Characterization and gene expression analysis of serum-derived extracellular vesicles in primary aldosteronism

Jacopo Burrello, Chiara Gai, Martina Tetti, Tatiana Lopatina, Maria Chiara Deregibus, Franco Veglio, Paolo Mulatero, Giovanni Camussi, Silvia Monticone.

Division of Internal Medicine 4 and Hypertension Unit, Department of Medical Sciences, University of Turin, Turin, Italy (J.B.; M.T.; F.V.; P.M.; S.M.). Molecular Biotechnology Center, Department of Medical Sciences, University of Torino, Torino, Italy (C.G.; T.L.; M.C.D.; G.C.).

Corresponding author: Silvia Monticone, Division of Internal Medicine, Department of Medical Sciences, University of Turin Via Genova 3, 10126 Torino, Italy. E-mail: silvia.monticone@unito.it

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ABSTRACT

Primary aldosteronism (PA) is associated with an increased risk of cardiovascular events compared to essential hypertension (EH). Endothelial dysfunction is described as a marker of aldosterone-dependent cardiovascular injury in patients with PA, as well as in EH. Circulating extracellular vesicles (EVs), reflecting endothelial cell activity, could represent one of the mediators of endothelial dysfunction in these patients. The aim of this study was to characterize for the first time circulating EVs from patients diagnosed with PA compared to controls with EH.

Serum EVs were isolated from 12 patients with PA and 12 with EH, matched by sex, age and blood pressure. At nanoparticle tracking analysis, EVs concentration was 2.2 times higher in PA patients (P=0.011) compared with EH and a significant correlation between EV number and serum aldosterone levels was identified; fluorescence-activated cell sorting analysis demonstrated that 42.1% of isolated EVs originated from leukocytes, 21.6% from endothelial cells, and 14.7% from platelets and that PA patients had a higher number of endothelial EVs compared to EH (P=0.005). Through EV mRNA profiling, 15 up-regulated and 4 down-regulated genes in PA patients compared to EH were identified; moreover, *EDN1* was expressed only in patients with PA. Micro-array platform was validated by qRT-PCR on 4 genes (*CASP1*, *EDN1*, *F2R*, *HMOX1*) involved in apoptosis, inflammation and endothelial dysfunction. qRT-PCR confirmed the up-regulation of *CASP1* and *EDN1* (fold-change 12.0/18.3; P=0.023 and P=0.035, respectively).

According to our results, circulating EVs may represent a marker of endothelial injury, involved in progression of organ damage in PA patients.

Keywords: extracellular vesicles, primary aldosteronism, endothelial dysfunction, endothelin-1, caspase-1.

ABBREVIATION LIST

NO, Nitric Oxide; ET-1 Endothelin-1; MR, Mineralocorticoid Receptor; PA, Primary Aldosteronism; BP, Blood Pressure; EVs, Extracellular Vesicles; EXOs, Exosomes; EH, Essential Hypertension; APA, Aldosterone Producing Adenoma; NTA, Nanoparticle Tracking Analysis; FACS analysis, Fluorescence-Activated Cell Sorting analysis;

INTRODUCTION

Endothelium is the inner layer of blood vessels and plays a key role in the regulation of peripheral arterial tone and vascular homeostasis[1]. Endothelial dysfunction results from the imbalance between vasodilators, such as nitric oxide (NO), and vasoconstrictors, such as angiotensin II and endothelin-1 (ET-1)[2]. In addition to impaired vasoreactivity, endothelial failure determines a pro-inflammatory and pro-coagulatory state, favouring initiation and progression of atherosclerosis[3]; consistently, several studies demonstrated the association between endothelial dysfunction and cardiovascular events[4].

It is well known that an inappropriate aldosterone production for sodium status can induce not only arterial hypertension, but also detrimental effects on endothelium and vascular remodelling[5]. In fact, it has been reported that aldosterone treatment inhibits NO release in rat vascular smooth muscle cells[6] and that aldosterone infusion promotes, in murine models, the impairment of endothelium-dependent vasodilatation and the development of severe arterial hypertension and inflammatory response, with perivascular leukocyte infiltrate and fibrinoid necrosis of the *tunica media*[7,8]. The acute administration of aldosterone inhibits acetylcholine-induced endothelium-dependent vasodilatation in healthy volunteers[9], whereas in patients with chronic heart failure (and hence secondary hyperaldosteronism), spironolactone administration improves endothelial function by increasing NO bioavailability[10]. Furthermore, patients with primary aldosteronism (PA) displayed aldosterone-related vascular inflammation and oxidative stress with endothelial dysfunction and

vascular damage[11,12], which translated into increased arterial stiffness and loss of vascular reactivity[11,13]. The administration of a MR antagonist significantly improved endothelial function both in patients with PA and in control patients with resistant hypertension, independently from blood pressure (BP) decrease[11].

Extracellular vesicles (EVs) are bilayer membrane structures classified according to size and biogenesis in microvesicles and exosomes (EXOs); EXOs are the smaller and best characterized EVs, with a diameter ranging from 30 to 150 nm[14]. EVs are constitutively released by cells and they are involved in mechanisms of autocrine, paracrine and endocrine signaling through the transfer of proteins, lipids, and nucleic acids. The ability to transfer genetic information, thus influencing the behavior of target cells, makes EVs key players in inter-cellular communication[14,15]. EVs reflect the activation state of parent cell and may represent a valuable resource for the assessment of cardiovascular disease[16]; considering EVs as surrogate markers of endothelial cell function/dysfunction, we hypothesized that they could be one of the key mediators of endothelial dysfunction and aldosterone-mediated cardiovascular injury.

PA is a frequent cause of endocrine hypertension with a prevalence of 5-10% among the hypertensive population[17,18]. Patients affected by PA display an increased prevalence of target organ damage and cardiovascular events compared to patients with EH[19]. Endothelial dysfunction may justify at least in part the increased cardiovascular risk in these patients; therefore, the aims of the present study were to characterize serum-derived EVs in patients with PA compared to controls with EH and to analyse their mRNA cargo in order to explore a potential role for EVs in aldosterone-mediated endothelial damage.

METHODS

A detailed description of patient selection, EV isolation and characterization protocols and gene expression analysis are provided in the online supplement.

Patient Selection

We recruited 12 patients with PA due to an aldosterone producing adenoma (APA) and 12 patients affected by EH matched by gender, age and BP values, diagnosed at the Division of Internal Medicine and Hypertension Unit, University of Torino. Each patient underwent medical examination and routine laboratory tests. Diagnosis of PA was made according to the Endocrine Society guideline[17]. The study was approved by the local ethical committee and fully informed written consent was obtained from each patient.

EVs isolation and characterization

EVs were isolated from aliquot of serum using a charge-based precipitation method, as previously described[20]. To further purify EVs samples, we performed a second isolation step through ultracentrifugation at 100,000 g[21]. Isolated EVs were then carefully characterized by Nanoparticle Tracking Analysis (NTA), Fluorescence-Activated Cell Sorting (FACS) analysis and Western Blot. FACS analysis and NTA described EVs derivation (endothelial cells, platelets or leucocytes) and the number of EVs per mL with their relative size distribution, respectively. A Western Blot on protein lysate was performed in order to confirm the presence of EVs markers on analyzed samples (CD63, TSG101 and Flotillin-1).

Gene expression analysis

RNA extraction from purified EVs and total RNA retro-transcription were performed through commercially available kits (mirVana Isolation Kit, Thermo Fisher Scientific, Waltham, Massachusetts, USA; RT^2 First Strand kit, Qiagen, Hilden, Germany), according to manufacturer's instructions. mRNA qRT-PCR array profiling was performed on 4 patients diagnosed with PA and 4 with EH, using a platform focused on the evaluation of 96 human genes involved in endothelial function regulation (Endothelial Cell Biology RT^2 Profiler PCR Array Format E384, *Qiagen*, Hilden, Germany). The results were subsequently validated with qRT-PCR on 10 patients with PA and 10 with EH (2 patients from each group were included also in qRT-PCR array analysis) for *CASP1*

(Caspase 1, Apoptosis-related Cysteine Peptidase), *EDN1* (Endothelin 1), *F2R* (Coagulation factor II [thrombin] receptor) and *HMOX1* (Heme Oxygenase [decycling] 1), selected considering their expression profile and available functional data in literature. Data were analyzed through a dedicated software, with the $2^{-\Delta\Delta CT}$ relative quantification (RQ) method, using *18SrRNA* (18S ribosomal RNA) as endogenous control. The RQ mean gene expression of PA patients was compared to EH and expressed as fold-change (FC). A gene was considered down-regulated for FC values between 0 and 1 or up-regulated for values greater than 1.

Statistical Analysis

IBM SPSS Statistics 22 (*IBM Corp., Armonk, New York, USA*) was used for statistical analyses. Data were analyzed with the Kolmogorov–Smirnov test to determine their distributions. Normally distributed variables (age, potassium, systolic and diastolic BP) are expressed as mean ± standard deviation; non-normally distributed variables (PRA, aldosterone, EVs-diameter and concentration, FACs analysis data and RQ) are expressed as median [interquartile range]. ANOVA one-way and Mann-Whitney's tests were used to compare variables with a normal or non-normal distribution, respectively. Correlations were evaluated by Pearson test (R coefficient) and regression curve analysis. *P*-values of less than 0.05 were considered significant.

RESULTS

EVs characterization

A total of 12 patients with PA and a subtype diagnosis of APA and 12 controls with EH, carefully matched by gender, age and BP values were included in the study; patient characteristics with hormonal parameters are described in Table 1. Each cohort was composed of 8 males and 4 females, with a mean age of 51 ± 8 years for PA patients and 50 ± 7 for EH patients ($P = 0.809$). As expected, BP values were similar (152/94 and 150/93 mmHg, respectively for patients with PA and EH; $P > 0.05$), whereas patients with PA had lower potassium and PRA levels and higher aldosterone levels

($P < 0.001$ for all comparison) compared with patients with EH. For each patient, serum-derived EVs were systematically characterized through NTA, FACS analysis and Western Blot for EV-markers (Figure 1).

At NTA, patients with PA and EH showed a similar EV-diameter (respectively 250 nm [176; 262] and 233 nm [203; 265]; $P = 0.874$; Figure 1A), whereas the number of EVs per mL was significantly higher for patients with PA compared with patients with EH (7.8E+11 [6.2E+11; 3.0E+12] versus 3.6E+11 [1.7E+11; 1.3E+12]; $P = 0.011$; Figure 1B).

To define the origin of isolated EVs, we performed FACS analysis for three different surface markers: CD31 for endothelial cells, CD42b for platelets, and CD45 for leucocytes. In cytofluorimetric analysis each EV passes through the flow cytometer and is detected as a distinct event; we expressed EVs concentration as the number of events detected in 60 seconds. Most EVs resulted positive for CD45 (42.1%), whereas 21.6% and 14.7% were positive for CD31 and CD42b, respectively, without significant differences in percentage distribution between the two group of patients. Patients with PA presented a higher absolute number of endothelial-derived EVs (1545 [1161; 2151] versus 645 [508; 1174] events per 60 seconds; $P = 0.005$), whereas we did not find differences for CD42b and CD45 between patients with PA and EH (808 [564; 979] versus 415 [313; 778] events per 60 seconds – $P= 0.052$ and 3091 [1970; 4531] versus 1460 [1108; 2542] events per 60 sec – $P = 0.075$, respectively). Consistent with NTA findings, the total absolute number of events per 60 seconds was significant higher in patients with PA compared with patients with EH (7642 [6375; 10391] vs 3450 [2464; 4931]; $P = 0.001$; Figure 1C).

The EV concentration was 2.2 times higher in patients with PA versus controls with both FACS analysis and NTA; furthermore, the number of EVs per mL directly correlated with aldosterone concentration ($R = 0.472$; $P= 0.020$). Conversely, we did not find a significant correlation with systolic ($R = 0.347$; $P= 0.097$) or diastolic BP ($R = 0.308$; $P = 0.144$, independently from the diagnosis (supplemental figure S1).

In order to confirm the presence of EVs in our samples, we also performed a Western Blot analysis that demonstrated the expression of EV markers CD63, TSG101 and Flotillin-1 (Figure 1D).

Gene expression analysis

To investigate a potential role for EVs in aldosterone-mediated endothelial damage in patients diagnosed with PA, we performed a qRT-PCR-array evaluating the expression level of a panel of 96 human genes involved in angiogenesis, vasoconstriction / vasodilatation, inflammatory response, apoptosis, cell adhesion, coagulation and platelet activation; we compared 4 patients with PA with 4 patients affected by EH. Considering both PA and EH cohorts, 63 genes were expressed in serum-derived EVs of at least one patient (Supplementary Table S1). Among the 19 transcripts found in EVs of at least 4 of the 8 patients included in the analysis, 4 genes were down-regulated (*CCL5* [Chemokine (C-C motif) ligand 5], *F3* [Coagulation factor III (thromboplastin)], *ITGB1* [Integrin, beta 1], *PDGFRA* [PLT-derived GF receptor, alpha polypeptide]) and 15 were up-regulated (*ANGPT1* [Angiopoietin 1], *BAX* [BCL2-associated X protein], *BCL2* [B-cell CLL/lymphoma 2], *CALCA* [Calcitonin-related polypeptide alpha], *CASP1* [Caspase 1, apoptosis-related cysteine peptidase], *COL18A1* [Collagen, type XVIII, alpha 1], *ENG* [Endoglin], *F2R* [Coagulation factor II (thrombin) receptor], *HMOX1* [Heme oxygenase (decycling) 1], *IL6* [Interleukin 6], *ITGA5* [Integrin, alpha 5], *PF4* [Platelet factor 4], *PGF* [Placental growth factor], *PTGIS* [Prostaglandin I2 synthase], *VEGFA* [Vascular endothelial growth factor A]); noteworthy, *EDN1* [Endothelin 1] was expressed in only 3 patients with a diagnosis of PA. The hierarchical clustering analysis is reported in Figure 2. After an initial unbiased transcriptional screening, we validated our results through a target gene approach; we performed qRT-PCR on 4 selected genes (*CASP1, EDN1, F2R, HMOX1*) in 10 patients with diagnosis of PA and 10 controls with EH. We selected the above-mentioned genes considering the expression profile in EVs and current available knowledge on their functional role.

After normalization for *18SrRNA* expression and correction for the number of EVs per mL, we calculated the fold-change (FC) as the ratio between the RQ (Relative quantification coefficient) of PA and EH patients (Figure 3). *CASP1* and *EDN1* were up-regulated in patients with PA compared with patients with EH (FC = 12.0, $P = 0.023$ and FC = 18.3, $P = 0.035$, respectively), whereas we did not find significant differences for *F2R* and *HMOX* (FC = 9.1, $P = 0.143$ and FC = 2.4, $P = 0.315$, respectively).

DISCUSSION

In this study, we systematically characterized, for the first time, serum-derived EVs and analyzed their gene expression profile in patients affected by PA compared to controls with EH. The total number of EVs per mL, mainly derived from leucocytes and endothelial cells, was significantly higher in patients with PA and directly correlated with the aldosterone concentration; in addition, analyzing their mRNA cargo, we demonstrated the up-regulation of *CASP1* and *EDN1* in EVs derived from PA patients compared to those derived from patients with EH.

In peripheral blood of healthy subjects, EVs mainly derive from platelets[22]; consistently with our results, the percentage of endothelial and inflammatory cell-derived EVs raises in pathological conditions such as atherosclerosis[23]. An increased number of circulating EVs has been reported in patients with acute coronary syndrome[24], chronic renal failure[25], diabetes[26], and pre-eclampsia[27]. The concentration of endothelial- and platelet-derived EVs has been observed to be higher also in hypertensive patients compared to normotensive controls and the number of EVs per mL was proportional to systolic and diastolic BP[28].

In the present study, where patients with PA and EH were matched for blood pressure levels, patients with PA displayed a higher serum-derived EVs number compared with patients affected by EH; this observation could be due to a direct effect of the increased aldosterone levels on EVs number, or, alternatively, this could be the consequence of a more severe PA-associated endothelial damage.

Consequently, EVs could be considered as markers of endothelial dysfunction, or as mediators of vascular damage in patients with PA. In agreement with this hypothesis, the number of EVs per mL was directly related to aldosterone concentration.

To further investigate the potential role of EVs in aldosterone-mediated endothelial damage, we performed a mRNA profiling analysis focused on genes involved in the regulation of endothelial function. Among genes differentially expressed in PA compared to EH patients, we confirmed by qRT-PCR in a larger cohort of patients, the up-regulation of *CASP1* and *EDN1* in EVs from patients with PA compared with patients with EH.

CASP1 encodes for caspase-1, a cysteine-aspartic acid protease, which plays a central role in the execution-phase of apoptosis. Recently, some studies demonstrated the involvement of caspase-1 in aldosterone-induced vascular endothelial damage[29] and renal tubulointerstitial fibrosis[30,31], through *in vitro* and *in vivo* studies. Aldosterone infusion resulted in an increased expression of caspase-1 and two inflammasome components, ASC (Apoptosis-associated Speck-like protein Caspase-recruitment domain) and NLRP-3 (Nucleotide-binding domain, Leucine-Rich-containing family, Pyrin domain-containing-3), whereas the mineralocorticoid receptor antagonist eplerenone reverted this effect[30]. The inflammasome complex activates caspase-1, which converts the precursors of IL-1β and IL-18 to their active forms, inducing an inflammatory response[32]. Confirming these results, leukocytes from a healthy volunteer treated in vitro with aldosterone displayed an increased expression of *NLRP-3* and the activation of caspase-1 with increased levels of IL-1β in the supernatant[29]; moreover, serum IL-1β levels were significantly higher in patients with PA compared to healthy controls, and polymorphonuclear cells from the same subjects exhibited the up-regulation of *NLRP-3* and an increased activity of caspase-1[29].

Chronic inflammation mediated by inflammasome complex and caspase-1 has been described in atherosclerosis, diabetes, and chronic renal failure[33-35] and may be responsible for an increased cardiovascular risk. Therefore, PA-derived EVs, carrying mRNA encoding for caspase-1, could be

associated with endothelial dysfunction and contribute to the increased cardiovascular risk observed in these patients[19].

EDN1 encodes a pre-pro-protein, which is processed through proteolysis in endothelial cells to the potent vasoconstrictor ET-1. ET-1 stimulates aldosterone production *in vitro* by primary culture of aldosterone-producing adenoma cells through binding to its receptors, ET-A and ET-B (Endothelin receptors Type A and B)[36]. Indeed, ET-1 was reported to be able to enhance angiotensin-II and ACTH-dependent up-regulation of *CYP11B2*, stimulating the synthesis of aldosterone[37]. On the other side, aldosterone induces the release of ET-1 from murine renal collecting duct cells through the epigenetic modification of chromatin structure around regulatory elements of *EDN1*, by MR activation[38]. In addition, murine models infused with aldosterone develop functional and structural vascular remodeling with increased levels of ET-1, systemic oxidative stress, *tunica intima* thickening, and deposition of collagen and fibronectin in the extra-cellular matrix[8]; spironolactone and an ET-A blocker prevented all these effects, suggesting that the detrimental action of aldosterone at vascular level is mediated not only by the aldosterone-mediated mineralocorticoid receptor activation but also by ET-1 and its receptors[8].

Nevertheless, the role of ET-1 in PA patients remains unclear; while some authors reported an increased production of aldosterone in subjects undergoing co-infusion of ACTH and ET-1[39], others did not find any difference in circulating levels of ET-1 in patients with PA compared to controls with EH[40]. Furthermore, there was no variation in the expression of genes encoding ET-1, ET-A, ET-B, ECE-1 and ECE-2 (Endothelin-Converting Enzyme -1 and -2) in APA compared to normal adrenal glands[41] and endothelial cells treated with aldosterone did not display differences in the expression of genes involved in renin-angiotensin-aldosterone system modulation, oxidative stress, and endothelial function[42].

Overall, studies attempting to demonstrate in humans a relationship between ET-1 and aldosterone-mediated endothelial damage produced contrasting results. One of the reasons for the difficulty to

reproduce the aldosterone-mediated detrimental effects in an experimental model could be the lack of permissive conditions, such as the chronic exposure to elevated aldosterone concentrations and the simultaneous effects of high blood pressure levels, high sodium and low potassium levels, oxidative stress, pro-inflammatory, and pro-thrombotic stimuli[42]. In this perspective serum-derived EVs, being able to transfer *EDN1* mRNA to endothelial cells, could represent one of the mediators of aldosterone-dependent vascular injury.

In conclusion, it is possible to speculate that concentration and content of EVs might reflect the increased endothelial damage displayed by patients with PA and that EVs can be actively involved in determining aldosterone-mediated vascular disease by delivering mRNA, cytokines, or other mediators.

PERSPECTIVES

Accumulating evidence highlights the contribution of EVs in the development of cardiovascular disease. For the first time we systematically characterized circulating EVs of patients diagnosed with PA compared to controls with EH. We found a significant correlation between serum aldosterone levels and EVs number and consistently a higher concentration of EVs in PA patients. PA-derived EVs are characterized by an enrichment of *CASP1* and *EDN1* transcripts, which encode for proteins involved in the pathogenesis of endothelial dysfunction and vascular damage. Therefore, a potential role for serum-derived EVs in determining the increased cardiovascular risk in PA can be hypothesized. Further in vitro and in vivo studies are warranted to investigate the potential functional and pathophysiological role of *CASP1* and *EDN1* overexpression and their link between aldosterone excess and endothelial dysfunction.

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clinical information and samples. J.B., C.G. and T.L. performed EVs characterization and gene expression analysis. J.B. performed statistical analysis. J.B. and S.M. wrote the manuscript with input from all authors. C.G., T.L., M.C.D., F.V., P.M. and G.C. critically revised the manuscript.

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NOVELTY AND SIGNIFICANCE

What is New?

This is the first study which systematically characterizes PA patients serum-derived EVs compared to EVs from patients with EH, matched by age, gender and BP values, through the analysis of diameter, number, surface markers and mRNA expression profiling.

What is Relevant?

Our results demonstrate that PA compared to EH patients have a higher number of circulating EVs, mainly derived from leucocytes and endothelial cells, and whose concentration directly correlates with serum aldosterone levels. PA-derived EVs are enriched in *CASP1* and *EDN1* transcripts, suggesting their involvement in the development of endothelial dysfunction displayed by these patients.

Summary

PA affects 5-10% of hypertensive patients and is associated with an increased prevalence of cardiovascular events; endothelial dysfunction is considered a pre-clinical marker of aldosterone-mediated cardiovascular disease.

Reflecting endothelial cells functional state, EVs could represent a marker of endothelial injury and also a mediator of the accelerated target organ damage, observed in patients with PA.

Table 1 *– Patients characteristics*

	PA (n = 12)	**EH** (n = 12)	***P*-value**
Sex (M/F)	8/4	8/4	1.000
Age (years)	51 ± 8.0	50 ± 7.0	0.809
Systolic BP (mmHg)	152 ± 13.2	150 ± 10.9	0.529
Diastolic BP (mmHg)	94 ± 7.4	93 ± 6.9	0.700
Potassium (mEq/L)	3.6 ± 0.4	4.3 ± 0.3	< 0.001
Aldosterone (ng/dL)	28.3 [23.5 - 42.9]	9.4 [6.0 - 14.8]	< 0.001
PRA (ng/mL/h)	0.3 [0.1 - 0.6]	1.3 [0.8 - 3.8]	< 0.001

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Legend to Table 1 – Clinical and biochemical characteristics of patients affected by PA (Primary Aldosteronism) or EH (Essential Hypertension). Sex (Male/Female) is expressed as absolute number. Age, Systolic BP (Blood Pressure), Diastolic BP and Potassium are reported as mean ± standard deviation. Aldosterone and PRA (Plasma Renin Activity) are reported as median [interquartile range].

Figure 1 *– Serum-derived extracellular vesicles (EVs) characterization*

Legend to Figure 1 – Characterization of serum-derived EVs from patients with a diagnosis of PA (Primary Aldosteronism; grey boxes) compared to patients with EH (Essential Hypertension; white boxes). (A) Diameter of EVs in nm at NTA (Nanoparticle Tracking Analysis); # *P*-value ≥ 0.05. (B) Quantification of EVs (EV n° per mL) at NTA; * $P = 0.011$ (C) FACS analysis for CD31, CD42b and CD45; the box plot represents the number of events in 60 seconds; # $P \geq 0.05$; * $P = 0.005$; § $P = 0.001$. (D) Western Blot Analysis of CD63 (35-55 kDa), TSG101 (45 kDa) and Flotillin-1 (45 kDa); representative blots are shown for 4 patients with PA and 4 with EH. In panel A, B and C, the horizontal line indicates the median and box and bar represent the 25^{th} to 75^{th} and the 5^{th} to 95^{th} percentiles, respectively.

Figure 2 *– mRNA qRT-PCR-array profiling*

Legend to Figure 2 – Heat map showing hierarchical cluster analysis of 63 genes expressed in serum-derived EVs from patients with diagnosis of PA (Primary Aldosteronism) compared to patients with EH (Essential Hypertension). Up-regulated transcripts are reported in green and down-regulated transcripts in red, with darker shades for intermediate values. In black are represented not expressed genes. Patients are represented on columns (4 patients with PA on the left and 4 patients with EH on the right), whereas genes are represented on lines and labeled on the right. The log color scale is shown on the top.

Figure 3 *– qRT-PCR*

Legend to Figure 3 – Validation of gene expression profile by real-time PCR. TaqMan gene expression assays were used in qRT-PCR, performed in triplicate, to determine fold changes (FC) of expression levels in PA (Primary Aldosteronism; grey boxes; n = 10) patients compared to EH (Essential Hypertension; white boxes; n = 10). *CASP1*, *EDN1*, *F2R* and *HMOX1* levels were evaluated using *18SrRNA* as endogenous reference gene. RQ (Relative Quantification) are corrected for the n° of EVs/mL for each sample. # $P \geq 0.05$; * $P = 0.023$; § $P = 0.035$. The horizontal line indicates the median and box and bar represent the 25^{th} to 75^{th} and the 5^{th} to 95^{th} percentiles, respectively.

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