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Increased extracellular vesicles (EVs) related to T cell-mediated inflammation and vascular function in familial hypercholesterolemia

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ABSTRACT

Background and aims: OxLDL modulates innate and adaptive immunity, and extracellular vesicles (EVs) released from both non-immune and immune cells are proposed key players in atherosclerosis development. In the present study, we aimed to investigate EVs expressing markers related to adaptive immunity-driven inflammation and endothelial activation/dysfunction in hypercholesterolemic patients.
Methods: EVs were phenotyped in thirty patients with familial hypercholesterolemia (FH) and twenty-three healthy controls using the Extracellular Vesicle (EV) Array with antibodies targeting proteins expressed on B and T cells, and endothelial cells.
Results: FH patients had a higher atherosclerotic burden, as determined by the mean carotid intima-media thickness (IMT) (0.64 ± 0.12 mm vs. 0.58 ± 0.07 mm; $p = 0.033$), higher oxLDL levels ($p < 0.0001$), and showed increased levels of EV-specific markers: CD9 ($p = 0.017$), CD63 ($p = 0.045$), CD81 ($p = 0.003$), Annexin V ($p = 0.018$), and EV markers related to adaptive/lymphocyte immunity: CD28 ($p = 0.034$), CD4 ($p = 0.049$), CD152 ($p = 0.029$), LFA-1 ($p = 0.024$), and endothelial function: CD62E ($p = 0.032$), CD144 ($p = 0.018$), tPA ($p = 0.017$), CD31 ($p = 0.024$). Linear regression revealed a positive relationship between carotid IMT and several of the increased markers observed within the FH group, including CD9 ($\beta = 0.33$; $p = 0.022$), CD63 ($\beta = 0.35$; p 225 $= 0.026$), CD28 ($\beta = 0.37$; $p = 0.026$), CD4 ($\beta = 0.40$; $p = 0.025$), CD152 ($\beta = 0.41$; $p = 0.017$), LFA-1 ($\beta = 0.42$; $p = 0.014$) and CD62E ($\beta = 0.38$; $p = 0.024$).
Conclusion: EVs associated with adaptive immunity and endothelial dysfunction are elevated in FH patients, and several markers related to a higher atherosclerotic burden.

1. Introduction

Familial hypercholesterolemia (FH) is an autosomal dominant genetic disorder of lipoprotein metabolism known to cause increased levels of low-density lipoprotein cholesterol (LDL-C), leading to accelerated atherogenesis and premature coronary heart disease, especially in the presence of high levels of the oxidized form of LDL-C (oxLDL) [1]. High levels of oxLDLs along with small endothelial injury cause low-grade inflammation which triggers both innate and adaptive immune responses involving monocytes, macrophages, neutrophils, T cells, and B cells [2]. Thus, innate and adaptive immunity both play fundamental roles in the development of atherosclerosis and its complications, and cells that mediate innate (antigen-independent) and adaptive immunity (antigen-specific and -dependent) localize in atherosclerotic lesions, as reviewed in Ref. [3]. While the innate immune response is considered fast-acting and non-specific, adaptive immunity develops antigen-specific receptors upon exposure to atherosclerosis-related antigens thereby playing a more prominent role in atherogenesis [4]. Cellular crosstalk between innate and adaptive immunity and the release of various soluble factors, including cytokines and chemokines, coordinate and drive inflammation. Another mechanism by which immune cells may exert their effect is through the release of small heterogeneous membrane-bound vesicles termed extracellular vesicles (EVs) into the extracellular fluid as part of the ongoing intercellular communication [5–7]. EVs

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have key functions in physiological and pathological processes and share a characteristic composition, which includes molecules such as proteins, lipids, DNA, RNA species (mRNAs, microRNAs, and long non-coding RNAs), and membrane receptors resembling the composition of the cell of origin [8]. Due to the difficulty of assigning composition and release pathways to EV subtypes, it has been agreed upon to differentiate them solely based on size. Therefore, EVs are categorized into two groups: small EVs (sEVs) and medium/large EVs (m/lEVs), with a size range of less than 200 nm and greater than 200 nm, respectively [9].

By our methodological approach, we focus on the smaller EVs, such as exosomes and exosome-like vesicles, with a diameter of up to ~150 nm. EVs can modulate the immune system to either enhance or suppress immune activities [7,10]. EVs involved in the regulation of the innate immune responses, e.g. released by mast cells [11], neutrophils [12], macrophages [13], eosinophils [14], and NK cells [15], are pro-inflammatory mediators acting on the innate immune system.

EVs involved in the acquired (adapted) immunity and released by B cells and T cells [16], and by dendritic cells (DCs) acting as messengers between the innate and the adaptive immune system [17], concentrates on the process of antigen presentation by expressing MHC-I, MHC-II and T cell co-stimulatory molecules/foreign antigens on their cell surfaces [18]. Depending on the cellular origin, EVs contribute to vascular inflammatory processes including endothelial activation, monocyte adhesion, and cell migration across the vascular endothelium [6,10]. Endothelial activation triggers the release of endothelial cell (EC)-derived EVs which share similarities to immune cell (IC)-derived EVs, as both content and biological functions can be modified under pathological conditions. An increasing body of evidence suggests a positive effect of certain EVs, particularly of endothelial origin, on vascular function and endothelial regeneration, as summarized in Ref. [6]. Yet, several studies suggest a more pathological role of EC-derived EVs including triggering of microvascular inflammation and intravascular calcification, atherosclerotic plaque formation, plaque rupture, thrombus formation after rupture, and the promotion of endothelial dysfunction, as reviewed in Ref. [19].

Regardless of the potential beneficial or detrimental effects of EVs on the regulation of vascular health, underlying mechanisms need to be addressed. In the present study, we aimed to investigate levels of small EVs expressing markers related to adaptive immunity-driven inflammation and endothelial activation/dysfunction in subjects genetically diagnosed with heterozygous FH. Furthermore, we investigated a possible association between small IC-derived EVs and carotid intima-media thickness (IMT) measurements, a noninvasive measure of subclinical atherosclerosis [20].

2. Materials and methods

2.1. Study population

The study population, including eligibility criteria, and clinical and metabolic characteristics was previously described in Ref. [21]. In brief, thirty patients (18 females and 12 males) genetically diagnosed with heterozygous FH and twenty-three healthy controls (15 females and 8 males) as indicated by a medical questionnaire, participated in the study (Table 1). None of the FH participants were taking medication at inclusion, except lipid-lowering therapy, which was withdrawn 8 weeks before study start.

Table 1
Characteristics of participants.

Characteristics	Controls (n = 23)	FH (n = 30)
Men/women	8/15	12/18
Age (years)	47.0 ± 10.1	45.5 ± 9.1
BMI (kg/m^2)	23.7 ± 3.3	25.5 ± 5.2
ALT (U/L)	19.8 ± 9.2	25.1 ± 12.3
Triacylglycerol (mmol/l)	1.0 ± 0.4	1.4 ± 0.8*
Fasting plasma glucose (mmol/l)	5.4 ± 0.5	5.5 ± 0.4
Total cholesterol (mmol/l)	5.1 ± 0.7	9.1 ± 1.8****
LDL-cholesterol (mmol/l)	2.9 ± 0.5	7.0 ± 1.8****
HDL-cholesterol (mmol/l)	1.7 ± 0.4	1.5 ± 0.4*
oxLDL-cholesterol (Units/L)	50.0 ± 11.8	100.3 ± 24.2****
ApoB (g/L)	0.8 ± 0.1	1.7 ± 0.4****

Data are means ± SD.
*p < 0.05, ****p < 0.0001.

Ethical approval

All experimental procedures were approved by the Central Denmark Region Committees on Health Research (2010–0147) and by the Danish Data Protection Agency (j. number 2010-41-4879) and conformed to the standards of the Declaration of Helsinki of the World Medical Association. All subjects were informed orally and in writing about the procedures and the aim of the study and gave written consent to participate.

2.2. Blood samples

Blood samples were collected into sodium citrate anticoagulant at a 3.2% (0.105 M) final concentration and processed within 1 h. Cell-free plasma was prepared by serial centrifugations (10 min at 1800×*g*, 15 min at 3000×*g*, and 5 min at 3000×*g*), frozen, and stored at −80 °C until analysis.

2.3. EV array analysis

EV surface marker profile in plasma samples was determined using the Extracellular Vesicle (EV) Array, a sandwich ELISA-based method, optimized to detect and phenotype the smaller types of EVs, such as exosomes and exosome-like vesicles, with a diameter up to ~150 nm, and from unpurified starting material in a high-throughput manner [22].

2.4. Antibody setup for phenotyping

A total of 27 commercially available antihuman (capture) antibodies were used to analyze the presence of selected marker proteins including: Annexin V (Cat. no. AF399, polyclonal), CD31/Platelet endothelial cell adhesion molecule 1 (PECAM-1) (Cat. no. AF806, polyclonal), CD4 (Cat. no. MAB379, clone 34930), CD45 (Cat. no. MAB1430, clone 2D1), tPA/Tissue plasminogen activator (Cat. no. AF7449, polyclonal), CD144/vascular endothelial cadherin (VE-Cadherin) (Cat. no. AF938, polyclonal), CD19 (Cat. no. MAB4867, clone 4G7-2E3), CD80 (Cat. no. MAB140), CD106/Vascular cell adhesion protein 1 (VCAM-1) (Cat. no. MAB809), CD83 (Cat. no. MAB1774, clone H15e), and CD16 (Cat. no. 555404) from R&D Systems (Europe); CD28 (Cat. no. 340975) and CD49d (Cat. no. 340976, clone L25) from BD Bioscience (New Jersey, USA); CD146/Melanoma cell adhesion molecule (MCAM) (Cat. no. ab24577, clone P1H12), Flotillin-1 (Cat. no. ab41927, polyclonal), Heat shock protein 90 (Hsp90) (Cat. no. ab13494, clone IGF1, polyclonal), and Tumor susceptibility gene 101 (TSG101) (Cat. no. ab117627, polyclonal) from Abcam (Cambridge, UK); CD81 (Cat. no. 302–020) and CD9 (Cat. no.156–020) from Ancell Corporation (MN, USA); Alix (Cat. no. 634501, clone 3A9) from Biolegend (San Diego, CA, USA); CD63 (Cat. no. MCA2142, clone MEM-259) from AbD Serotec (Raleigh, North Carolina); CD152/Cytotoxic T-lymphocyte-

associated protein 4 (CTLA4) (Cat. no. LC-C134750, clone ANC152.2/8H5) from LifeSpan BioSciences (Seattle, USA), CD62E/CD62 antigen-like family member E (E-selectin) (Cat. no. MA1-22165) from Thermo Scientific (Carlsbad, USA), Heat shock protein 70 (Hsp70) (Cat. no. SPA-811, polyclonal) from Assay design (Michigan, USA), CD54/Intercellular Adhesion Molecule 1 (ICAM-1) (Cat. no. BMS1011, clone R6.5) from eBioscience (San Diego, USA), and LFA1/Lymphocyte function-associated antigen 1 (Cat. no. 250944, clone HI111) from Abbiotec, (San Diego, USA).

Briefly, capture antibodies were diluted with PBS with 5% glycerol and printed in triplicates at 200 μg/mL together with positive (100 μg/mL of biotinylated human IgG) and negative (PBS with 5% glycerol) controls on epoxy-coated slides (75.6 × 25.0 mm; SCHOTT Nexterion, Lyngby, Denmark) using a SpotBot Extreme Protein Edition Microarray Printer with a 946 MP4 pin (ArrayIt Corporation, Sunnyvale, CA). After printing, the slides were left to dry at room temperature overnight before further analysis. The dried printed slides were blocked with 50 mM ethanolamine, 0.1 M Trisbase, and 0.1% SDS, pH 9.0 for 1 h before insertion into multi-well cassettes (ArrayIt, CA, USA).

2.5. Antibody setup for quantification

A cocktail of biotin-conjugated anti-tetraspanin antibodies (CD9 (Cat. no. LS-C35419), CD81 (Cat. no. LS-C35761), and CD63 (Cat. no. LS-C35717), LifeSpan BioSciences, Seattle, USA) diluted in PBS (1:1500) was used to detect and visualize the captured EVs.

2.6. Catching and visualization

The EV Array procedure was carried out as described in Baek and Jorgensen [22,23]. In short, 10 μL of cell-free plasma samples were applied after being diluted (1:10) in washing buffer (PBS, 0.2% Tween®20), whereafter they were incubated with mild agitation at 2 h at room temperature following 18 h at 4 °C.

After incubation, the slides were removed from the multi-well cassettes and washed for 10 min in a high throughput washing station (ArrayIt). A cocktail of biotinylated detection antibodies (described above) was applied and incubated with mild agitation for 2 h at room temperature.

After incubation, the slides were washed for 10 min before the addition of streptavidin-Cy5 (Life Technologies, CA, USA) diluted in PBS 1:1500. After 30 min of incubation at room temperature with mild agitation, the slides were washed for 10 min followed by a 10 min wash in MilliQ water. Before scanning, the slides were dried with compressed air.

2.7. Scanning and data analysis

Detection/scanning was performed on an Innoscan 710AL scanner (Innopsys, Carbonne, France) with a 635 nm laser and the settings: 60% PMT, 10 mV, scan speed at 35 lines per second, and a resolution at 5 μm. Images were analyzed using Mapix ver. 6.3.0 (Innopsys, Carbonne, France), and spots were automatically detected and then verified manually. The total intensity of the spots was calculated and exported for data analysis. The EV Array has an inter assay coefficient of variance (CV) of 10.5% using plasma samples [24], and the positive to negative ratio was accepted if: (Pos-neg)/(pos) was above 0.97. A sketch of the print setup and examples of raw scanning images are shown in Supplementary Fig. 1. Heat maps were generated in Genesis 1.7.6 (IGB TU Graz, Austria).

2.8. Measurement of carotid intima-media thickness

Ultrasonographic measurements of the carotid artery intima-media thickness were performed as described in Ref. [21]. In brief, subjects were placed in the supine position, the neck mildly extended, and the head rotated contra-laterally to the side. Images were obtained by manual measurement using an ultrasound system with a 14 MHz linear transducer (Preirus Hi-vision Hitachi, Tokyo, Japan). To measure carotid IMT, three 10 mm segments were scanned longitudinally: the distal portion of the common carotid artery (CCA), the carotid bifurcation (BIF), and the proximal portion of the internal carotid artery (ICA). Carotid IMT was measured at the posterior (far) walls of the left carotid artery as the distance between the luminal-intimal interface and the medial-adventitial interface, and values were calculated as means of the three measurements.

2.9. Statistics

Statistical analyses were carried out using the STATA 16 statistical program (StataCorp LP, Texas, USA). Continuous variables were tested for normality by Shapiro-Wilk's W test. Normally distributed data with equal variances between groups were compared using Student's t-test. Heteroscedastic or non-normally distributed variables were compared using the Mann-Whitney *U* test. Regression analysis, adjusted for age and gender, was performed to identify independent risk factors for the development of subclinical atherosclerosis. The Breusch-Pagan/Cook-Weisberg test for heteroscedasticity was used to test the residuals for constant variance. Robust regression was applied if the assumption of homoscedasticity was violated. Added variable plots (partial regression leverage plots) illustrate the fit of the regression models. $p \leq 0.05$ was considered statistically significant.

3. Results

3.1. Patient characteristics

As previously reported in Ref. [21], FH patients had a significantly higher atherosclerotic burden, as determined by the mean carotid IMT (0.64 ± 0.12 mm vs. 0.58 ± 0.07 mm, $p = 0.033$), and significantly higher cholesterol levels, including oxLDL ($p < 0.0001$), except for HDL-C levels which were significantly lower in FH patients (Table 1). All other laboratory measurements were within age- and gender-specific reference intervals and no significant differences were observed between groups.

3.2. Increase in markers related to vesicles, endothelial dysfunction, and immune response

A heat map presenting data of all markers analyzed by the EV Array is depicted in Fig. 1. Univariate analysis of each marker showed several deregulated markers in patients with FH compared with healthy controls. Vesicle-related markers increased in FH include the tetraspanins CD9 ($p = 0.017$), CD63 ($p = 0.045$), CD81 ($p = 0.003$), known to be involved in cell penetration, invasion and fusion events; and Annexin V ($p = 0.018$), responsible for membrane transport and fusion. Boxplots of vesicle-related markers significantly increased in FH are shown in Fig. 2. EVs expressing heat shock proteins HSP70 and HSP90, as part of stress response during antigen binding and presentation, were comparable in both groups, like EVs expressing Alix and TSG101, internal markers involved in cargo sorting and exosome release; and flotillin-1, a lipid raft-associated protein involved in exosome biogenesis (along with Alix and TSG101). EVs expressing markers related to adaptive/

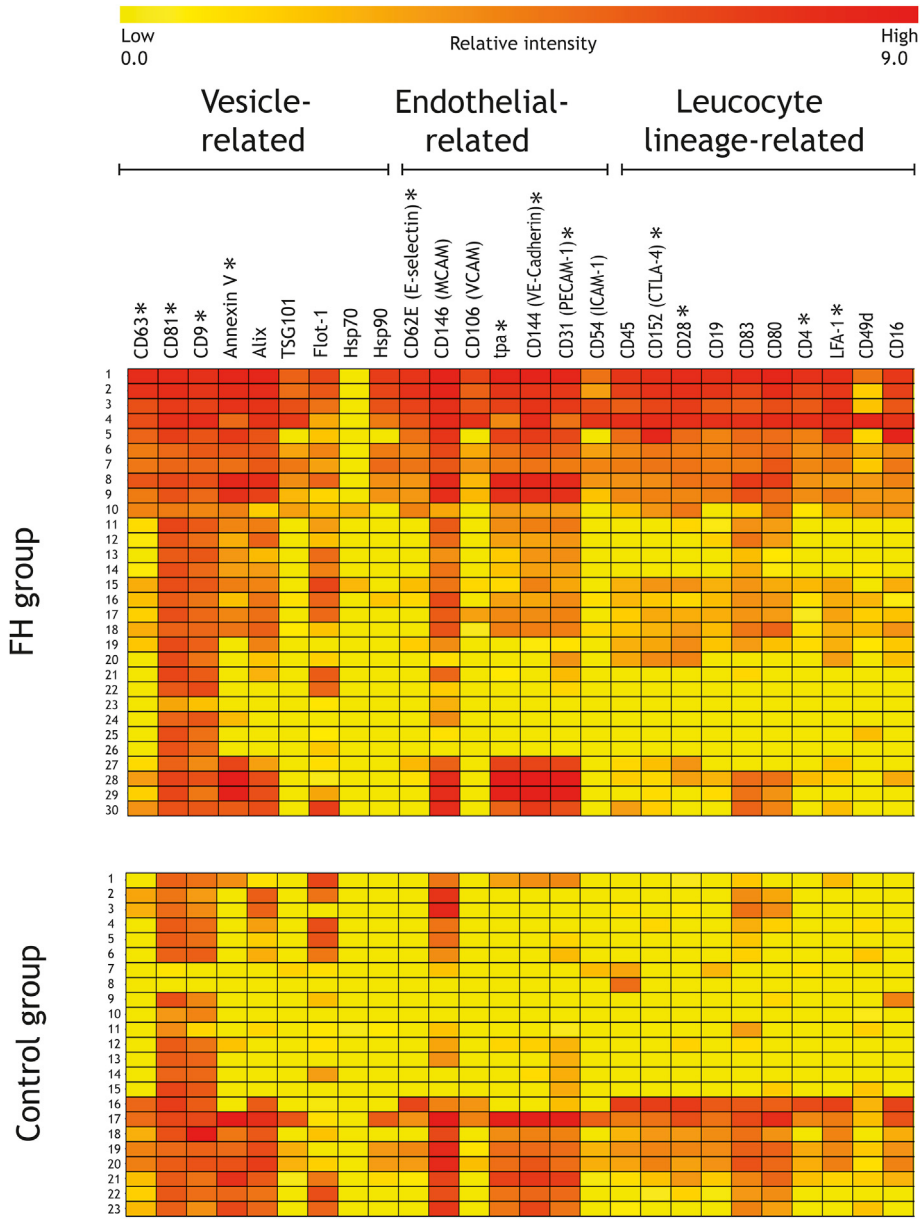

Fig. 1. Heat map presenting data from the EV Array. Shown are data from 9 markers related to vesicles, 7 markers related to endothelial cells and 11 markers related to the leukocyte lineage.
Significantly elevated markers in FH are indicated by *. Data are shown as log2-transformed relative fluorescence intensities. Abbreviations and alternative names of selected CD markers: CD62E, E-selectin; CD146, MCAM; CD106, VCAM; CD144, VE-Cadherin; CD31, PECAM-1; CD54, ICAM-1; CD152, CTLA-4 (cytotoxic T-lymphocyte–associated antigen 4); CD49d, VLA-4 (very late antigen-4) alpha chain.

lymphocyte immunity were similarly increased in FH. Significantly increased markers also found on the surface of immune cells include CD28 ($p = 0.034$), CD4 ($p = 0.049$), CD152 ($p = 0.029$) and LFA-1 ($p = 0.024$), whereas CD45, CD80, CD83, CD16, CD19 and CD49d were comparable in both groups. Boxplots of EV markers related to adaptive/lymphocyte immunity and significantly increased in FH are shown in Fig. 3. Further, FH patients demonstrated increased levels of EVs expressing markers related to endothelial function, including CD62E ($p = 0.032$), CD144 ($p = 0.018$), tPA ($p = 0.017$) and CD31 ($p = 0.024$), but not CD54, CD106 and CD146. Boxplots of EV markers related to endothelial function and significantly increased in FH are shown in Fig. 4.

Linear regression adjusted for age and gender revealed a relationship between carotid IMT and several of the significantly increased markers observed within the FH group. Vesicle-related markers showing significance include CD9 ($\beta = 0.33$; $p = 0.022$) and CD63 ($\beta = 0.35$; $p = 0.026$), but not CD81 and Annexin V. Vesicles carrying markers related to adaptive immunity showing significance include CD28 ($\beta = 0.37$; $p = 0.026$), CD4 ($\beta = 0.40$; $p = 0.025$), CD152 ($\beta = 0.41$; $p = 0.017$) and LFA-1 ($\beta = 0.42$; $p = 0.014$). Finally, EC-derived vesicles showing significance include CD62E ($\beta = 0.38$; $p = 0.024$), but not CD144, tPA and CD31. The added variable plots (partial regression leverage plots) in Fig. 5 illustrate the fit of each regression model.

4. Discussion

EVs have emerged as an important mechanism of cellular

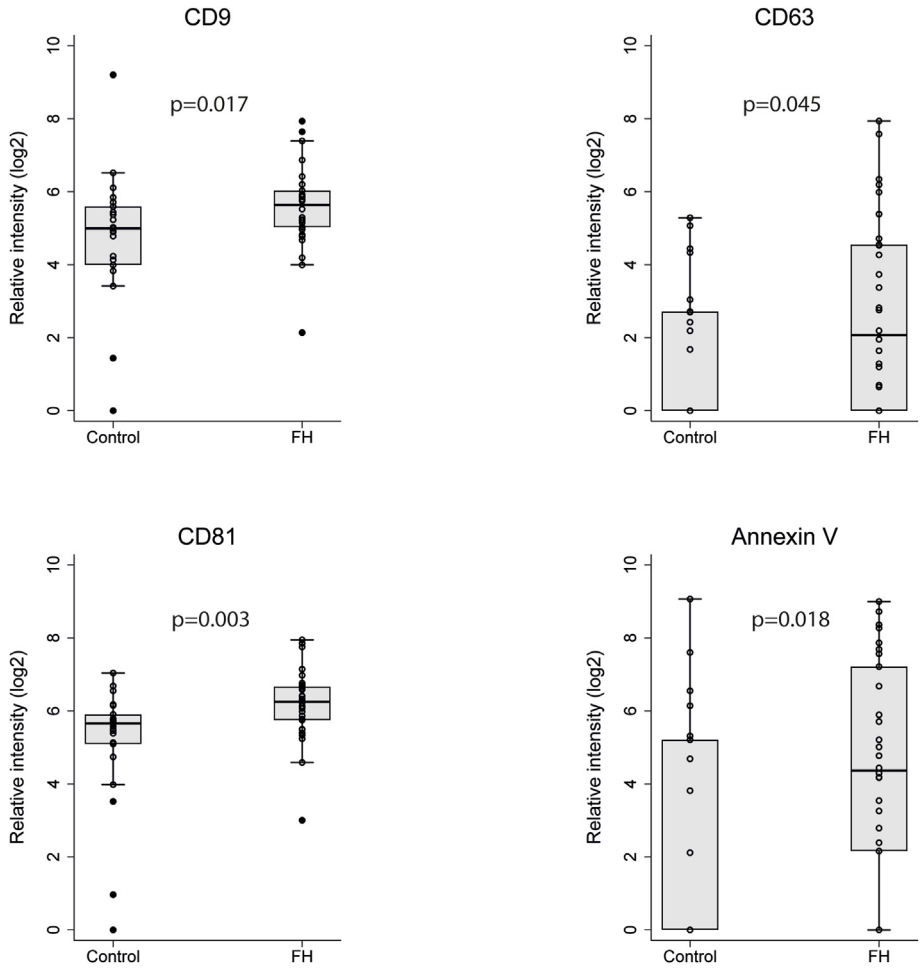

Fig. 2. Boxplots of significantly increased vesicle-related markers. The two-sample Wilcoxon rank-sum (Mann-Whitney) test was used to create statistical data.

interchange of bioactive molecules carrying a great potential as diagnostic and therapeutic agents. Increasing evidence suggest a key role of EVs in a variety of physiological processes [25], and immune responses [10], as well as several pathologies, including cancer [26], cardiovascular diseases [27], and metabolic disorders [28]. Moreover, the diverse impact of EVs biological functions on the immune and vascular systems are now being recognized [6,7], but the biological relevance and possible role in atherosclerosis need further attention. Herein, we investigated levels of small EVs related to adaptive immunity and endothelial function/dysfunction in FH patients prone to a condition of oxidative stress and compared their association to a surrogate marker of early atherosclerosis development. EV-specification was achieved by genuine EV-markers in combination with EV-components related to the cell of origin. Although present in different amounts on EV subpopulations [29], small-sized EVs are usually enriched in tetraspanin proteins, mostly CD9, CD63, and CD81, specific markers because of the endosomal pathway [30]. Thus, as general markers of small-sized EVs, it was not surprising to find elevated levels of these markers in patients previously identified with higher plasma levels of circulating EVs [21,31]. The numerous investigated vesicle-related markers (Alix, Annexin V, flotillin-1, TSG101, and heat shock proteins) are all associated with EVs, however contrary to expectations only Annexin V showed significance in FH. As to date, reliable markers to distinguish between EV subpopulations are missing, and their overall contribution to the total EV population is still an ongoing debate among scientists. Furthermore, markers such as flotillin-1, TSG101, and the heat shock proteins may not belong to the same group of small EVs (nor exosomes), as reported in Refs. [29,32], which may explain discrepancies among EV markers assumed to be highly specific.

EVs derived from both immune and non-immune cells are active regulators of immunity, by mediating immune stimulation/suppression and by driving inflammatory, autoimmune, and infectious disease pathology, as reviewed in Ref. [10]. High levels of lipids, such as cholesterol and triglycerides, directly activate both innate and adaptive immunity in all stages of atherogenesis [33], and it is widely known that different lymphocyte subsets play important roles in cellular and humoral immunity. In FH, immune cells play specific pro-atherosclerotic activities, from the initial leukocyte recruitment to the artery wall to plaque rupture/erosion, as reviewed in Ref. [33].

Classifying EVs to either B or T cell origin is often hampered by the overlap of markers among lymphoid progenitor cells which are adding further complexity to the interpretation of our data and could explain some of the contradictory data presented herein. CD80 and CD83 are examples of such markers, as they are expressed on both activated B and T cells [34,35], as well as on DCs [36], known to link the innate and adaptive immune systems. Along with EVs expressing CD19, they showed similar plasma levels in FH and controls, suggesting steady levels of EVs carrying these markers. B cells are known to infiltrate the adventitial layer of human coronary lesions where they may form lymphoid follicles containing inflammatory cells, mainly B cells with some T cells and

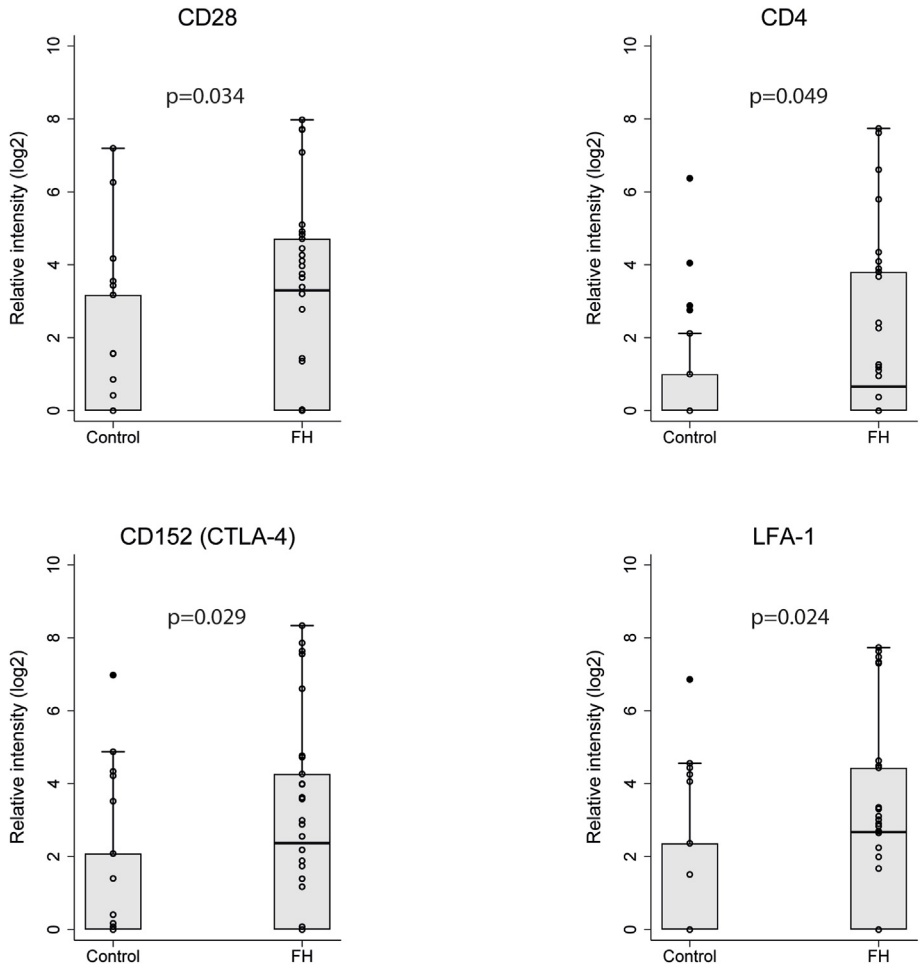

Fig. 3. Boxplots of significantly increased EV markers related to adaptive/lymphocyte immunity. The two-sample Wilcoxon rank-sum (Mann-Whitney) test was used to create statistical data.

DCs [37], and anti-oxLDL IgM antibodies binding the oxidized phospholipids on oxLDL are produced by B1 cells [38]. Thus, although not confirmed by our data, we expected increased B cell activity in FH resulting in higher plasma levels of EVs carrying B cell markers. Further insight into T or B cell-specific EVs could be revealed by assessing the dual expression of specific T and B cell markers in combination with activation markers such as CD20, CD69, and PD-L1/L2; however, this was not the scope of the current study.

In addition to being part of the adaptive immune system, T cells participate in the formation of atherosclerotic lesions as early as monocytes, stimulated to enter lesions by several of the same adhesion molecules and chemokines that promote monocyte recruitment to the artery wall [39]. Moreover, EVs released by activated T cells may represent an important atherogenic factor, as suggested in Ref. [40]. Interestingly, in this regard, numerous T (and B) cell-derived EVs determined herein were significantly increased in FH after adjusting for sex and age, including EVs carrying cell-specific markers CD28, LFA-1, CD4 and CD152, suggesting an activation of the adaptive immune response, as discussed in Refs. [41–43]. Contrary to this, levels of EVs carrying CD45, a marker expressed by all hematopoietic cells including lymphocytes, monocytes, and macrophages, were similar in both groups. As such, it is likely that some EV contributions from lymphocytes are masked by other EV populations sharing the same markers, i.e. of monocyte and/or macrophage origin, which specific markers, CD16 and CD49d, showed comparable levels in both groups. However, further studies are needed to clarify this.

T and B cell-derived EVs may regulate some of the immunomodulatory processes, and it is likely that T cell-derived EVs, target innate immune cells, particularly at inflammatory sites where activated T cells and different immune cell subsets are present simultaneously, as suggested in Ref. [5]. This is in line with an earlier report of EVs isolated from the supernatants of activated human CD4(+) T cells contributing to the formation of atherosclerotic plaque by inducing cholesterol accumulation in monocytes [40]. Future studies are needed on this topic to further explain the potential function/role of the different EVs originating from immune cell subsets.

Vascular endothelial damage leads to endothelial activation/dysfunction and apoptosis, and subsequent atherosclerotic lesion formation, which triggers the release of EC-derived EVs [27], and their putative biological functions have been a field of interest by others [6,44,45]. Our data suggest increased vascular activity in FH, as four (CD62E, CD144, tPA, and CD31) of the seven investigated markers related to endothelial cell function/dysfunction were significantly increased when compared to healthy controls, and thus in support of previous observations on larger EVs (microvesicles) in FH [31].

The noninvasive determination of the artery wall thickness (Carotid IMT) is a well-accepted surrogate marker for subclinical cardiovascular disease and is often used as a variable predictive of

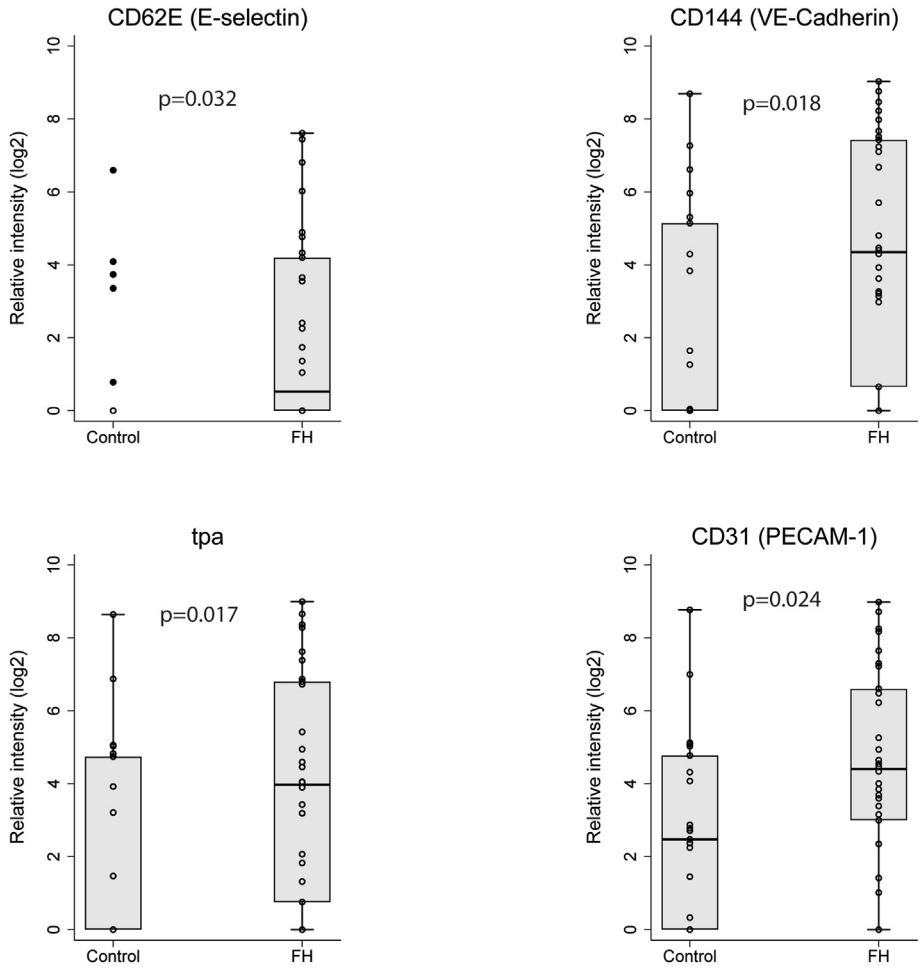

Fig. 4. Boxplots of significantly increased EV markers related to endothelial function. The two-sample Wilcoxon rank-sum (Mann-Whitney) test was used to create statistical data.

cardiovascular events [20]. Most of the significantly increased vesicle- and T cell-related markers, but only a single EC-related marker, were positively related to carotid IMT in FH. Our study cannot prove causality because of its cross-sectional nature. However, the results presented herein may provide information about the steady-state condition in the vascular compartment during prolonged exposure to high cholesterol levels.

In a previous study on the same study population and based on a flow cytometric approach we reported on increased concentrations of total microvesicles (MVs), endothelial cell-derived MVs, erythrocyte-derived MVs, and monocyte-derived MVs in FH, of which MVs of endothelial and monocyte origin were associated with lipoprotein-associated oxidative stress [31]. The results presented herein resemble our previous findings of increased vesicle levels in FH compared to healthy controls but differ concerning methodical setup and types of investigated EV marker proteins. A few of the many benefits of using flow cytometry include estimation of size and number, as well as discovering cells of origin in all kinds of body fluids. Nevertheless, the methodical approach used herein has the advantage of being a more time- and cost-efficient method when investigating a broader panel of EV markers and using smaller amounts of a sample [22].

The ability of the EV Array to capture sEVs quantitatively has previously been proven within other studies, where detailed molecular analyses of small-sized EVs were performed by western blotting [46], nanoparticle tracking analysis (NTA) [22], and transmission electron microscopy (TEM) [47,48]. We intended to optimize an already established and verified technology, which is why we chose not to focus on the EV characteristics despite the recommendations by the MISEV guidelines [9]. As for limitations, we acknowledge, that our testing method is unable to distinguish subpopulations of EVs, and using a cocktail of antibodies against the tetraspanins CD9, CD63, and CD81 to detect sEVs captured on the EV Array [22], and excluding the detection of other types of EVs, makes our data highly selected towards the general assumption of tetraspanin-enriched vesicles, although the use of tetraspanin as a marker has been questioned by others [29,49,50]. Only vesicles carrying markers simultaneously present with either of the three tetraspanin proteins are detected by the EV Array, thus our results are based on a highly selected population of sEVs and should be interpreted with care. Moreover, it should be emphasized that the EV Array data reported herein is only semi-quantitative and the intensities on the heatmap shown in Fig. 1 indicate the abundance of EVs expressing a particular marker. Lastly, the lack of sufficient statistical power due to the small sample size might have contributed to the nonsignificant findings presented herein. Additionally, we did not account for the potential problem of multiple comparisons by controlling the family-wise error rate. As a result, it is imperative to carry out larger and more comprehensive studies to confirm and validate our findings.

To the best of our knowledge, this is the first study addressing levels of EC- and IC-derived EVs in FH and their relationship to atherosclerosis development. We herein demonstrate significantly higher levels of EVs, as well as EVs related to adaptive immunity

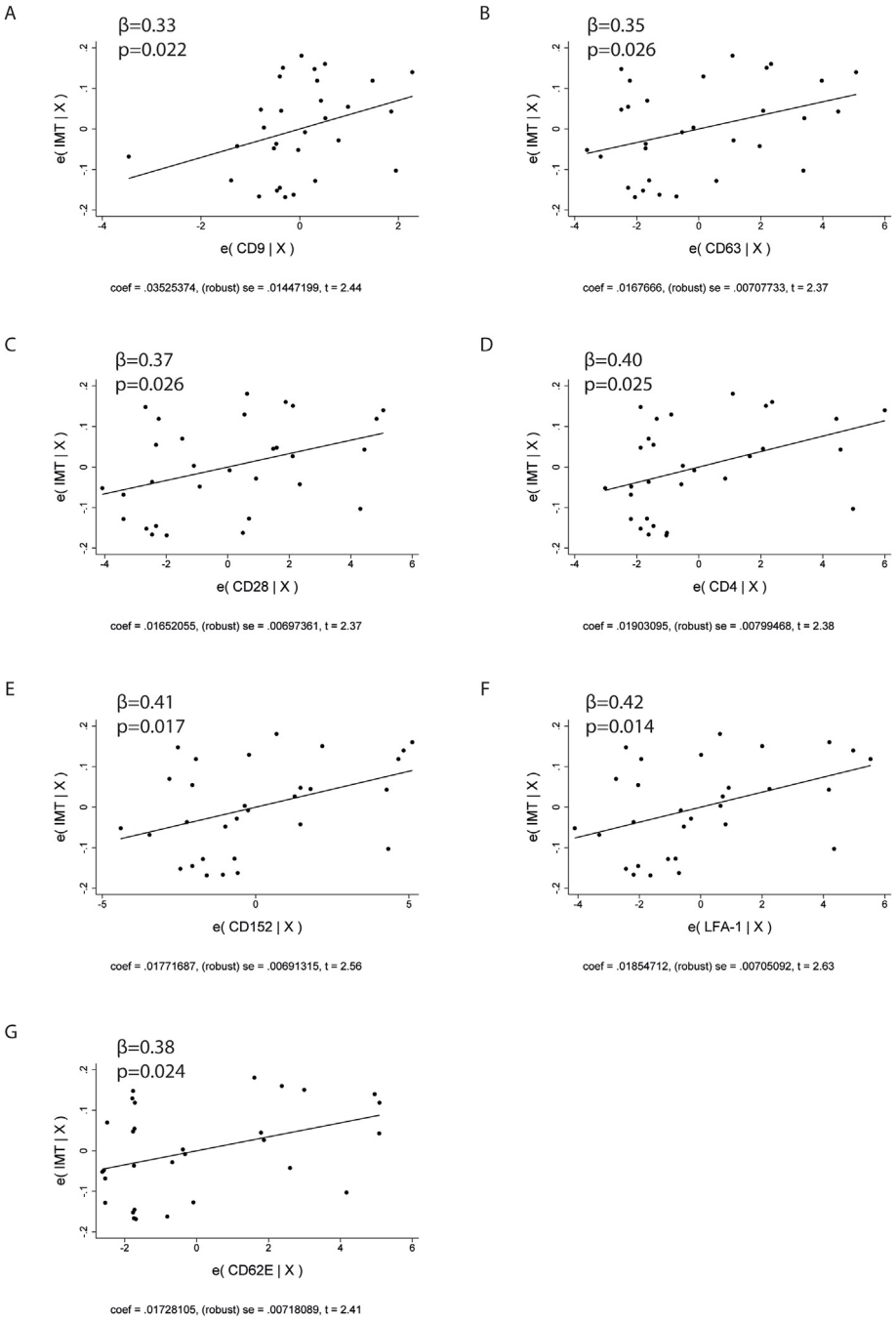

Fig. 5. Linear regression (added variable plots/partial regression leverage plots) between intima-media thickness of the common carotid artery and significantly increased EV markers in FH. Vesicle-related markers (A–B), markers related to adaptive immunity (C–F) and markers related to endothelial cell activation (G).

and endothelial function/dysfunction in FH patients when compared to healthy controls. Several of these EVs were related to carotid IMT, a measure of early atherosclerosis development. The present study does not show a clear relationship between atherosclerosis progression and the origin of EVs. However, this may not be unexpected as EVs derived under pathological conditions can exert both protective and pathological effects [6]. Our findings of an FH-related increase in small-sized EVs carrying markers linked to vascular endothelium and adaptive immunity may have some value as indicators for the ongoing processes within the vascular compartment, and although their correlation with carotid IMT does not necessarily imply causation, and should be interpreted with some care, our finding adds additional observational evidence to the field. Far from all investigated markers were increased, nor correlated to carotid IMT, thus emphasizing the complex nature of i.e. immune cells. We suggest further research in this field to fully elucidate the complexity and role played by EVs in the ongoing process of atherosclerosis.

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Author contributions

MHN and AH contributed to the conception and design of the analysis. RB and MMJ performed the experiments. MHN, AH and MM completed analysis, interpretation of the data and drafting of the manuscript. All authors critically reviewed and revised the manuscript during development and approved the final document for submission.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.athplu.2023.06.004.

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