarcoidosis is known to cause inflammation of the lungs, as does smoking. So our findings that the level of CD9 on EVs are higher in the group of smokers (due to

inflammation) correlates with the results observed by Qazi and coworkers when comparing inflammatory patients with non-inflammatory healthy individuals. Cigarette smoke was found to reduce the amount of CD9 and CD81 present on the surface of macrophages from COPD patients [42]. It could then be speculated whether the decrease of CD9 and CD81 on the cell surface could be due to the fact that the macrophages are diverting/redirection the molecules to the surface of EVs instead.

Cigarette smoke contains more than 4000 different chemicals, most of which are generated during the combustion process in the cigarette [43]. This study has shown that the level of markers carried by EVs in plasma is greatly influenced by the smoking status of the individual. However, the response to smoking was also seen to be influenced by the gender, as females tend to have lower amount of certain EV markers whereas males tend to have increased the amount of other EV markers.

To our knowledge this is the first extensive study of the protein profiles of plasma EVs on a large cohort of healthy individuals. With this study we have elucidated that certain markers of plasma EVs are influenced by demographic variations such as gender, age and smoking status (as summarized in Table 2). When the purpose is to use EVs in a clinical setting, it should be emphasized how important it is to choose the correct demographic group when comparing marker levels of plasma EVs.

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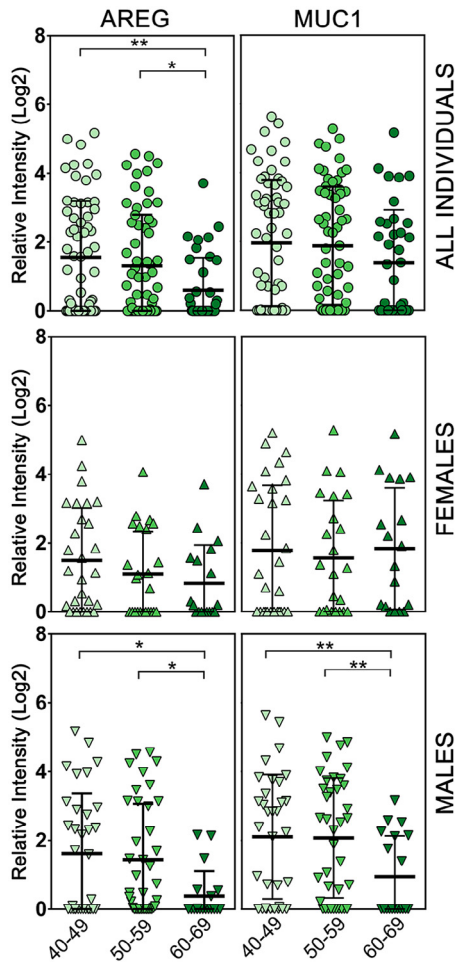


Fig. 5. The influence of aging on the EV phenotypes with respect to AREG and MUC1. The levels are shown for all individuals with no regard to gender as well as for the genders individually. Mean \pm SD is shown and significant differences are marked with asterisks, $p < 0.05$ (*), $p < 0.01$ (**).

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