**Potential role of plasma extracellular vesicles in prediction of cardiovascular risk and alterations in response to statin therapy in hypertensive patients: findings from *in-vitro* studies and a nested case-control study from the Anglo-Scandinavian Cardiac Outcomes (ASCOT) Trial**

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**Supplementary figures**

**Figure S1. Schematic representation of the study.** In this nested case-control study (nCCS), plasma samples were obtained at two years from the randomizations from cases and controls (see Supplementary Figure S1). Cases (n, 20) were patients who had a major adverse CV event [MACE] (defined as either coronary event or non-fatal stroke, or death) during a median 3.5 years of follow-up subsequent to collection of the samples. Controls (n, 20) were propensity score matched patients with similar age, duration of in-trial treatment and who did not have any CV event during the subsequent follow-up. Both case and control groups had the same number of patients who were initially randomized to a statin or a placebo arm.

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**Figure S2. Characterization of monocyte EVs.** Monocytes were isolated using the RosetteSep™ purification protocol. Cells (1x106/ml) were incubated with vehicle (V) or TNF-α (50 ng/ml), in presence or absence of Iloprost (1 µM; PGI2) for 60 min. EV generation in cell-free supernatants was quantified by nanoparticle tracking analysis as both size distribution of isolated EVs (a) and mean and mode of size (b). EV phenotypic characterization was quantified following the described gating strategy (c) and Bodipy staining for total vesicles with; monocyte CD14+ EVs ; platelet CD41+ EVs and double positive CD14/CD41+ vesicles were measured (d). (\*p<0.05, \*\*p<0.01, \*\*\*p< 0.001; one-way ANOVA post Bonferroni test, mean ± SEM, n=5 distinct preparations). (e) Western blot analysis of the vesicle showing positive controls Annexin A1 (AnxA1), CD9, negative control Calnexin (Cnxn) and loading control Actin-β1 (ACTB1) (f) Visualization of EVs by TEM. Representative of n=5 distinct cell preparations.



**Supplementary Tables**

**Table S1. Baseline characteristics and investigated EV markers amongst hypertensive patients who did and did not have a major adverse CV event (MACE) during the median follow-up of 3.1 years.**

|  |  |  |
| --- | --- | --- |
| **Characteristic** | **Control\* (N=20)\*** | **Case (N=20)\*** |
| Age mean (SD), years | 69.94 (5.21) | 69.94 (5.23) |
| Female, n (%) | 8 (40) | 1 (5) |
| Male, n (%) | 12 (60) | 19 (95) |
| SBP mean (SD), mmHg | 167.25 (16.89) | 170.35 (14.46) |
| DBP mean (SD), mmHg | 89.20 (10.69) | 92.72 (11.57) |
| Total Cholesterol mean (SD), mmol/l | 5.73 (0.75) | 5.59 (0.72) |
| BMI mean (SD), kg/m2 | 28.17 (3.83) | 27.70 (4.05) |
| Current smoker, n (%) | 10 (50) | 16 (80) |
| Diabetes status, n (%) | 3 (15) | 6 (30) |
| Randomised to amlodipine-based treatment, n (%) | 10 (50) | 6 (30) |
| **EV markers** |  |  |
| Mode median , nm | 155.80  | 142.20  |
| CD14 median , objects x105/mL | 6.80  | 14.20 |
| CD146 median , objects x105/mL | 15.65  | 18.40  |
| CD14/CD41 median , objects x105/mL | 2.34  | 4.65  |
| CD14 median , | 1.11  | 1.22  |
| CD146 median  | 2.84  | 2.67 |
| CD14/CD41 median  | 0.31 | 0.51  |
| Note: there is no missing data |   |   |

\* Cases are defined as those who had a MACE during a median 3.1 years of follow-up. Controls are matched for age, duration of treatment (n, 20) and statin/placebo treatment allocation, and did not have a MACE during follow-up

**Table S2: descriptive analysis by LL allocation.**

|  |  |  |
| --- | --- | --- |
| **Characteristic** | **Placebo (N=20)** | **Atorvastatin (N=20)** |
| Age mean (SD), years | 70.50 (5.52) | 69.38 (4.83) |
| Female, n (%) | 6 (30) | 3 (15) |
| Male, n (%) | 14 (70) | 17 (85) |
| SBP mean (SD), mmHg | 169.32 (12.49) | 168.28 (18.51) |
| DBP mean (SD), mmHg | 89.95 (9.84) | 91.97 (12.48) |
| Total Cholesterol mean (SD), mmol/l | 5.77 (0.88) | 5.55 (0.55) |
| BMI mean (SD), kg/m2 | 27.37 (3.80) | 28.51 (4.01) |
| Current smoker, n (%) | 12 (60) | 14 (70) |
| Diabetes status, n (%) | 5 (25) | 4 (20) |
| Randomised to amlodipine-based treatment, n (%) | 8 (40) | 8 (40) |
| **EV markers** |  |  |
| Mode median, nm | 156 | 138.70 |
| CD14+ median, objects 105/mL | 7.37 | 8.37 |
| CD146+ median, objects 105/mL | 34.40 | 14.55 |
| CD14+/CD41+ median, objects 105/mL | 3.26 | 2.34 |
| CD14+ median, % | 1.08 | 1.25 |
| CD146+ median, % | 4.15 | 1.54 |
| CD14+/CD41+ median, % | 0.51 | 0.21 |

Note: there are no missing data.

**Table S3:** Risk of MACE (odds ratios [95% CI]) with increase in one unit of EV markers stratified by allocation to placebo or a statin, interaction test.

|  |  |  |  |
| --- | --- | --- | --- |
| **EV marker** | **Placebo arm (n,20)** | **Statin arm (n,20)** | **Interaction p-value** |
| **odds ratio\*\*** **(95% CI)** | **p-value** | **odds ratio\*\*\*****(95% CI)** | **p-value** |
| Mode, per nm | 0.98 (0.95, 1.02) | 0.377 | 0.93 (0.85, 1.01) | 0.088 | 0.211 |
| CD14+, log2 (objects/mL)\*\* | 6.35 (0.57, 70.93) | 0.133 | 2.94 (0.77, 11.21) | 0.115 | 0.584 |
| CD146+, log2 (objects/mL)\*\* | 1.46 (0.75, 2.84) | 0.269 | 1.30 (0.45, 3.75) | 0.631 | 0.855 |
| CD14+/CD41+, log2 (objects/mL)\* | 1.99 (0.87, 4.53) | 0.101 | 1.20 (0.82, 1.82) | 0.394 | 0.283 |
| \* Note that these variables have been transformed to log base 2, so a 1 unit increase represents a doubling of the variable on the original scale. \*\*risk of MACE for one unit raise amongst patient taking placebo\*\*\*risk of MACE for one unit raise amongst patient taking statin  |

**Table S4:** Difference in mean level of EV markers in plasma samples between statin treatment allocation groups of ASCOT patients.

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| --- | --- | --- | --- |
| **EV marker** | **Difference in means placebo-statin in CONTROLS****(p-value from unpaired t-test)** | **Difference in means placebo-statin in CASES****(p-value from unpaired t-test)** | **Interaction p-value** |
| Mode, nm | 12.26 (p=0.289) | 28.51 (p=0.017) | p=0.319 |
| CD14, objects x105/mL | 0.95 (p=0.913) | -13.19 (p=0.137) | p=0.256 |
| CD146, objects x105/mL | 9.97 (p=0.320) | 31.93 (p=0.003) | p=0.125 |
| CD14/CD41, objects x105/mL | 0.25 (p=0.951) | -2.69 (p=0.509) | p=0.609 |

Table S5 Mean differences in EV markers between cases and controls, or those allocated to atorvastatin or placebo, with further respective stratification using 2X2 factorial allocation.

Table S5a: Overall mean difference between case and control, and stratified mean levels amongst those on atorvastatin and placebo regardless of whether case or control.

|  |  |
| --- | --- |
| EV marker | Case and control group analysis with overall mean differences, and using 2X2 stratification the mean differences between cases and control amongst those on statin or placebo  |
| EV marker | **Overall mean difference between cases/controls (n=40)\*** | **Mean differences between cases and controls on those on atorvastatin (n,20) [10 each from cases and controls]\*** | **Mean differences between cases and controls on those on placebo(n,20) [10 each from cases and controls]\*** | **Interaction p-value** |
| Mode, nm | -16.07 (p=0.055) | -25.97 (p=0.016) | -2.43 (p=0.839) | p=0.132 |
| CD14, objects x105/mL | 12.72 (p=0.070) | 19.58 (p=0.030) | 3.25 (p=0.749) | p=0.216 |
| CD146, objects x105/mL | 11.62 (p=0.159) | -0.11 (p=0.992) | 27.78 (p=0.023) | p=0.071 |
| CD14/CD41, objects x105/mL | 7.10 (p=0.025) | 8.81 (p=0.032) | 4.74 (p=0.309) | p=0.494 |

**\*Adjusted for age at baseline and sex**

Table S5b : Overall mean difference between those allocated to atorvastatin or placebo, and stratified mean differences amongst those that are cases or control, regardless of whether previously allocated to statin or a placebo.

|  |  |
| --- | --- |
| EV marker | Allocation to statin or placebo groups with overall mean differences, and using2X2 stratification, the average mean differences amongst those on statin and placebo amongst those who are cases or control  |
|  | **Overall mean difference between Statin/placebo(n=40)\*** | **Mean difference between statin/placebo amongst cases (n,20) [10 each from those on statin and placebo]\*** |  **Mean difference between statin and placebo amongst those who are control (n,20) [10 each from those on statin and placebo]\*** | **Interaction p-value** |
| Mode, nm | -21.39 (p=0.007) | -32.32 (p=0.003) | -8.78 (p=0.430) | p=0.132 |
| CD14, objects x105/mL | 5.04 (p=0.431) | 12.62 (p=0.156) | -3.71 (p=0.694) | p=0.216 |
| CD146, objects x105/mL | -20.03 (p=0.011) | -32.98 (p=0.002) | -5.09 (p=0.641) | p=0.071 |
| CD14/CD41, objects x105/mL | 0.40 (p=0.889) | 2.29 (p=0.566) | -1.78 (p=0.678) | p=0.494 |

\*Adjusted for age at baseline and sex