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Original articles

Microparticles in nasal lavage fluids in chronic rhinosinusitis; potential biomarkers for diagnosis of Aspirin Exacerbated Respiratory Disease

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Abstract:

Background: Microparticles (MPs) are submicron sized shed membrane vesicles released from activated or injured cells and are detectable by flow cytometry. MP levels have been utilized as biomarkers to evaluate cell injury or activation in patients with pathological conditions.

Objective: To compare MP types and levels in nasal lavage fluids (NLFs) from controls and patients with chronic rhinosinusitis without nasal polyps (CRSsNP), CRS with NP (CRSwNP) and aspirin exacerbated respiratory disease (AERD).

Methods: We collected NLFs from CRSsNP (n=33), CRSwNP (n=45), AERD (n=31), and control (n=24) subjects. Standardized flow cytometry methods were used to characterize the following MP types; endothelial MPs, epithelial MPs (EpCAM(+)MPs, E-cadherin(+)MPs), platelet MPs (CD31(+)CD41(+)MPs), eosinophil MPs (EMR1(+)MPs), mast cell MPs (FcεRI(+)c-kit(+)MPs) and basophil MPs (CD203c(+)c-kit(-)MPs). Basophil activation was evaluated by the MFI of CD203c (CD203cMFI) on basophil MPs.

Results: Activated mast cell MPs (CD137(+)FcεRI(+)c-kit(+)MPs) were significantly increased in NLFs compared to controls in CRSsNP (2.3-fold, $p<0.02$), CRSwNP (2.3-fold, $p<0.03$) and in AERD (7.4-fold, $p<0.0001$). Platelet MPs (3.5-fold, $p<0.01$) and basophil MPs (2.5-fold, $p<0.05$) were increased only in AERD. CD203cMFI on MPs was increased in CRSwNP ($p<0.002$) and AERD ($p<0.0001$), but not CRSsNP. EpCAM(+)epithelial MPs in CRSwNP were no different from control ($p=0.91$) and lower than those in CRSsNP ($p<0.02$) and AERD ($p<0.002$).

Conclusion: Based on released MPs, mast cells, platelets, and basophils were more highly activated in AERD than in CRS. Epithelial injury was lower in CRSwNP than in CRSsNP and AERD. MP analysis may help identify phenotypes of CRS, and in distinguishing AERD from CRSwNP.

(251 words)

Capsule Summary

Microparticles are released into the nasal cavity from activated immune cells and injured structural cells in CRS and AERD. Assessment of microparticle levels in nasal lavage fluid may help distinguish the various phenotypes, especially AERD.

Key messages

- Microparticles may be useful to assess the degree of cell injury, activation, and apoptosis relevant to the pathophysiology of CRS and AERD.
- Analysis of MP subsets suggests that mast cells, platelets, basophils and endothelial cells are all more highly activated in AERD than in CRS.
- Platelet MPs, epithelial MPs and mast cell MPs may have utility as biomarkers to distinguish AERD from CRSwNP in both clinical and research settings.

Key words

Microparticles, epithelial injury, eosinophil activation, apoptosis, mast cell activation, basophil activation, CD137, CD69, chronic rhinosinusitis, Aspirin Exacerbated Respiratory Disease

Abbreviations

CRS; Chronic rhinosinusitis

CRSsNP; CRS without nasal polyps

CRSwNP; CRS with nasal polyps

- 76 NP; Nasal polyps
- 77 AERD; Aspirin Exacerbated Respiratory Disease
- 78 MPs; microparticles
- 79 FcεRI; high-affinity IgE receptor
- 80 EpCAM; Epithelial cell adhesion molecule
- 81 EMR1; EGF-like module-containing mucin-like hormone receptor-like 1
- 82 MFI; mean fluorescence intensity
- 83 NLFs; Nasal lavage fluids
- 84 ActMPs; activated MPs
- 85 ApoMPs; apoptotic MPs
- 86 EndoMPs; endothelial MPs
- 87 EpiMPs; epithelial MPs
- 88 EoMPs; eosinophil MPs
- 89 MCMPs; mast cell MPs
- 90 BasoMPs; basophil MPs
- 91 FM; lipid-FM specific dye,
- 92 CFSE; protein- Carboxyfluoresceinsuccinimidyl ester (CFSE) specific dye
- 93 ATA; Aspirin tolerant asthma
- 94

Introduction

Chronic rhinosinusitis (CRS) is an inflammatory disease of the nose and paranasal sinuses commonly divided into a form with nasal polyps (CRSwNP) and a form without nasal polyps (CRSSNP). There is a particularly severe form of CRSwNP in which subjects have comorbid asthma and sensitivity to aspirin and other COX1 inhibiting drugs referred to as Aspirin Exacerbated Respiratory Disease (AERD). However, there is still an ongoing debate about the underlying pathological mechanisms of this phenotype of disease. Distinguishing AERD subjects from CRSwNP subjects is important because asthma exacerbations secondary to aspirin sensitivity have high morbidity in AERD and because the pathophysiology of these two phenotypes appears to be distinct^{1,2}. Diagnosis based on patients' recall of a history of aspirin sensitivity is not always reliable. It was reported that 15- 34 % of rhinosinusitis subjects with asthma were unaware of their aspirin intolerance until they developed symptoms during a monitored aspirin challenge, and up to 15% of asthmatic subjects who reported a history of aspirin intolerance had negative aspirin challenge test results^{1,3,4}. However, performing aspirin challenges to diagnose AERD are not routinely performed in a clinical setting given the length of time needed to perform the challenge and the potential risk of inducing a severe reaction. New biomarkers are needed to clarify the diagnosis and pathophysiology of CRSSNP, CRSwNP and AERD, especially to objectively distinguish AERD from CRSwNP.

Microparticles (MPs) are extracellular vesicles ranging in size from 100 to 1000 nm that are released by shedding from the plasma membrane of various cell types, especially when they are activated or undergo apoptosis in response to injury⁵. Growing evidence has suggested that the levels and markers of released MPs are biomarkers of the degree of injury, activation, and apoptosis of cells from which they are derived in various diseases. For example, endothelial MPs

are increased in subjects with disorders such as cardiovascular diseases⁶⁻⁸, renal failure⁹, metabolic diseases¹⁰, and COPD¹¹ in response to endothelial injury. The release of E-selectin endothelial MPs is an indicator of endothelial activation, whereas Annexin V positive PECAM positive endothelial MPs have been used as an indicator of endothelial apoptosis^{12, 13}. MPs are distinct from exosomes that are smaller extracellular vesicles released from cells through exocytosis of multivesicular bodies.⁵ In the case of MPs, the markers expressed on the cell of origin are expressed on the membrane of the MPs since they are formed by blebbing of plasma membrane. Thus, MP analysis can be used to evaluate the type of cells that are injured and the state of activation or apoptosis of injured cells. Assay of MPs has been shown to have significant value in quantifying the degree of cell injury and activation in clinical samples^{8, 14}.

We report here the successful use of MP assessment of nasal lavage fluids (NLFs) to evaluate the pathophysiology of CRS using flow cytometry with a standardized MP gating strategy^{15, 16}. Disruption of the epithelial barrier has been speculated to initiate chronic inflammation in CRS¹⁷⁻¹⁹. Eosinophils and mast cells have been reported to be involved in the pathophysiology of CRS^{20, 21}. Elevated basophil counts were reported in polyps of subjects with CRSwNP²². Abnormal platelet activation was reported in AERD^{23, 24}. Our present study demonstrates that measuring MPs in NLFs may be an effective tool to study pathogenesis of CRS, showing consistency with many of these previously published findings. Furthermore, we demonstrate that some MP types have potential use as biomarkers that distinguish AERD subjects from CRSwNP subjects independently of comorbid asthma and ongoing steroid use.

Methods

Patient Population

See Online Repository.

Nasal lavage fluid sampling and processing

See Online Repository.

Measurement of MPs in nasal lavage by flow cytometry

MPs in NLFs were measured by flow cytometry as previously described¹¹. NLF samples were incubated with each specific antibody (Table E1) were analyzed using a BD FACS LSRII flow cytometer and BD FACS DIVA software version 8.0 (BD Biosciences, Erembodegem Belgium). The MP gate was determined using Megamix plus SSC beads (BioCytex, Marseille, France) with a standardized MP gating strategy^{15, 16}(Fig.E1). The gating strategies of previously unstudied MP types were verified using positive control MPs isolated from culture supernatant of each cell type or plasma (Fig.E8). MP levels were evaluated as absolute MP numbers per one microliter of NLF (/μl NLF) using flowcount beads. Preliminary experiments showed that our MP assay has good repeatability (Fig.E7). Further details are provided in the Online Repository materials.

Characterization of MPs

We quantified MPs from seven different cell types, including some previously unstudied MP types (epithelial MPs, eosinophil MPs, mast cell MPs, and basophil MPs), with activation or apoptosis markers, for a total of 16 different data sets as shown in Table 1. MPs were defined as particles that ranged from 0.1 to 1.0 μm in size and were positively labelled with specific antibodies. Epithelial MPs (E-cadherin(+))MPs: E-cad+EpiMPs, EpCAM(+))MPs: EpCAM+EpiMPs), eosinophil MPs (EMR1(+))MPs: EoMPs), mast cell MPs (FcεRI(+))c-

kit(+)-MPs: MCMPs) and basophil MPs (CD203c(+)-kit(-)-MPs: BasoMPs) are abbreviated as shown and were defined using cell specific markers as indicated and previously reported²⁵⁻³⁰. Activated eosinophil MPs (CD69(+)-EoMPs: ActEoMPs) and activated mast cell MPs (CD137(+)-MCMPs: ActMCMPs) were defined as previously reported³¹⁻³⁷. Basophil activation was evaluated using CD203c mean fluorescence intensity (CD203cMFI) on basophil MPs. We did not use CD63, another basophil activation marker, because platelets express CD63 and adhere to leukocytes in AERD subjects³⁸. Apoptotic eosinophil MPs (Annexin V(+)-EoMPs: ApoEoMPs), apoptotic basophil MPs (Annexin V(+)-BasoMPs: ApoBasoMPs) and apoptotic or degranulated mast cell MPs (Annexin V(+)-MCMPs: AV+MCMPs) were defined using Annexin V. Further details are provided in the Online Repository.

Statistics

All data were analyzed using non-parametric methods unless stated otherwise. A p-value of less than 0.05 was considered significant. Further details are provided in the Online Repository.

Results

Subject demographics

The demographics of the subjects in this study are shown in Table 2. There were no significant differences in age, gender, ethnicity, atopy or past smoking status among the groups. In addition, there were no significant differences in the distribution of comorbid atopy among CRSsNP, CRSwNP, and AERD subjects. The proportion of comorbid asthma was significantly higher in CRSwNP than in CRSsNP ($p<0.03$), and was significantly higher in AERD than in CRSsNP ($p<0.0001$) or CRSwNP ($p<0.0001$). Furthermore, when compared with CRSsNP, the proportion

of oral steroid use was significantly higher in AERD than in CRSsNP ($p<0.002$) or CRSwNP ($p<0.05$), but there was no significant difference in the prevalence of intranasal steroid use.

Comparisons of endothelial MP (EndoMP), and epithelial MP (EpiMP) levels in NLFs

EndoMPs (PECAM endothelial MPs) were measured as a marker for endothelial injury^{9, 8}.

ActEndoMPs (E-selectin endothelial MPs) were measured as a marker for endothelial activation^{7, 11, 12, 39, 40}. Compared with control, ActEndoMPs were significantly increased in CRSsNP (1.8-fold vs. control median, $p<0.02$), CRSwNP (1.7-fold, $p<0.03$), and AERD (3.2-fold, $p<0.0001$) (Fig.1.A). EndoMPs were also significantly increased in CRSsNP (1.7-fold, $p<0.002$), CRSwNP (1.4-fold, $p<0.03$) and AERD (1.6-fold, $p<0.0005$) (Fig.1.B). EpCAM is located on the basolateral side of epithelial cells, whereas E-cadherin is localized in adherens junctions⁴¹. Loss of intercellular junctions and cellular detachment from basement membranes are observed during epithelial injury^{42, 43}. We measured both EpCAM+EpiMPs and E-cad+EpiMPs to evaluate degrees and conditions of epithelial injury. Compared with control, EpCAM+EpiMPs were significantly increased in CRSsNP (1.6-fold, $p<0.05$) and AERD (2.4 fold, $p<0.001$) (Fig.1.C) but were not increased in CRSwNP ($p=0.91$). Levels in CRSwNP were lower than in CRSsNP ($p<0.02$) and AERD ($p<0.0003$). E-cad+EpiMPs were significantly increased in CRSsNP (2.9-fold, $p<0.0003$), CRSwNP (1.5-fold, $p<0.05$) and AERD (2.3-fold, $p<0.0001$) (Fig.1.D). Similarly, E-cad+EpiMPs in CRSwNP were significantly lower than in CRSsNP ($p<0.004$) and AERD ($p<0.002$).

Comparisons of platelet MP (PltMP) levels in NLFs

Platelet MPs are released from activated platelets, associated with phosphatidylserine externalization, and regarded as markers for platelet activation⁴⁴⁻⁴⁶. Compared with control, PltMPs were significantly increased only in AERD (3.5-fold, $p<0.0003$) (Fig.1.E).

Comparisons of eosinophil MP (EoMP), mast cell MP (MCMP), and basophil MP (BasoMP) levels in NLFs

When compared with control, EoMPs were significantly increased in CRSsNP (1.8-fold, $p<0.0001$), CRSwNP (1.6-fold, $p<0.0001$) and AERD (1.9-fold, $p<0.0001$) (Fig.1.F). ActEoMPs were also significantly increased in CRSsNP (2.2-fold, $p<0.01$), CRSwNP (2.3-fold, $p<0.03$) and AERD (3.1-fold, $p<0.001$) (Fig.1.G). ApoEoMPs were significantly increased in CRSsNP (1.6-fold, $p<0.0001$), CRSwNP (2.2-fold, $p<0.0001$) and AERD (2.4-fold, $p<0.0001$) (Fig.1.H). Compared with control, MCMPs were significantly increased only in AERD (4.3-fold, $p<0.002$) and were not increased in CRSsNP or CRSwNP (Fig.1.I). In contrast, ActMCMPs were significantly increased in CRSsNP (2.4-fold, $p<0.03$), CRSwNP (2.4-fold, $p<0.02$), and AERD (7.4-fold, $p<0.0002$) (Fig.1.J). AV+MCMPs were significantly increased only in AERD (4.9-fold, $p<0.0003$) (Fig.1.K). When compared with control, BasoMPs were significantly increased only in AERD (2.5-fold, $p<0.05$) (Fig.1.L). However, CD203cMFI (the mean fluorescence intensity of CD203c on BasoMPs) was increased in both CRSwNP ($p<0.002$) and AERD ($p<0.0001$) (Fig.2). ApoBasoMPs were increased only in AERD (2.3-fold, $p<0.006$) and were not increased in either CRSsNP or CRSwNP (Fig.1.M).

Comparisons of Annexin V positive MP (AV+MP), erythrocyte MP (ErythroMP), and membrane dye positive MP (FM+CFSE+MP) levels in NLFs

When compared with control, the total level of Annexin V(+)MPs (AV+MPs), regardless of cell type of origin, was significantly increased in CRSwNP (1.4-fold, $p<0.02$) and AERD (1.4-fold, $p<0.02$) but not in CRSsNP (Fig.E2.A). When compared with control, ErythroMPs were not increased in CRSsNP, CRSwNP or AERD subjects (Fig.E2.B). To observe the trends of total MP levels in NLFs, we measured membrane dye positive MPs (FM(+))CFSE(+)MPs: FM+CFSE+MPs) as previously described⁴⁷. When compared with control, FM+CFSE+MP levels were significantly increased in CRSsNP (1.6-fold, $p<0.0007$), CRSwNP (1.3-fold, $p<0.003$) and AERD (2.0-fold, $p<0.0001$). In addition, FM+CFSE+MP levels were higher in AERD than in CRSsNP ($p<0.005$) and CRSwNP ($p<0.0003$) (Fig.E2.C).

Receiver Operating Characteristic (ROC) curve analysis of MP levels for distinguishing AERD from CRSwNP

We assessed the feasibility of using levels of MPs as biomarkers to distinguish subjects with AERD from subjects with CRSwNP (45 CRSwNP and 31 AERD subjects) using area under the curve (AUC) analysis. There were significant differences in levels of ActEndoMPs, PltMPs, EpCAM+EpiMPs, E-cad+EpiMPs, MCMPs, ActMCMPs, AV+MCMPs, BasoMPs and ApoBasoMPs between CRSwNP and AERD. The results of this analysis are summarized in Table 4. There were significant differences in distribution of oral corticosteroid use and asthma between the two groups. The multivariate logistic analysis showed that the following MP types had cut-off values that were significantly associated with AERD, independent of asthma and oral steroid use (Table E2): cut-off values of PltMPs, 206/ μ L (AUC 0.77, sensitivity 68%, specificity, 84%) (Fig.3.A); EpCAM+EpiMPs, 3687/ μ L (AUC 0.75, sensitivity 52%, specificity 91%) (Fig.3.B); E-cad+EpiMPs, 1027/ μ L (AUC 0.72, sensitivity 74%, specificity 64%) (Fig.3.C);

MCMPs, 854/ μ L (AUC 0.70, sensitivity 74%, specificity 64%); ActMCMPs, 424/ μ L (AUC 0.71, sensitivity 58%, specificity 80%); AV+MCMPs, 192/ μ L (AUC 0.72, sensitivity 81%, specificity 60%) (Fig.3.D); and ApoBasoMPs, 0.69/ μ L (AUC 0.69, sensitivity 61%, specificity 73%).

Comparisons of MP levels between CRSwNP subjects without asthma and CRSwNP subjects with aspirin tolerant asthma, and comparison between CRSwNP subjects with aspirin tolerant asthma and AERD subjects

Although the proportion of females was significantly higher in CRSwNP without asthma than CRSwNP with aspirin tolerant asthma (ATA) ($p<0.03$), there was no significant difference in the other patient demographic endpoints between these two groups (Table E5). There was no significant difference in levels of any MP types between CRSwNP without asthma and CRSwNP with ATA (Table E6). There was no difference in the patient demographic endpoints between CRSwNP with ATA and AERD. Even when compared with the more narrowly defined group of CRSwNP with ATA, levels of PltMPs ($p<0.0003$), EpCAM+EpiMPs ($p<0.0005$), E-cad+EpiMPs ($p<0.02$), MCMPs ($p<0.02$), ActMCMPs ($p<0.02$), AV+MCMPs ($p<0.02$) and ApoBasoMPs ($p<0.006$) were still higher in AERD. There was no significant difference in levels of the other MP types between these two groups (ActEndoMPs: $p=0.061$, EndoMPs: $p=0.588$, EoMPs: $p=0.386$, ActEoMPs: $p=0.241$, ApoEoMPs: $p=0.698$, BasoMPs: $p=0.165$ and CD203cMFI on BasoMPs: $p=0.116$).

Discussion

This is the first study to evaluate the pathophysiology of CRS and AERD by analyzing released MP levels and types in NLFs. The findings are summarized in Table 3. MPs were detected in NLFs of both control and CRS subjects, and there were similarities as well as significant

differences in MP types and levels comparing NLFs from subjects with CRSsNP, CRSwNP, and AERD. MPs contain various bioactive effectors originating from the parent cells that can actively modulate immune responses under both normal healthy and pathological conditions^{5, 48, 49}. Our results indicate that MPs were released from activated immune cells and injured structural cells into the nasal cavity under pathological and healthy normal conditions and may play roles in cell to cell communication in the nose and paranasal sinuses. Furthermore, we demonstrate that several MP types, have potential use as biomarkers to distinguish AERD from CRSwNP subjects independently of asthma and steroid intake, especially PltMPs (AUC 0.77, sensitivity 68%, specificity, 84%), EpCAM+EpiMPs (AUC 0.75, sensitivity 52%, specificity 91%), and AV+MCMPs (AUC 0.72, sensitivity 81%, specificity 60%). Current estimates of AERD prevalence among CRS patients and among the general population are variable due to variations in diagnostic methods and definitions¹. In this study, aspirin intolerance was diagnosed based on clinical history (reports from patients and/or in patient charts). However, clinical history alone may not be sufficient to diagnose all AERD subjects⁵⁰. As such, oral aspirin challenges remain the gold standard for confirming this disease^{1, 3, 4}. Future analysis of MPs in a large cohort of subjects with challenge-confirmed AERD is essential to establish the usefulness of MP levels in NLFs as biomarkers. If validated in such a study, the use of MP may help to better estimate AERD prevalence and help standardize the diagnosis of AERD.

Convincing evidence implicates epithelial barrier disruption in the pathogenesis of asthma, eczema and CRS^{19, 51}. However, there have been no reports evaluating the degree of epithelial injury in CRS and comparing CRSsNP, CRSwNP and AERD. In this study, both EpCAM+EpiMPs and E-cad+EpiMPs were significantly increased in CRSsNP and AERD when compared with control subjects (Fig.1.C,D). E-cadherin is localized only in adherens junctions⁴¹.

Thus, the increased E-cad+EpiMPs in the nasal cavity may reflect a loss of intercellular adherens junctions in CRSsNP and AERD as observed during epithelial injury⁴³. In contrast, EpCAM is located on the basolateral side⁴¹. The increased EpCAM+EpiMPs may reflect detachment of epithelial cells from basement membranes in CRSsNP and AERD as observed during epithelial injury⁴². Our results indicate that epithelial barrier disruptions are induced by loss of both intercellular junctions and cellular attachment to the basement membrane in CRSsNP and AERD. On the other hand, EpCAM+EpiMPs and E-cad+EpiMPs were both significantly lower in CRSwNP when compared with CRSsNP or AERD, indicating a lower degree of epithelial injury in CRSwNP than in CRSsNP or AERD. Interestingly, although E-cad+EpiMPs were significantly increased, EpCAM+EpiMPs were not increased in CRSwNP. Epithelial barrier disruption may be induced mainly by loss of intercellular junctions in CRSwNP. Variable epithelial barrier disruption may reflect heterogeneity of inflammatory mechanisms and histological changes among CRSwNP, CRSsNP and AERD.

The accumulation of eosinophils in polyps has been known for over a century^{22, 52, 53}. In our studies, ECP levels in NLFs were significantly increased in CRSwNP and AERD when compared with CRSsNP (Fig.E5), indicating that a high degree of eosinophil degranulation occurs in CRSwNP and AERD, but not CRSsNP. However, we were surprised to find that there was no significant difference in EoMP and ActEoMP levels among CRSsNP, CRSwNP and AERD (Fig.1.F,G). Because eosinophils accumulate in most polyps, our results may indicate that the number of released EoMPs and ActEoMPs per eosinophil was higher in CRSsNP, suggesting that the degree of activation per eosinophil was higher in non-polypoid diseases than in polypoid disease. Eosinophils may be also activated in non-polypoid diseases without eosinophil accumulation and degranulation. Although correlations between ActEoMPs and ECP levels were

weak (CRSwNP: $r_s = 0.358$, $p < 0.04$, AERD: $r_s = 0.111$, $p = 0.63$), one recent article reported relationships between exosome release and eosinophil degranulation⁵⁴. Exosomes in eosinophils contain EPO, MBP, and ECP, and are secreted after induction of degranulation *in vitro*⁵⁴. In contrast with MP release, exosome secretion can occur both spontaneously and constitutively, and is controlled in different cell types by various environmental influences^{5, 54}. Eosinophil degranulation may be a specific feature of eosinophil activation under the microenvironment of polyps. Analysis of extracellular vesicles may improve our understandings and quantification of eosinophil activation in CRS.

Tryptase positive mast cells are increased in CRS, along with increased IgE levels in many cases, and have been implicated in pathogenesis^{21, 55}. ActMCMPs were increased 2-fold in CRSsNP and CRSwNP, and increased an impressive 7.4-fold in AERD when compared with control (Fig.1.J). In addition, ActMCMPs showed strong correlations with ActEoMPs (Fig.E4). These results indicate that mast cells are profoundly activated in AERD subjects, more so than in CRSsNP and CRSwNP. Furthermore, the correlations of ActMCMPs with ActEoMPs suggest that the activation pathways for these two cells may be similar, or related. Interestingly, AV+MCMPs were significantly increased (4.9-fold) only in AERD and were not increased in CRSsNP or CRSwNP. Because phosphatidylserine externalization occurs in the process of mast cell degranulation⁵⁶, our results may indicate that mast cell activation, rather than apoptosis, is what we are detecting with this MP analysis. There was no significant difference in mast cell MP levels between AERD subjects with and without atopy (data not shown). Non-IgE dependent mast cell activation was also reported to play an important role in the pathophysiology of AERD⁵⁷. However, there are no established mast cell activation cell surface markers specific for IgE independent pathways. Discovery of markers unique to non-IgE dependent mast cell

activation and evaluation of non-IgE pathway activation using MP assays could be useful in future studies.

In this study, MP findings indicate that basophils are not activated in CRSsNP, but are activated in CRSwNP and AERD (Fig.2). This may be in concordance with the Type 2 cytokine pattern in CRSwNP and AERD. In addition, ApoBasoMPs were significantly increased only in AERD but not in any other subject group (Fig.1.M), indicating that apoptosis is induced in basophils only in AERD. Furthermore, BasoMP levels in AERD were higher than in CRSwNP (Fig.1.L), indicating that the degree of basophil activation is likely higher in AERD than CRSwNP. In a recent study, we reported lower basophil counts in polyps in AERD compared with CRSwNP and speculated that the basophils are likely migrating to AERD polyps and then become undetectable due to either degranulation or apoptosis²². The finding in the present study suggests that the highest level of activation and apoptosis in basophils occurs in AERD polyps, supporting this previous speculation.

PltMPs were significantly increased only in AERD, and not in the other phenotypes of CRS in this study (Fig.1.E). In addition, PltMPs were strongly correlated with ActEoMPs in AERD (Table E3) (Fig.E3). Platelet MPs have previously been reported to be shed from activated platelets and are regarded as both effectors and biomarkers for platelet activation^{44, 45}. Our results provide further support to the concept that platelets are activated in AERD subjects, even without treatment with COX1 inhibitor drugs. Laidlaw and Boyce reported that platelets adhere to eosinophils and neutrophils and can be found within nasal polyp tissue in AERD subjects to a greater extent than in CRSwNP³⁸. The present studies support their conclusion, and suggest that the high degree of platelet activation is unique to AERD among the types of CRS that we studied here. The use of MPs to assess the level of activation of platelets within NLF

may provide an important new tool to study disease prevalence, progression and remission in AERD.

It should be noted that there are some interesting patterns of baseline MP levels in NLFs of control subjects. Although platelet MPs and endothelial MPs are the major populations of MPs in the plasma^{58, 59}, baseline PltMP levels were very low in NLFs (about 4% of EndoMP). In addition, although basophils are rare populations in both the nasal cavity and blood, baseline BasoMP levels were the highest (as high as EndoMPs) among the MP types in NLFs. We also measured baseline BasoMPs and EndoMPs in the plasma (n=9), and found that baseline BasoMP levels were around 40% of the level of EndoMPs (Table E4). MP levels in NLFs and plasma reflect the balance between MPs that are released from the cells of origin and MPs that are taken up by target cells. We speculate that this balance may be different between the nasal cavity and the circulation, or among MP types. Differences in the rate of turnover among the cell types may also influence baseline levels of each MP type. Mechanisms of MP release and uptake in *in vivo* models have not been clarified yet and should require further study.

There are a number of limitations of this study. Although there was no difference in the prevalence of nasal steroid use among CRSsNP, CRSwNP and AERD, the number of subjects using oral corticosteroids in AERD was significantly higher than in the other forms of CRS. Glucocorticoids can induce apoptosis in immune cells such as eosinophils⁶⁰, and basophils⁶¹. However, there was no significant difference in levels of any MP types between AERD subjects with and without oral steroid use in this study (data not shown). The increased ApoEoMPs, and ApoBasoMPs may be specific features of AERD. In addition, platelet MPs in plasma were reported to be significantly increased in subjects with asthma, which is one of the triad of conditions defining AERD⁶². Although growing evidence has suggested that abnormal platelet

activation is uniquely associated with the pathophysiology of AERD independently of asthma²³,
further studies are necessary to clarify this point. However, PltMP levels in NLFs were higher
in AERD than in CRSwNP with aspirin tolerant asthma, and the multivariate logistic analysis
showed our cut-off values of platelet MPs, epithelial MPs, and mast cell MPs were significantly
associated with AERD independently of asthma in this study. Furthermore, MP assay in NLFs is
representative of inflammation in the entire nasal cavity and NLFs have not yet been isolated
from specific anatomic structures within the nose and sinuses. Thus, it is impossible to clarify the
parts of the nasal cavity from which MPs in NLF are originating. To address this weakness, we
are now developing a new assay using a sponge based approach⁶³ to collect MPs released from
specific anatomic structures in nasal cavity.

In conclusion, we found evidence for activation of endothelium, epithelium, mast cells,
basophils, eosinophils and platelets in the various forms of CRS and also found clear differences
among the phenotypes of disease. Furthermore, we found that some MP types, especially
EpCAM+EpiMPs, PltMPs, and AV+MCMPs, are potential biomarkers for distinguishing AERD
from CRSwNP in the clinic. Although platelet activation markers were reported to be potential
diagnostic biomarkers for AERD in blood²³, MP analysis in NLF may make diagnosis of AERD
more robust by evaluating both EpiMPs and AV+MCMPs in addition to PltMPs. Recently, type
2 innate lymphoid cells have been detected in nasal polyp tissues and interest has focused on
their role in the pathophysiology of CRS^{64,65}. At present, definitively detecting ILC2 requires a
cocktail of several cell surface markers, making detection of ILC2-derived MP challenging.
Developing reliable MP analysis of these cells is of great interest to us. We measured NLF
samples collected from patients taken in the operating room in this study. However, in results not
shown, we have recently found that MPs were also detectable in NLFs collected from patients

during a routine clinical office visit. We believe that MPs in NLFs may become an invaluable tool in the study of CRS and AERD in the laboratory and provide new biomarkers for diagnosis and treatment of CRS in the clinic.

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429 **Table 1. Definition of MPs**

MP type	Abbreviation	Definition
Endothelial MPs	Endothelial MPs	
E-selectin endothelial MPs	ActEndoMPs	E-selectin(+)MPs
PECAM endothelial MPs	EndoMPs	CD31(+)CD41(-)MPs
Epithelial MPs	EpiMPs	
EpCAM EpiMPs	EpCAM+EpiMPs	EpCAM(+)MPs
E-cadherin EpiMPs	E-cad+EpiMPs	E-cadherin(+)MPs
Platelet MPs	PltMPs	CD31(+)CD41(+)MPs
Eosinophil MPs	EoMPs	EMR1(+)MPs
Activated EoMPs	ActEoMPs	CD69(+)EMR1(+)MPs
Apoptotic EoMPs	ApoEoMPs	Annexin V(+)EMR1(+)MPs
Mast cell MPs	MCMPs	FceRI(+)c-kit(+)MPs
Activated MCMPs via IgE pathway	ActMCMPs	CD137(+)MCMPs
Apoptotic or degranulated MCMPs	AV+MCMPs	Annexin V(+)MCMPs

Basophil MPs	BasoMPs	CD203c(+)c-kit(-)MPs
Activated basophil	CD203cMFI	CD203c MFI on BasoMPs
Apoptotic BasoMPs	ApoBasoMPs	Annexin V(+)BasoMPs
Annexin V positive MPs	AV+MPs	Annexin V(+)MPs
Erythrocyte MPs	ErythroMPs	CD235a(+)MPs
Membrane dye positive MPs	FM+CFSE+MPs	FM(+)CFSE(+)MPs

430 MFI; Mean fluorescence intensity, FM; lipid-FM specific dye, CFSE; protein-

431 Carboxyfluoresceinsuccinimidyl ester (CFSE) specific dye

432

433 Table 2. Demographics of the 133 subjects

	Control	CRSsNP	CRSwNP	AERD
	(n=24)	(n=33)	(n=45)	(n=31)
Gender, male/female	16/8	17/16	30/15	14/17
Ethnicity, White/ non-White	19/5	29/4	34/11	22/9
Age (years), mean $\pm$ standard deviation	44.5 $\pm$ 14.2	42.1 $\pm$ 15.0	46.9 $\pm$ 11.3	46.7 $\pm$ 11.7
Smoking status, never/ex-smokers ¹⁾	21/3	25/8	34/11	23/8
Comorbidity				
Asthma (%)	-	8 (24%)	22 (49%)*	31 (100%)*,#
Atopy (%)	5 (21%) ²⁾	16 (49%)	28 (62%)	20 (65%)
Steroid use				
Oral (%)	-	0 (0%)	4 (9%)	8 (25%)*#
Dose (prednisone mg/day), median [Range]	-	-	7.5 [5-10]	7.5 [5-30]

Intranasal (%)	-	6 (18%)	9 (20%)	8 (26%)
Fluticasone propionate	-	6	6	5
Mometasone furoate	-	0	3	2
Dexamethasone	-	0	0	1

¹⁾; Current smokers were excluded and all subjects stopped smoking more than 6 months before enrollment.

²⁾; None were on medication for atopy at the time of enrollment.

*; $p < 0.05$, vs. CRSsNP, #; $p < 0.05$, vs. CRSwNP

440 **Table 3. Comparisons to controls of MPs in nasal lavage among CRSsNP, CRSwNP and**
 441 **AERD**

	Control	CRSsNP	CRSwNP	AERD
Endothelial MPs (EndoMPs)				
ActEndoMPs	-	1.8-fold	1.7-fold	3.2-fold
	653 [243-3301]	1198 [283-4823]	1103 [223-7180]	2078 [401-10231]
EndoMPs	-	1.7-fold	1.4-fold	1.6-fold
	2206 [869-4657]	3634 [1495-6869]	3055 [1146-7924]	3588 [1990-8840]
Epithelial MPs (EpiMPs)				
EpCAM+EpiMPs	-	1.6-fold	NS	2.4-fold
	1534 [266-3301]	2383 [403-9769]	1606 [343-6680]	3687 [463-8995]
E-cad+EpiMPs	-	2.9-fold	1.5-fold	2.3-fold
	586 [150-3182]	1669 [249-8202]	886 [146-3252]	1326 [404-8542]
Platelet MPs (PltMPs)	-	NS	NS	3.5-fold
	77 [0-535]	112 [0-450]	65 [0-874]	267 [12-2462]

Eosinophil MPs (EoMPs)	-	1.8-fold	1.6-fold	1.9-fold
	1867 [486-3017]	3342 [1136-7651]	2938 [1105-8100]	3577 [832-8154]
ActEoMPs	-	2.2-fold	2.3-fold	3.1-fold
	142 [0-1050]	312 [14-3306]	326 [20-1787]	443 [52-2373]
ApoEoMPs	-	1.6-fold	2.2-fold	2.4-fold
	589 [99-1005]	961 [276-2757]	1284 [260-3527]	1401 [60-3762]
Mast cell MPs (MCMPs)	-	NS	NS	4.3-fold
	461 [59-3912]	536 [35-4003]	557 [73-5275]	1989 [58-6176]
ActMCMPs	-	2.4-fold	2.4-fold	7.4-fold
	59 [0-1211]	143 [0-1558]	140 [0-1619]	437 [0-2407]
AV+MCMPs	-	NS	NS	4.9-fold
	138 [0-682]	145 [0-2175]	152 [0-1446]	675 [29-3521]
Basophil MPs (BasoMPs)	-	NS	NS	2.5-fold
	2786 [227-5274]	2825 [305-6953]	2519 [873-9578]	3497 [1289-18841]

CD203cMFI	- 1.35 [1.02-2.58] X10 ³	NS 1.30 [0.70-3.50] X10 ³	Increased 1.91 [0.93-8.73] X10 ³	Increased 2.56 [1.09-6.81] X10 ³
ApoBasoMPs	- 162 [2-924]	NS 161 [33-934]	NS 195 [0-1708]	2.3-fold 375 [0-3308]
Annexin V positive MPs (AV+MPs)	- 4358 [1684-8147]	NS 5044 [2015-8316]	1.4-fold 6242 [2437-15629]	1.4-fold 6293 [2784-22001]
Erythrocyte MPs (ErythroMPs)	- 2154 [475-4556]	NS 1547 [538-4405]	NS 1732 [336-4198]	NS 2186 [514-4181]
Membrane dye positive MPs (FM+CFSE+MPs)	- 20794 [13242- 37119]	1.6-fold 33913 [16302- 53933]	1.3-fold 28587 [12000- 68764]	2.0-fold 43271 [19218- 77955]

442 All data of MP levels are presented as median [range].

443 NS; not significant vs. control

444 ##-fold; significantly higher by ##-fold vs. control

445 Increased; significantly higher in MFI vs. control

446

447

448

Table 4. Receiver operating characteristic (ROC) curve analysis of MP levels for distinguishing AERD from CRSwNP

	Cut-off value	AUC [95% CI]	Sensitivity	Specificity
ActEndoMPs	2079	0.68 [0.54-0.79]	52	80
PltMPs	206	0.77 [0.64-0.86]	68	84
EpCAM+EpiMPs	3687	0.75 [0.63-0.85]	52	91
E-cad+EpiMPs	1027	0.72 [0.59-0.82]	74	64
MCMPs	854	0.70 [0.57-0.81]	74	64
ActMCMPs	424	0.71 [0.57-0.82]	58	80
AV+MCMPs	192	0.72 [0.59-0.82]	81	60
BasoMPs	6869	0.64 [0.50-0.76]	32	91
ApoBasoMPs	338	0.69 [0.56-0.81]	61	73

Figure Legends**Fig.1. Comparison of MPs among CRSsNP, CRSwNP, AERD, and control subjects**

(A) ActEndoMPs, (B) EndoMPs, (C) PltMPs, (D) EpCAM+EpiMPs, (E) E-cad+EpiMPs, (F) EoMPs, (G) ActEoMPs, (H) ApoEoMPs (I) MCMPs, (J) ActMCMPs, (K) AV+MCMPs, (L) BasoMPs, (M) ApoBasoMPs

*, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$

Fig.2. Comparison of CD203c mean fluorescence intensity (MFI) on BasoMPs among CRSsNP, CRSwNP, AERD, and control subjects

*, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$

Fig.3. Receiver operating characteristic (ROC) curve of PltMP, EpCAM+EpiMP, E-cad+EpiMP, AV+MCMP levels for distinguishing AERD subjects from CRSwNP subjects

(A) PltMPs, (B) EpCAM+EpiMPs, (C) E-cad+EpiMPs, (D) AV+MCMPs

Fig.4. Released MP types and levels in CRSwNP and AERD subjects

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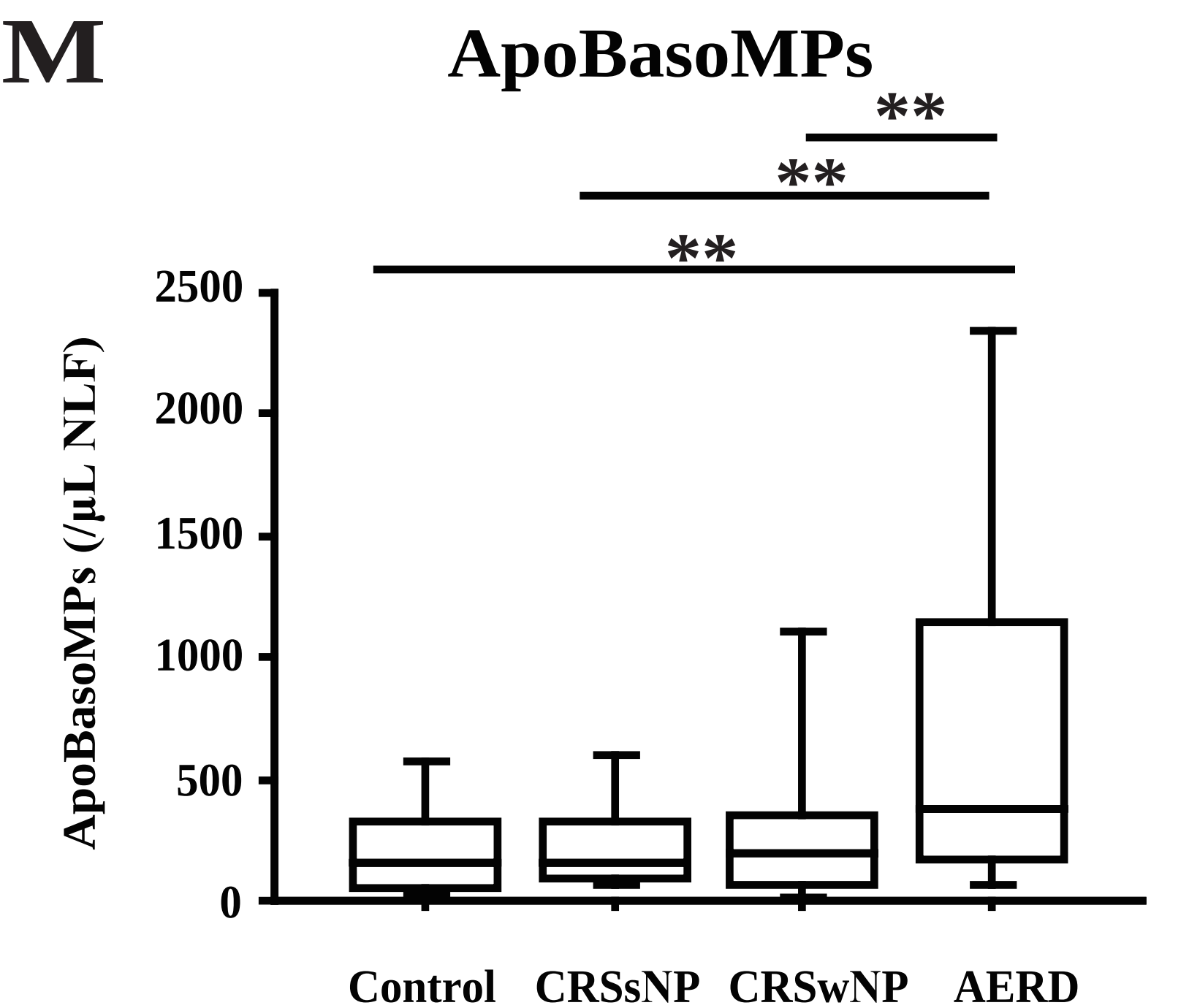
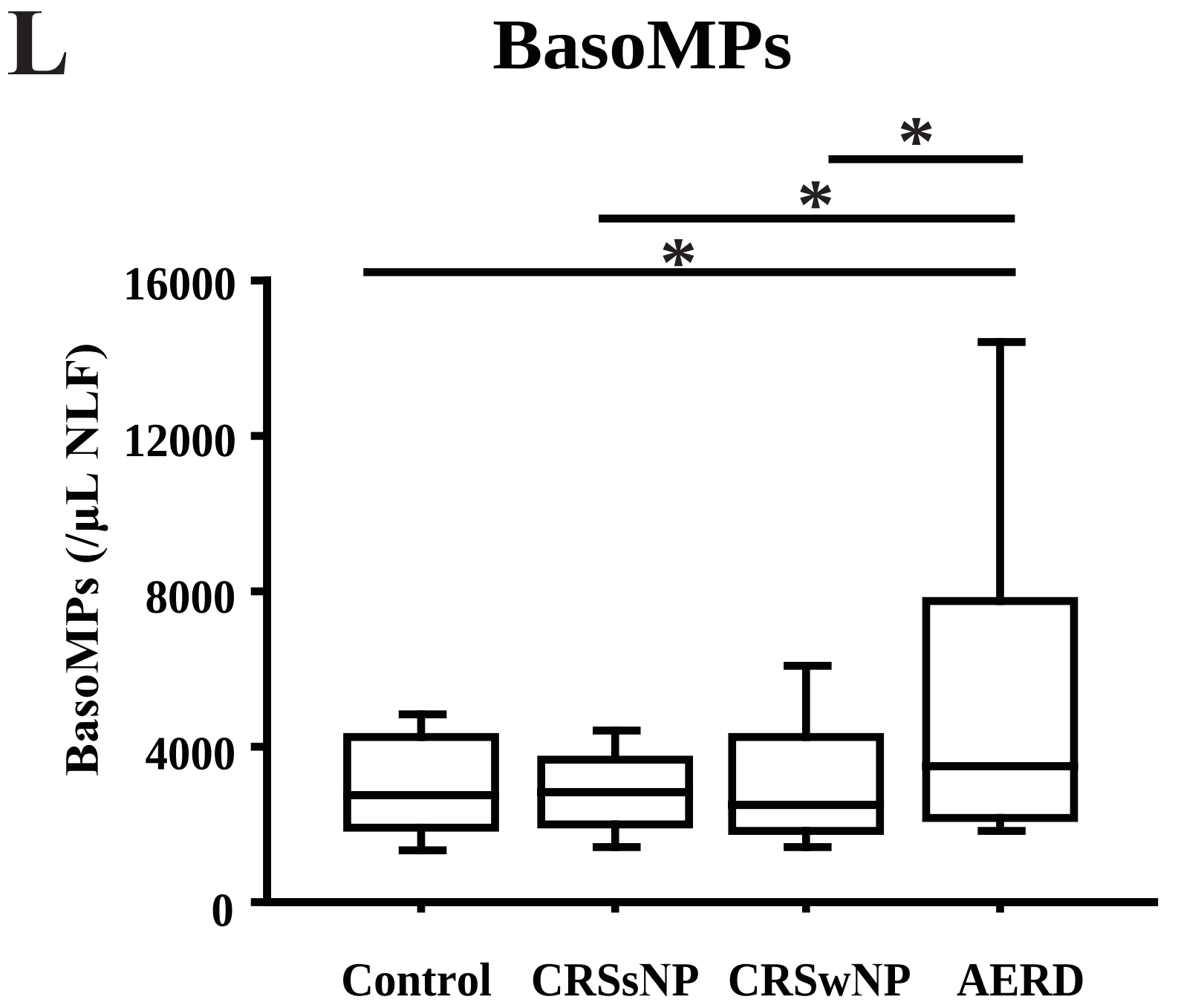
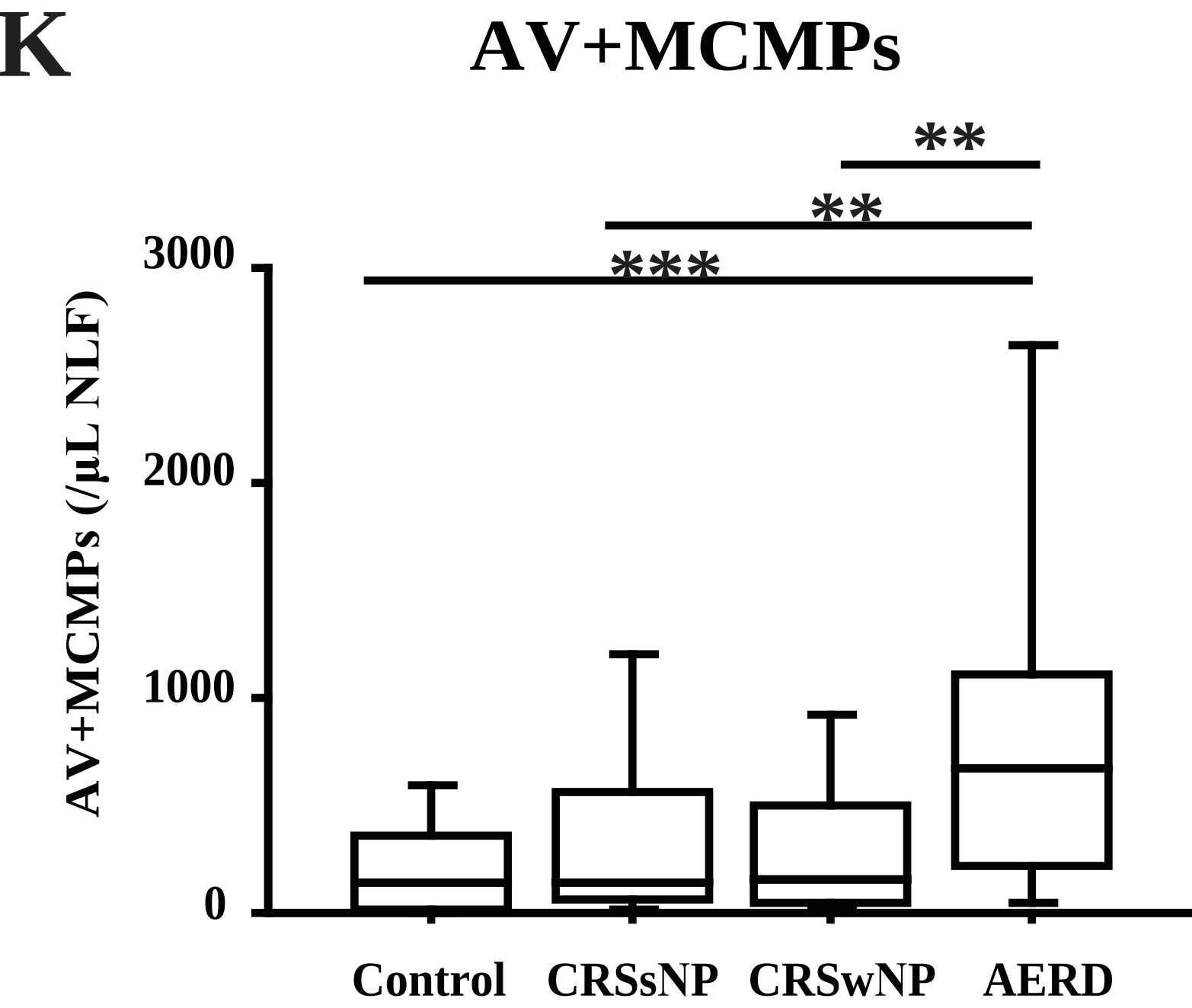
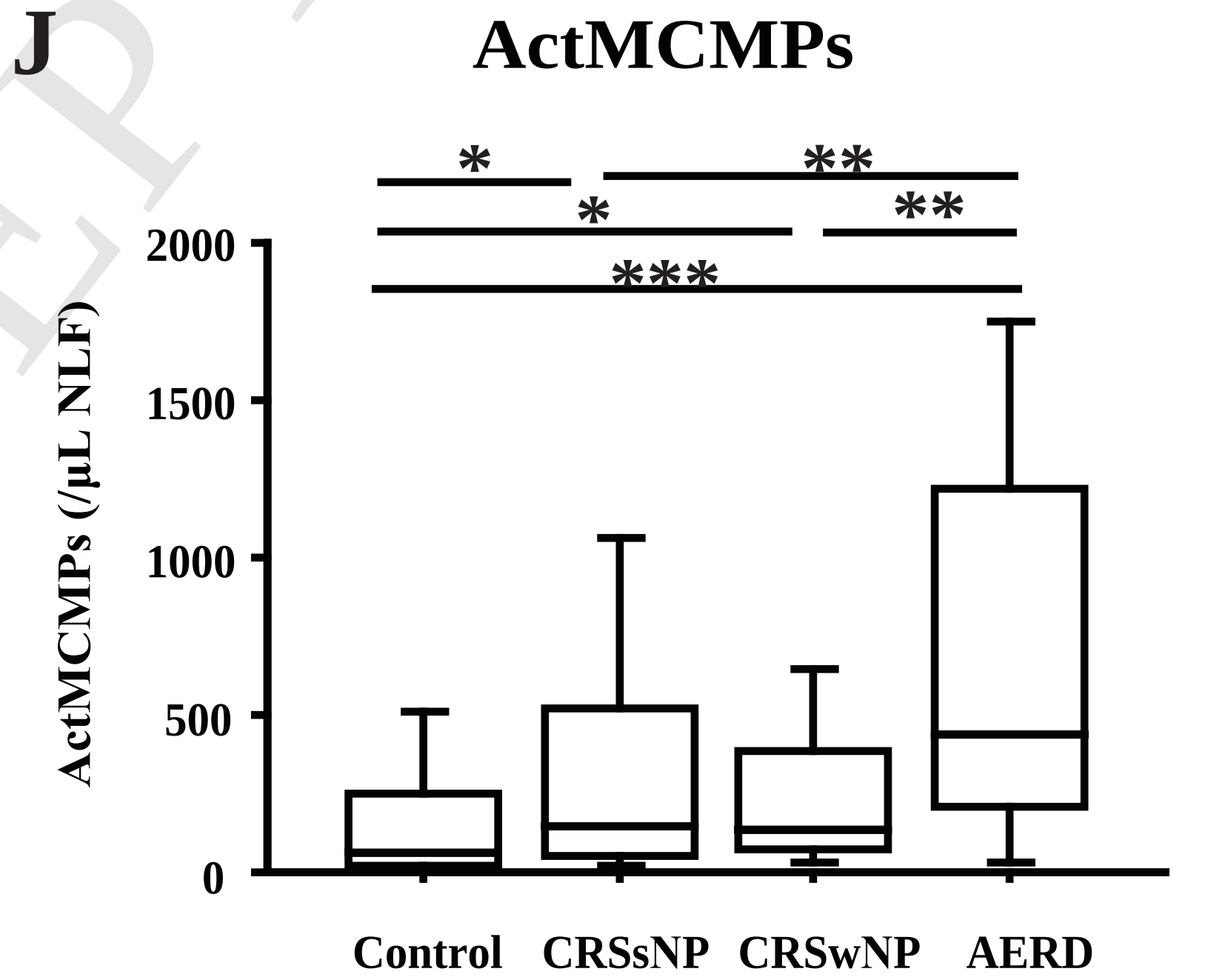
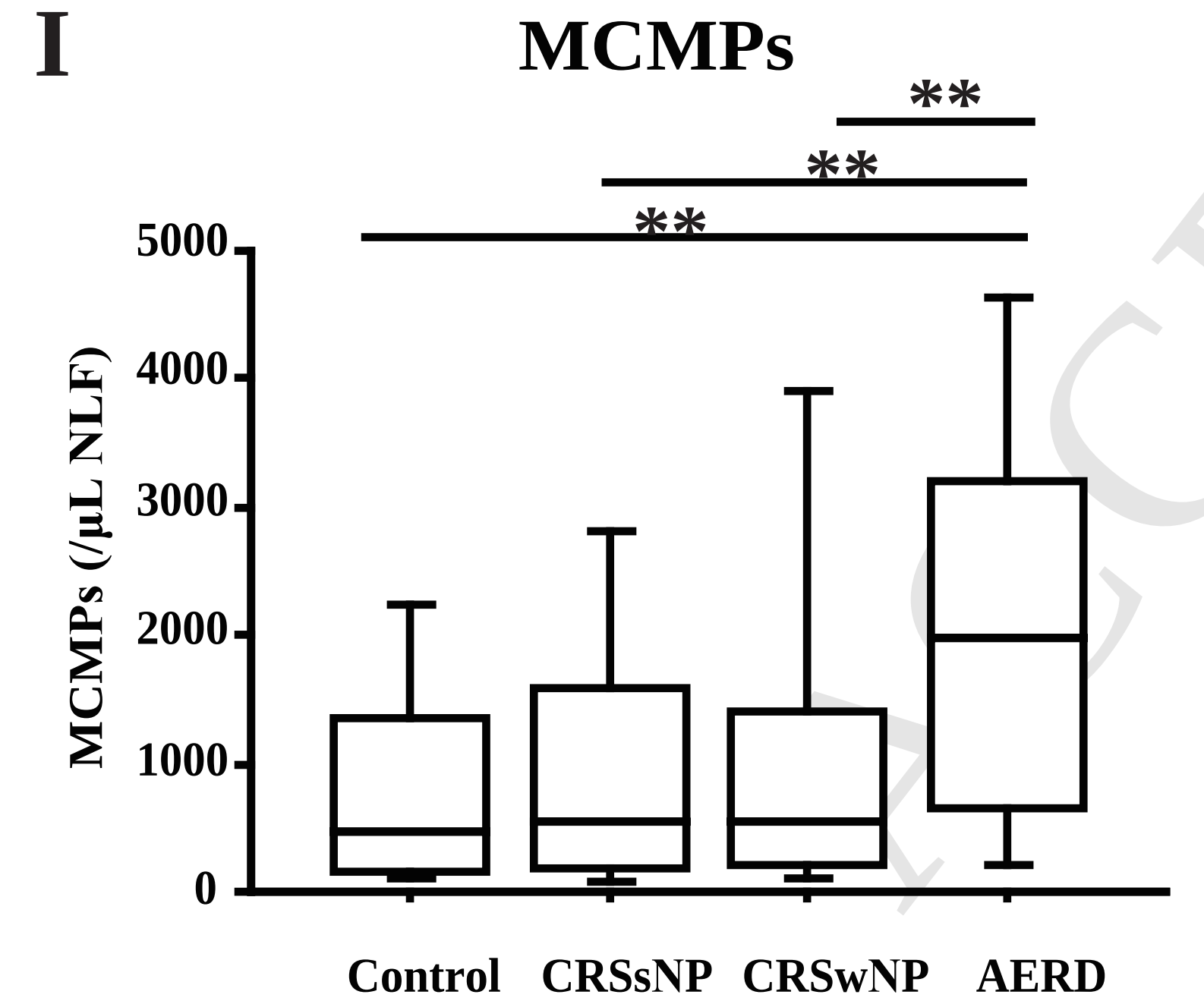
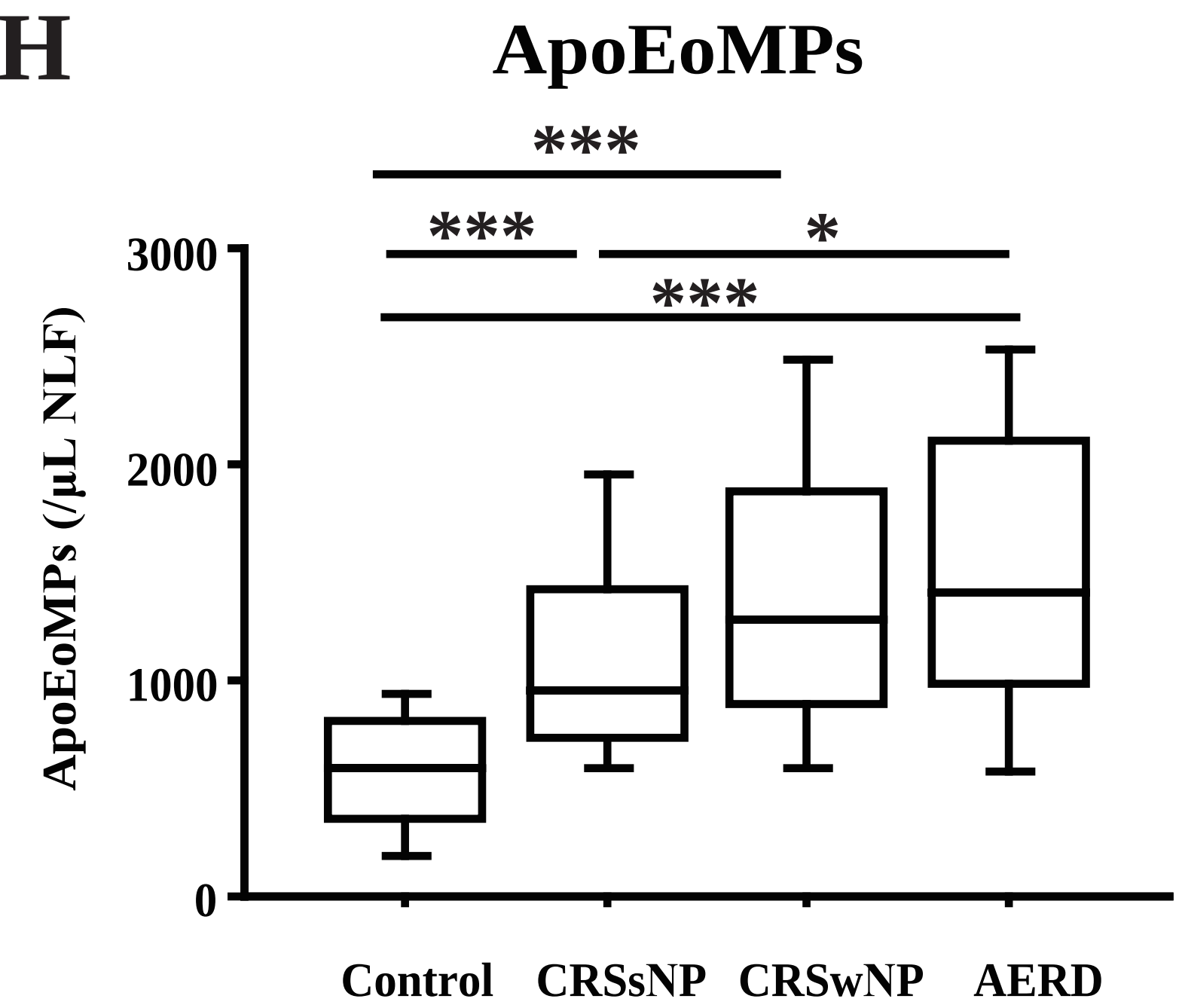
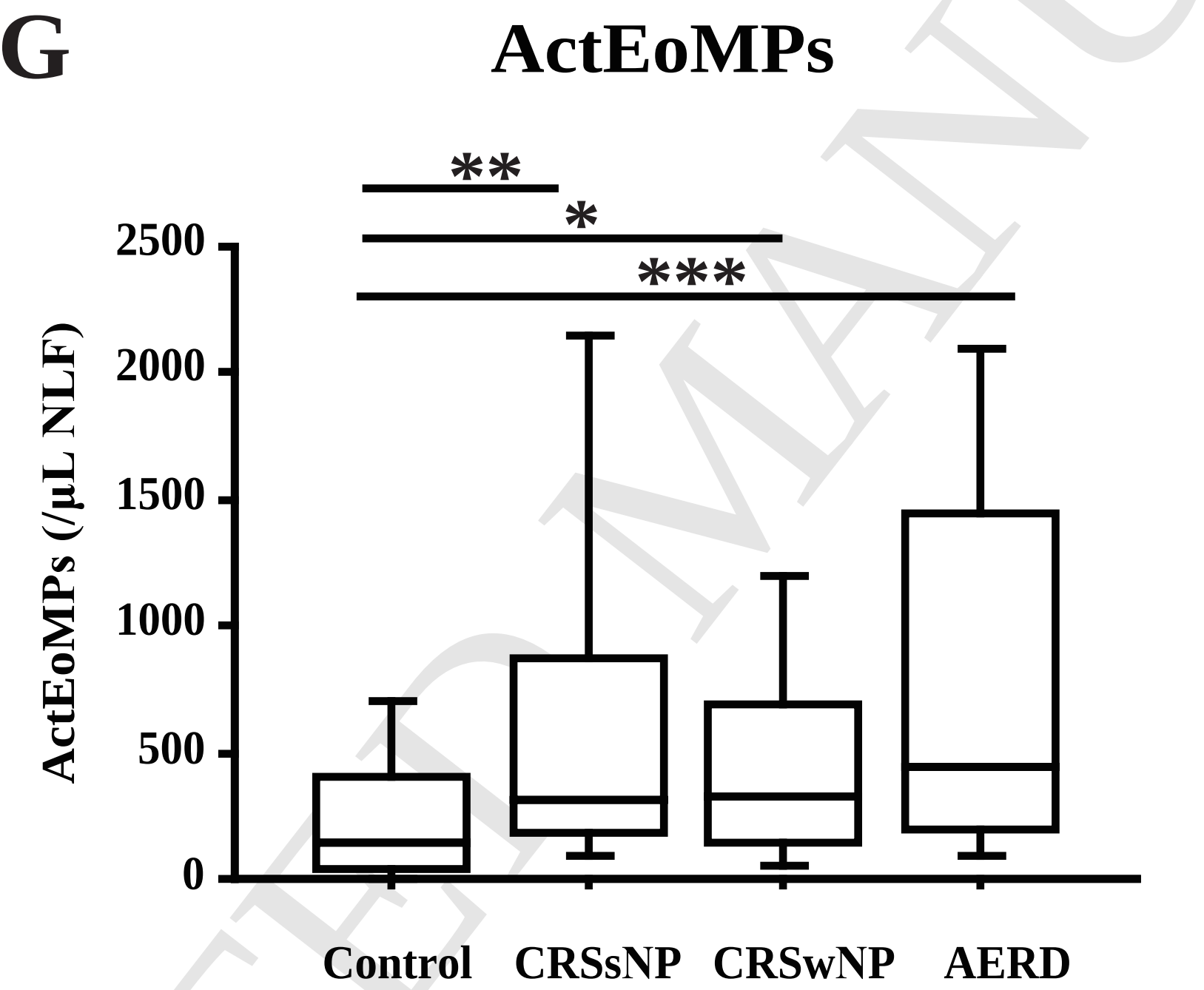
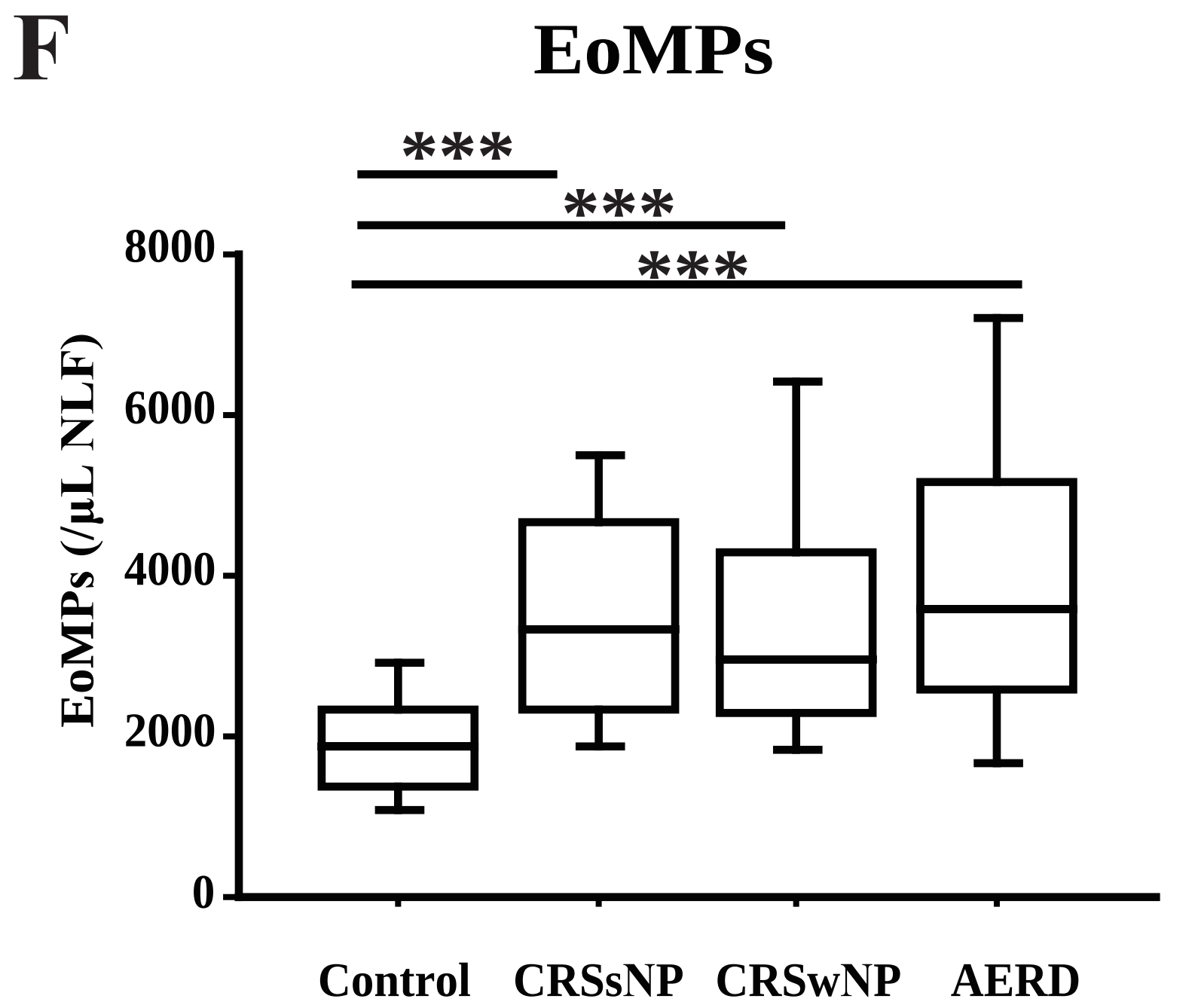
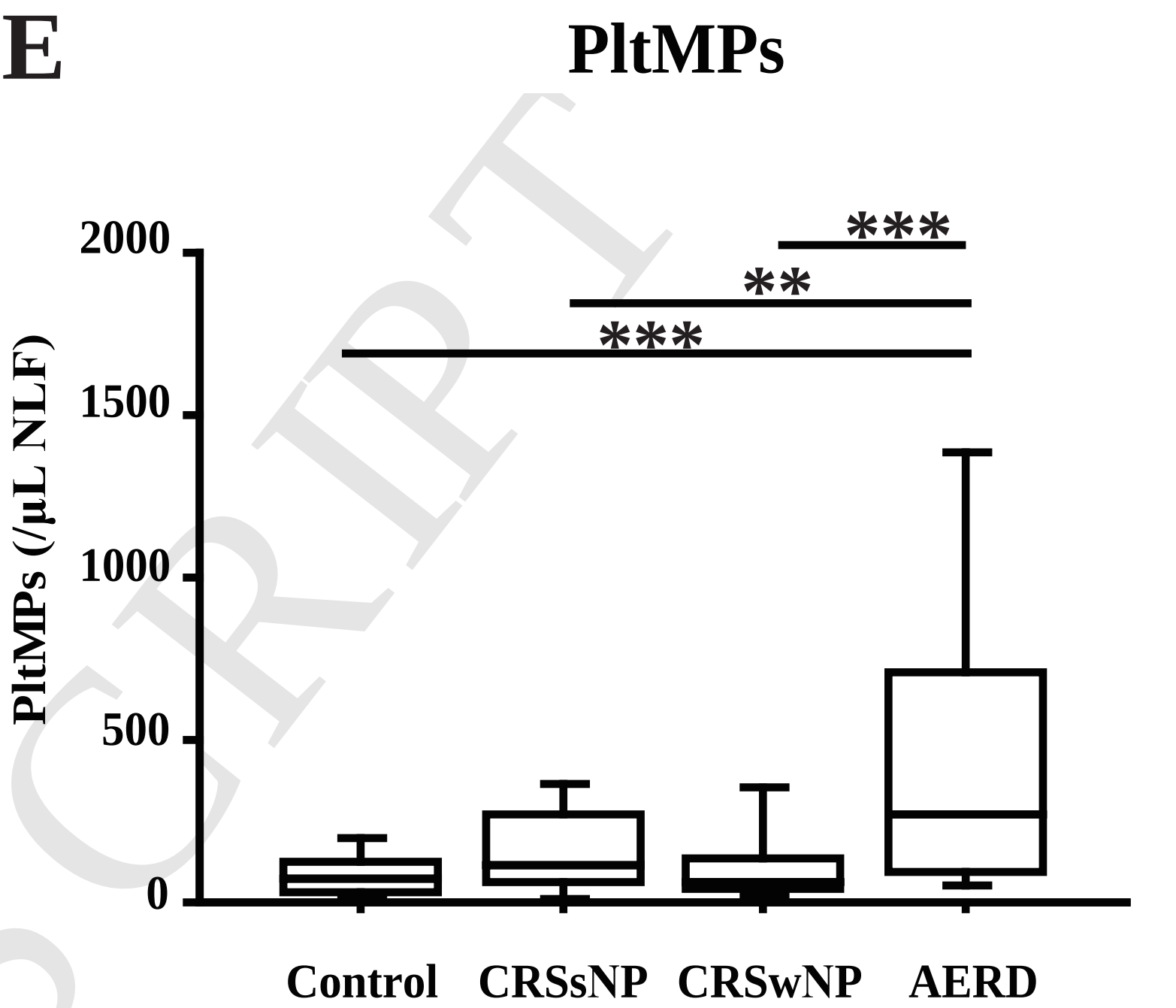
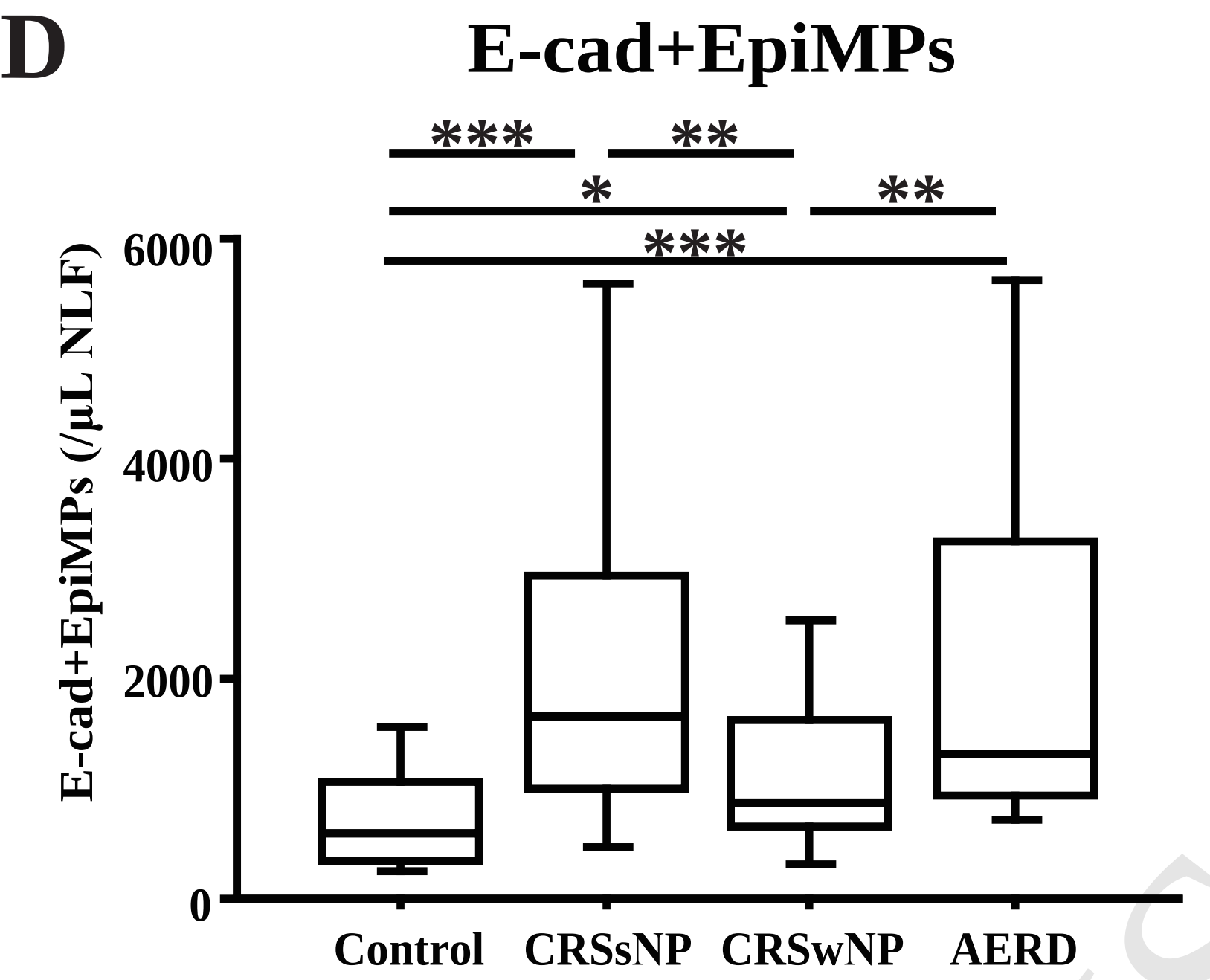
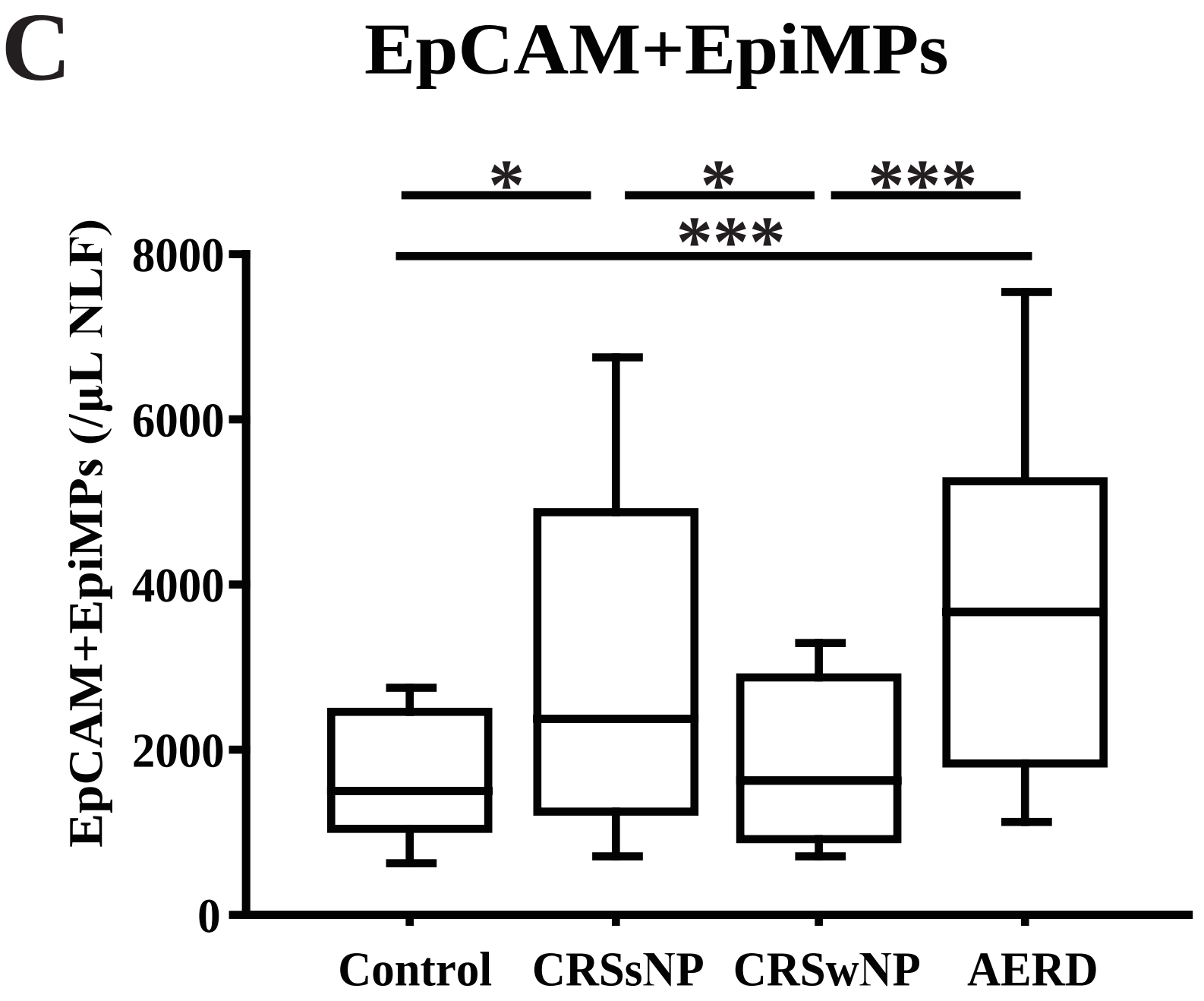
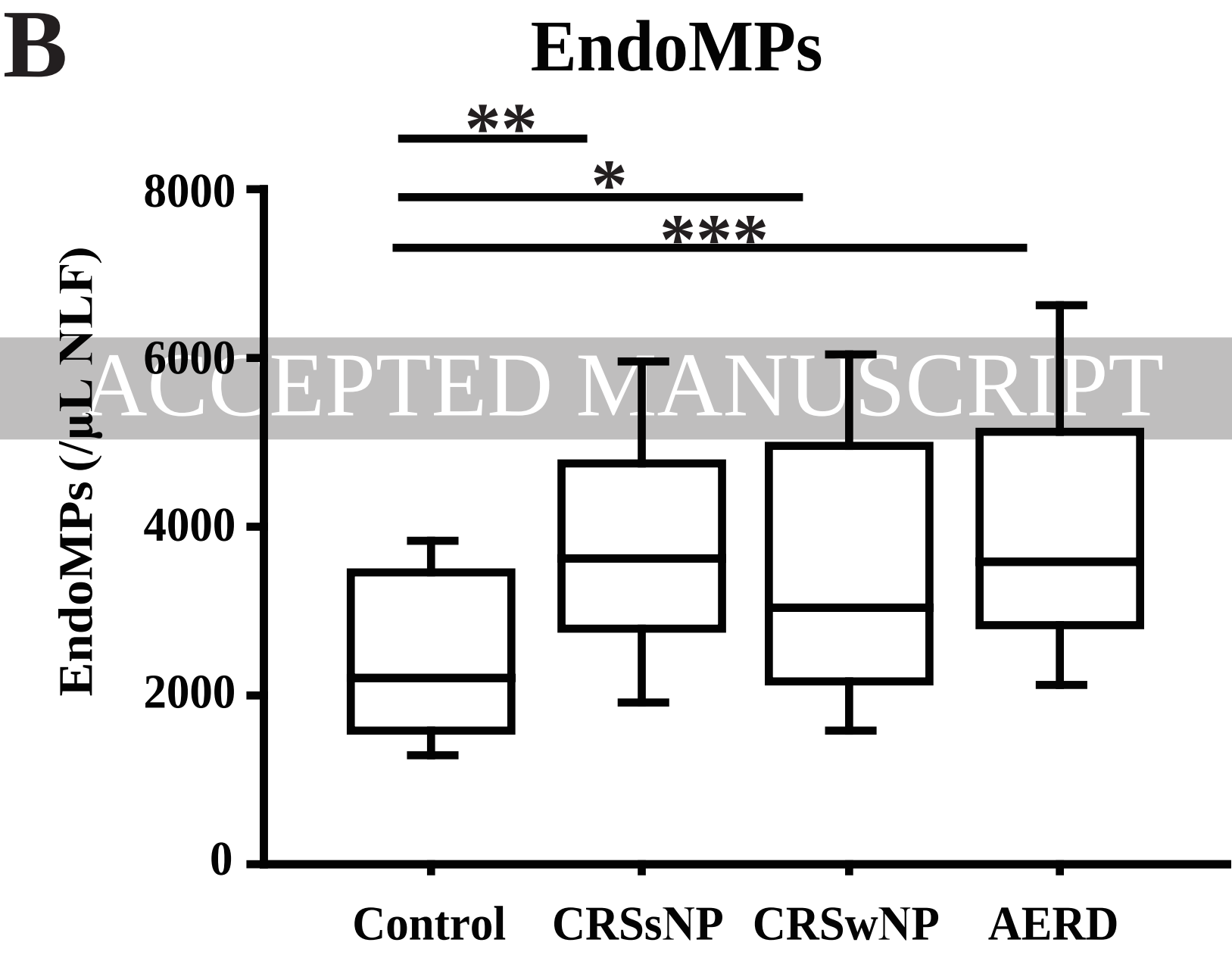
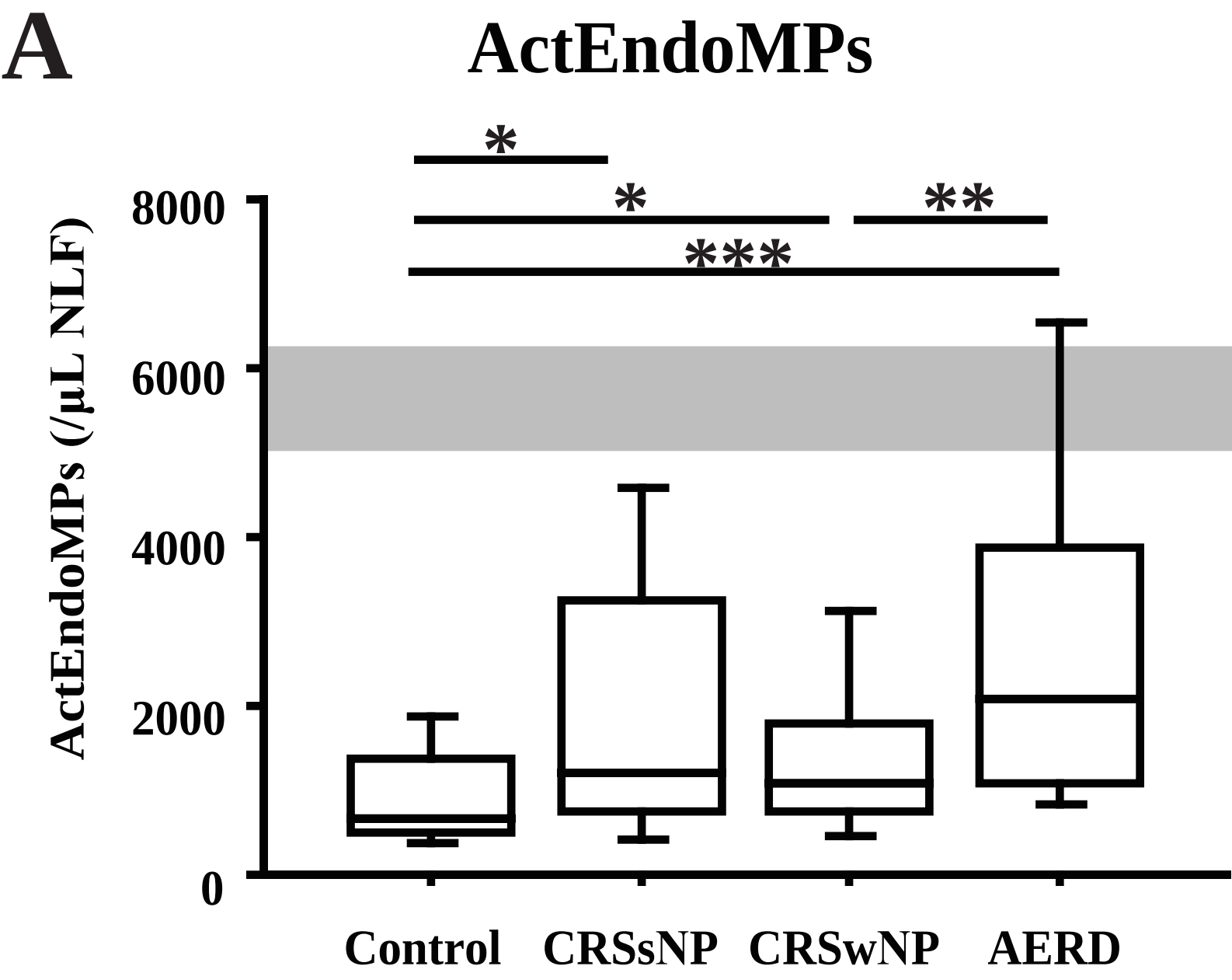
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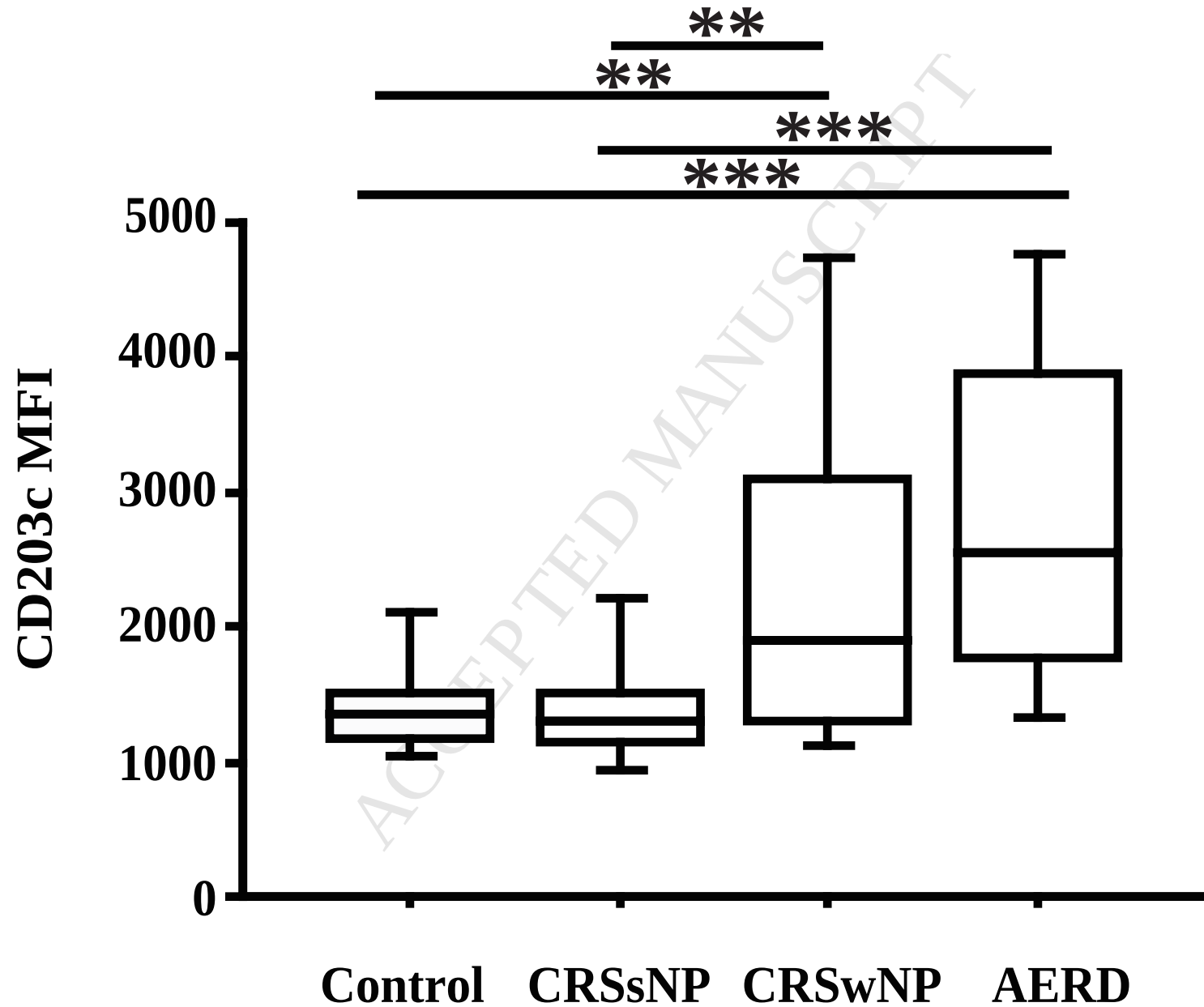
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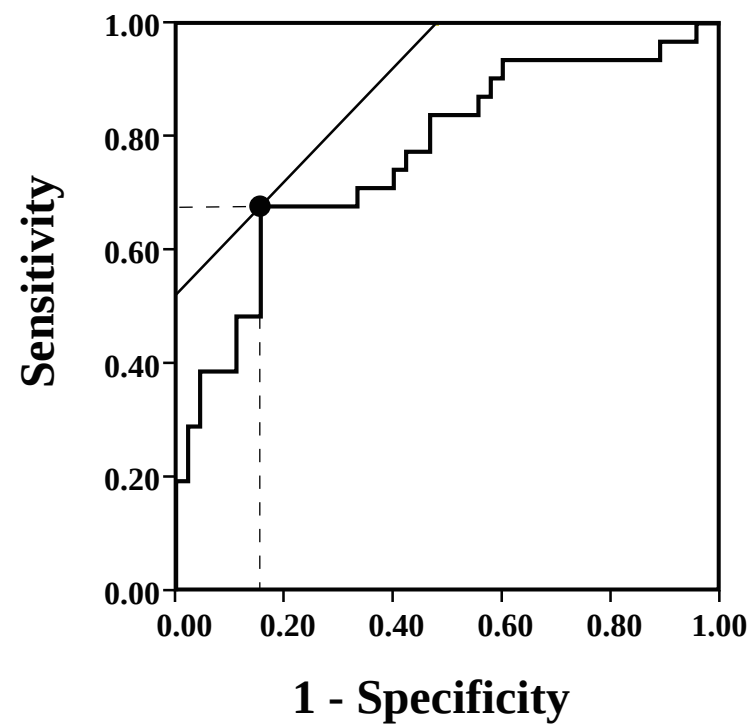
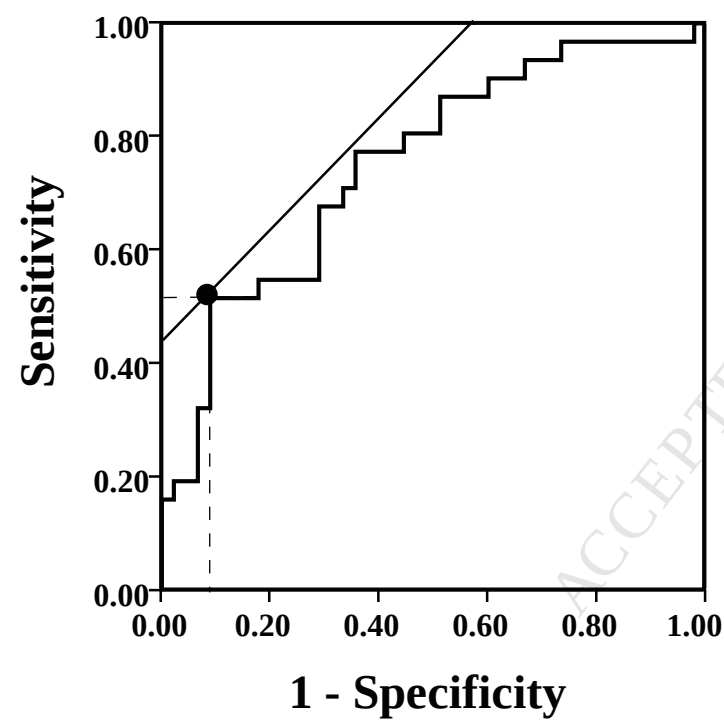
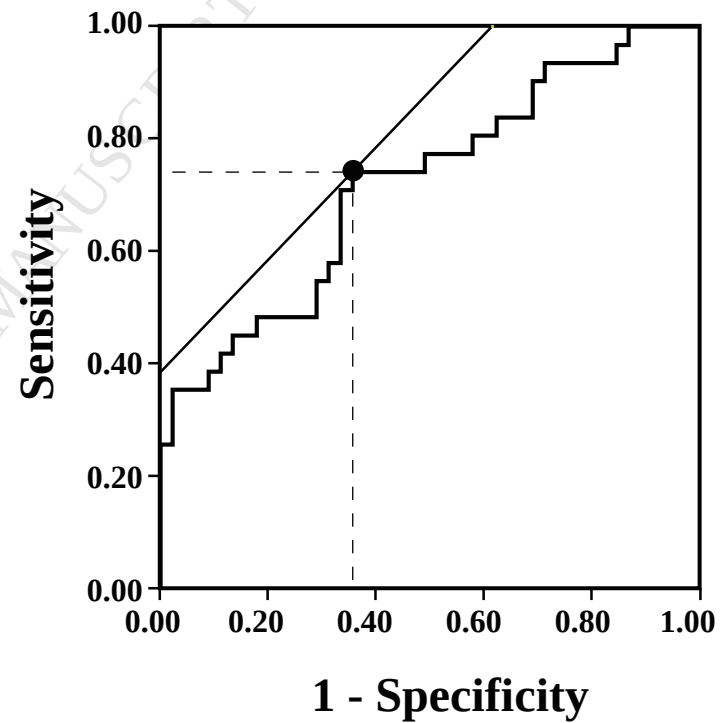
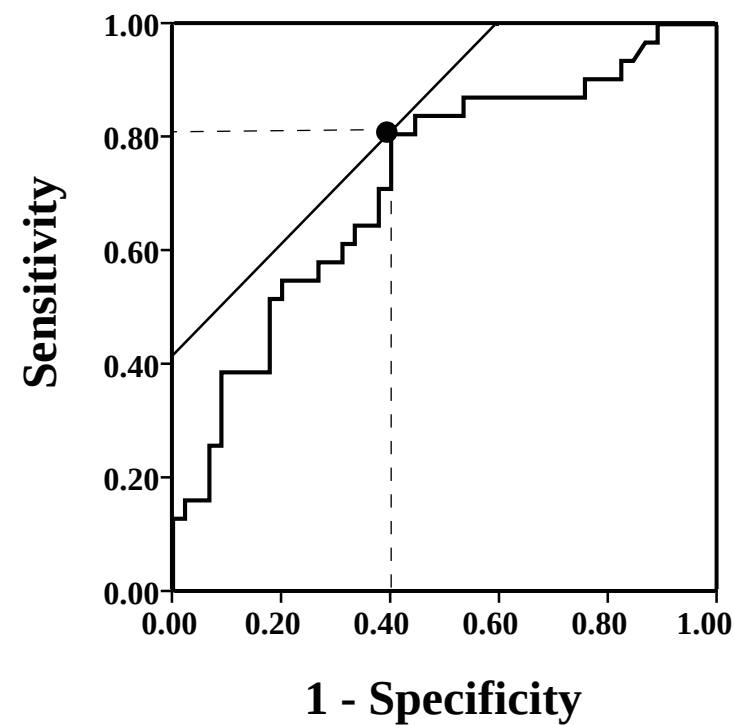
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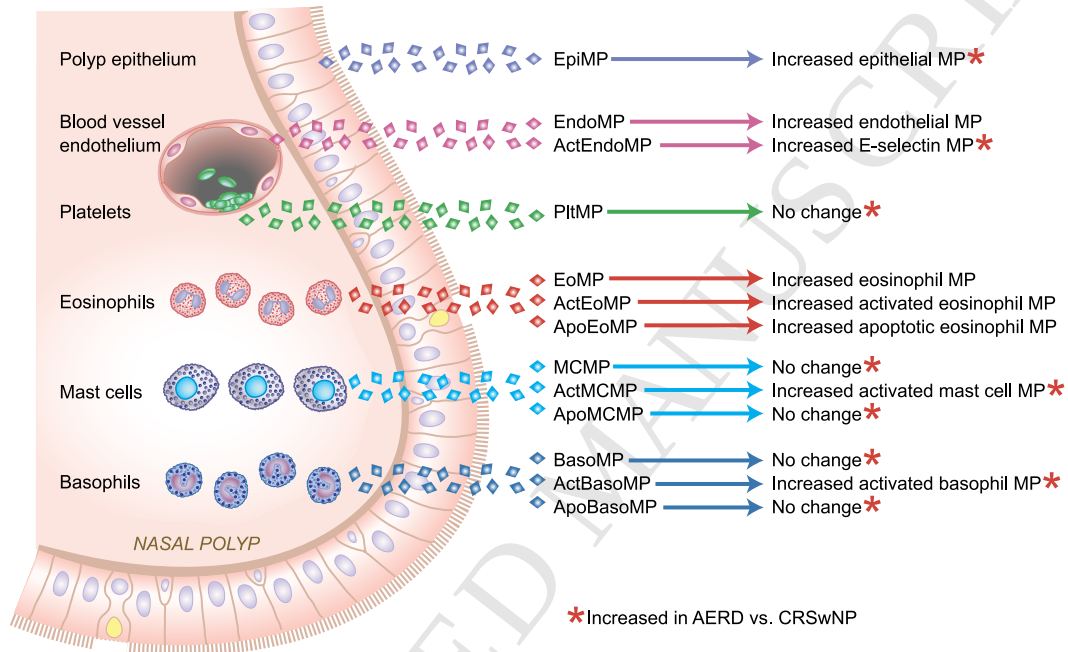
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CD203cMFI



A**PltMPs****B****EpCAM+EpiMPs****C****E-cad+EpiMPs****D****AV+MCMPs**



Online Repository materials

Microparticles in nasal lavage fluids in chronic rhinosinusitis; potential biomarkers for diagnosis of Aspirin Exacerbated Respiratory Disease

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Methods

Patient Population

Subjects with CRSsNP, CRSwNP, or AERD and control were recruited from the Division of Allergy and Immunology and Department of Otolaryngology clinics at the Northwestern University Feinberg School of Medicine. All subjects provided written informed consent, and the Institutional Review Board of the Northwestern University Feinberg School of Medicine approved this study. The presence of sinusitis or bilateral nasal polyposis was confirmed in subjects with CRS and AERD by endoscopy or computed tomography imaging. CRS subjects met the criteria as previously defined by the American Academy of Otolaryngology–Head and Neck Surgery Chronic Rhinosinusitis Task Force^{1,2}. AERD was diagnosed based on physician-diagnosed asthma, CRSwNP, and history of respiratory reaction (at least once) following ingestion of a non-steroidal anti-inflammatory drug. AERD subjects with history of aspirin desensitization treatment did not participate in this study. Subjects with established immunodeficiency, a systemic coagulation disorder, cystic fibrosis, an antrochoanal polyp, or unilateral nasal polyp were excluded from the study. The use of steroids (oral and/or intranasal), history of asthma and atopy, and smoking status were also documented. Control subjects underwent endoscopic sinus surgery for other indications, such as skull base surgery without either sinus disease or pathological lesions in the nasal cavity. All control subjects had no history of sinonasal inflammation, asthma or use of steroid. Five out of 24 control subjects had history of atopy without medication at enrollment. Current smokers were excluded and all subjects had ceased smoking for more than 6 months before enrollment in this study.

Nasal lavage fluid sampling and processing

Nasal lavage fluids (NLFs) were collected at the time of surgery according to the protocol previously described³. After secretions were removed from the nasopharynx, phosphate buffered saline (PBS: 8 mL) was sprayed with a syringe towards the middle meatus. And then, the fluid was collected with a suction collector connected to a 15-ml conical tube. The collected NLF was centrifuged for 10 minutes at 3000 rpm to remove cells and debris, and then, centrifuged in an Amicon Ultra-4 10K Centrifugal Filter Unit (EMD Millipore, Billerica, MA) at 3,000 rpm for 10 minutes at 4°C. The core sizes of Amicon Ultra-4 10K Centrifugal Filter Unit range from 1 to 50nm, and this filter is available for extracellular vesicle purification⁴. There was no clotted NLF sample in this study. The resulting retentate was concentrated 2-fold, collected, divided into small aliquots, and stored at -80°C until use.

Measurement of MPs in nasal lavage fluid by flow cytometry

MPs in nasal lavage were measured according to the modified protocol used in plasma as previously described⁵. Antibodies used for flow cytometry are summarized in Table E1. Ten microliters of NLFs was incubated with each specific antibody at room temperature. After 30 min of incubation, samples were diluted in 300 µl of a 0.9% saline salt solution. Equal volumes of sample and flowcount beads (Beckman-Coulter) were then added, and analyzed using a BD FACS LSRII flow cytometer using BD FACS DIVA software version 8.0 (BD Biosciences, Erembodegem Belgium). The MP gate was set using Megamix plus SSC beads (BioCytex, Marseille, France) for cytometer settings used in MP analysis to standardize MP flow cytometry assay⁶⁻⁸(Fig. E1). Filtered 0.9% saline salt solution, unstained controls or fluorescence minus one (FMO) controls were used to distinguish true positive events from noise and increase the specificity of MP detection⁹. The flow rate was set to the lowest rate to decrease noise. However,

due to the limitation of flow cytometry technology, noise appeared in the MP gate in the FSC vs. SSC scatter-gram and in the low FITC and PE intensity area (nonspecific fluorescence background) even when the filtered water sample was analyzed at the lowest flow rate^{7, 10}(Fig.E1.B). We thus detected MPs with fluorescent labelling. The gating strategy of previously unstudied MP types were verified using positive control MPs isolated from culture supernatant of each of the cell types or plasma isolated from fMLP stimulated blood (Fig.E8). The absolute MP numbers (/μl NLF) were calculated with the following formula; (MP positive events / Flowcount bead events) X 30 [Ten microliter samples were diluted with 300 μl of a 0.9% saline salt solution] X 1000 [Concentration of flowcount beads /μL]. The preliminary experiment shows that our MP flow cytometry assay has good repeatability (Fig.E7).

Characterization of MPs

● Endothelial MPs and platelet MPs

We measured E-selectin endothelial MPs (E-selectin(+)MPs: ActEndoMPs) and PECAM endothelial MPs (CD31(+)CD41(+)MPs: EndoMPs) according to previous articles^{5, 8, 11-13}. The PECAM endothelial MPs (EndoMPs) is one of the main endothelial MP subtypes studied, and plasma levels of this MP have been reported to be associated with the presence of cardiometabolic risk factors in a large community study and are regarded as markers for endothelial injury^{5, 8, 12}. E-selectin endothelial MPs (ActEndoMPs) have been measured as markers for endothelial activation in various diseases^{5, 14-17}. We measured platelet MPs (CD31(+)CD41(+)MPs: PltMPs) according to previous articles^{11, 12}. Platelet MPs are released from activated platelets with phosphatidylserine externalization, and are regarded as markers for platelet activation¹⁸⁻²⁰.

● Epithelial MPs

We defined epithelial MPs (EpiMPs) as EpCAM EpiMPs (EpCAM+EpiMPs) and E-cadherin EpiMPs (E-cad+EpiMPs), using specific markers for epithelial cells. EpCAM is located on the basolateral side of epithelial cells, whereas E-cadherin is localized in adherens junctions²¹. Loss of intercellular junctions and cellular detachments from basement membranes are observed during epithelial injury^{22, 23}. We previously reported that levels of circulating EpCAM+EpiMPs and E-cad+EpiMPs were significantly increased in COPD subjects when compared with control in response to epithelial injury probably due to disruptions of endothelial barriers²⁴. We measured both EpCAM+EpiMPs and E-cad+EpiMPs to evaluate degrees and conditions of epithelial injury in CRS.

● Eosinophil MPs

We defined eosinophil MPs (EoMPs) as EMR1(+)MPs using the specific marker for human eosinophils^{25, 26}. We also defined activated EoMPs as CD69(+)EoMPs (ActEoMPs) using the CD69 eosinophil activation marker²⁷⁻³¹. In addition, we also defined apoptotic EoMPs as Annexin V(+)EMR1(+)MPs (ApoEoMPs).

● Mast cell MPs

We defined mast cell MPs as FcεRI(+)c-kit(+)MPs (MCMPs) because mast cells express both FcεRI and c-kit³². We also defined activated MCMPs via IgE dependent pathways as CD137(+)MCMPs (ActMCMPs) using CD137, a mast cell activation marker expressed after IgE dependent activation^{33, 34}. Because phosphatidylserine externalization was reported to occur in the process of mast cell degranulation via an IgE dependent pathway as well as during mast cell apoptosis³⁵, we defined apoptotic or degranulated MCMPs as Annexin V(+)MCMPs (AV+MCMPs).

● Basophil MPs

We defined basophil MPs as CD203c(+)c-kit(-)MPs (BasoMPs) using CD203c which is selectively and constitutively expressed by mast cells and basophils, and c-kit to distinguish between mast cells and basophils. To ensure that CD203c(+)c-kit(-)MPs are derived from basophils, we co-stained with anti-human IL-3R α antibody (CD123-PE, clone: 6H6, BioLegend, San Diego, CA, USA) or anti-human CCR3 antibody (CD193-PE, clone: 61828, R&D Systems, Minneapolis, MN, USA), both of which are expressed on basophils^{36,37}. About 80% of CD203c(+)c-kit(-)MPs were CD123 positive, and about 80% of them were CCR3 positive (n=40, data not shown). We also co-stained with anti-human Siglec 6 antibody (CD327-PE, clone: 767329, R&D Systems, Minneapolis, MN, USA) which is expressed on mast cells, but not on basophils^{38,39}, and CD203c(+)c-kit(-)MPs were Siglec 6 negative (n=40, data not shown). These results indicated that CD203c(+)c-kit(-)MPs are mainly derived from basophils. In addition, we evaluated the degree of basophil activation using CD203c mean fluorescence intensity (MFI) on BasoMPs because CD203c is rapidly up-regulated when basophils are activated⁴⁰⁻⁴². We did not use CD63, another basophil activation marker as well as a platelet marker, because platelets were reported to adhere to leukocytes in AERD subjects⁴³. Furthermore, we defined apoptotic BasoMPs as Annexin V(+)BasoMPs (ApoBasoMPs).

● Erythrocyte MPs, Annexin V+MPs

We measured erythrocyte MPs defined as CD235a(+)MPs (ErythroMPs) as previously described⁴⁴. We measured ErythroMPs as a general control in our studies. In addition, we also measured Annexin V positive MPs (Annexin V(+)MPs: AV+MP) regardless of cell type of origin.

● Membrane dye positive MPs

Due to nonspecific fluorescence background (Fig.E1.B)^{7,10}, fluorescent labelling is essential to detect MPs by flow cytometry. Thus, to observe the trends of total MP levels, we labeled MPs fluorescently with both FMTM 4-64FX lipid-specific dyes and Carboxyfluoresceinsuccinimidyl ester (CFSE) protein-specific dyes (ThermoFisher Scientific, Waltham, MA) and measured membrane dye positive MPs (FM+CFSE+MPs) by flow cytometry as previously described⁴⁵. The lipophilic FM dye fluoresces intensely when it binds to the outer leaf of the plasma membrane. To exclude contaminants such as protein aggregates or micelles formed by unbound lipophilic FM dyes⁴⁵, we also co-stained with CFSE. CFSE easily crosses plasma membranes. Once inside the cells, esterases in extracellular vesicles cleave the acetate groups of CFSE and yield the fluorescent carboxyfluorescein molecule⁴⁵. We combined these two staining approaches to discriminate true positive MP events from false positive events, and to avoid overestimation of MP levels. However, the important limitation of this staining is that detection of MPs depends on the binding capacity of FM to membrane of MPs and the capacity of the relevant esterase in MPs to cleave acetate groups of CFSE. Therefore, we evaluated FM+CFSE+MP level only as an indicator of total MP level.

ELISA assay

Eosinophil cationic protein (ECP) was measured using the MESACUP ECP TEST (MBL International, Woburn, MA) according to the manufacturer's protocols. ECP levels are evaluated as nanograms of ECP per microliter of nasal lavage fluid.

In vitro experiments for establishing a gating strategy of EpiMPs, EoMPs, and MCMPs

Although we set the MP gate using calibration beads, the shapes of the beads may not be the same as for each MP type. Thus, to set and verify the gating strategy of the new MP types EpiMPs, EoMPs and MCMPs, we performed *in vitro* studies to isolate positive control MPs from culture supernatants of A549 cells, BEAS2B cells, human primary eosinophils and human primary cultured mast cells. All culture media were filtered using 0.22 μ m membrane filters before use to exclude MP in the medium. A549 cells (American Type Culture Collection, CCL-195) were cultured in DMEM/F12 (Thermo Fisher Scientific Inc. Waltham, MA, USA) with 10% FBS and penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA) at 37 °C in 5% CO₂. A549 cells were stimulated with 500 μ M H₂O₂ for 24 hours. BEAS2B cells (American Type Culture Collection, CRL 9609) were cultured in DMEM/F12 with 5% FBS and penicillin/streptomycin at 37 °C in 5% CO₂. BEAS2B cells were stimulated with 500 μ M H₂O₂ for 24 hours. Human eosinophils were isolated from blood of human healthy donors as previously described⁴⁶. The isolated eosinophils were cultured for 24 hours in RPMI 1640 medium (Thermo Fisher Scientific Inc. Waltham, MA, USA) containing 10% FCS, 30 ng/mL IL-5 (PeproTech, Rocky Hills, NJ) and 2.5 μ g/mL anti-Siglec-8 (clone 2C4) at 37 °C in 5 % CO₂ according to the protocol previously described⁴⁶. The primary cultured human mast cells were obtained as described previously⁴⁷. The cultured primary mast cells were stimulated with 100 ng/ml IL-33 for 6 hours. The culture supernatants of A549, primary human eosinophils, and primary cultured human mast cells were centrifuged at 300 g for 10 min to pellet and discard the cells and debris, and then again centrifuged at 21,000 g for 60 minutes to pellet the MPs, as previously described^{17, 48}. MPs were then resuspended in 300 μ l of a 0.9% saline salt solution, incubated with antibodies for 30 min, diluted in 300 μ l of a 0.9% saline salt solution and analyzed by flow cytometry. The MCMP scattergram showed a typical double staining pattern

of MP analysis as shown in the manual of the Megamix calibration beads⁴⁹ and previously reported^{50, 51}. Each new MP type (EpCAM+EpiMPs, E-cad+EpiMPs, EoMPs, and MCMPs) in NLF and the correspondent *in vitro* samples were detected using the same gating strategy without inconsistencies (Fig.E8)

Basophil activation test for establishing gating strategy of BasoMPs

To set and verify the gating strategy for BasoMPs, blood from healthy donors was collected in heparinized tubes, and incubated with 4 μ M Formylmethionyl-leucyl-phenylalanine (fMLP, Sigma, St Louis, MO, USA) at 37 °C for 30 min according to the modified basophil activation test protocol previously described⁵². Basophil MPs released from activated basophils in blood were then analyzed by flow cytometry. After fMLP stimulation, the treated blood was centrifuged for 10 min at 170g, and further centrifuged for 20 min at 1,500g to obtain platelet-free plasma. Negative control blood was prepared with incubation of PBS. Ten microliters of platelet free plasma was incubated with antibodies for 30 min, diluted in 300 μ l of a 0.9% saline salt solution, and analyzed by flow cytometry. Our gating strategy detected increased BasoMP numbers with higher CD203c intensity in plasma isolated from fMLP stimulated blood when compared with plasma isolated from unstimulated blood (Fig.E8). These results indicated that BasoMPs in nasal lavage samples and the correspondent plasma samples were detected using the same gating strategy without inconsistencies.

Statistics

All data are presented as the median $\pm$ interquartile range (25th-75th IQR), and were analyzed using non-parametric methods unless otherwise stated. Categorical data were analyzed using the

chi-square test. Sensitivity, specificity and respective areas under the curve (AUC) values were determined by development of receiver operating characteristic (ROC) curves. The optimum cut-off value of MP levels for distinguishing AERD from CRSwNP was calculated to maximize the sum of sensitivity and specificity by minimizing the distance of the cut-off value to the top-left corner of the ROC curve. Multiple logistic regression analysis was used to identify independent factors that distinguish AERD from CRSwNP. The repeatability of MP measurement (EpCAM+EpiMPs, PltMPs, ActEndoMPs, EoMPs, MCMPs, BasoMPs, ActBasoMPs: CD203cMFI) was analyzed according to the method reported by Bland and Altman for twice comparing MP measurements (Test1 and Test 2) of one sample⁵³. The differences in the MP numbers between Test 1 and Test 2 [Test 1 – Test 2] were plotted against the mean of the twice MP measurements [(Test 1+ Test 2) / 2]. The limits of agreement were the mean difference $\pm$ 1.96 Standard deviation (SD). We evaluated significant decreases or increases between the mean and difference of the twice MP measurements using the regression line. A p-value of less than 0.05 was considered significant and calculations were performed using JMP Pro 11.0.0 (SAS Institute Inc, Cary, CA). A multivariate analysis of MP levels was performed using principal component analysis (PCA) on variation-covariation matrices of MP levels from all subjects utilizing the PAST v2.17b statistical package.

Results

Principal Component Analysis (PCA) of MPs in nasal lavage fluids

PCA was performed to assist in the interpretation of MP type profiling of control, CRSsNP, CRSwNP, and AERD samples. The first principal component (PC1) explained 42.27% of the variance in the dataset, which discriminated between control and the three CRS phenotypes.

CRSwNP and AERD were best characterized by increased BasoMPs and AV+MPs, which was also the greatest discriminator in the second principal component (PC2). Higher BasoMP level distinguished CRSwNP and AERD from CRSsNP, and higher EpCAM+EpiMP, E-cad+EpiMP and ActEndoMP levels distinguished CRSsNP from CRSwNP. Other MP types did not discriminate control, CRSsNP, CRSwNP and AERD subjects clusters (Fig.E6).

Repeatability of MP measurements

Fig.E7 shows a Bland-Altman plot of independent (Test 1 and Test 2) measurements of EpCAM+EpiMPs, PltMPs, EndoMPs, EoMPs, MCMPs, BasoMPs, and CD203cMFI in an independent set of NLFs from 10 control and 40 CRS subjects not used for the studies reported. The Shapiro-Wilk test showed that differences [Test 1 – Test 2] of all 6 MP types and CD203cMFI were normally distributed ($p > 0.05$). Dots were randomly scattered above and below the mean of the difference [Test 1 – Test 2] line over a wide range of MP levels and the regression line did not show significant trends for either decrease or increase (EpCAM+EpiMPs: $r = -0.054$, $p = 0.711$, PltMPs: $r = 0.008$, $p = 0.959$, ActEndoMPs: $r = 0.017$, $p = 0.907$, EoMPs: $r = 0.041$, $p = 0.777$, MCMPs: $r = 0.060$, $p = 0.681$, BasoMPs: $r = 0.008$, $p = 0.959$ and ActBasoMPs: $r = -0.024$, $p = 0.872$). In addition, because the same method was used in Test 1 and Test 2, the means of differences [Test 1 – Test 2] of 6 MP types and CD203cMFI were nearly zero as expected (EpCAM+EpiMPs: 18.6 [95% confidence interval (CI), -24.9 to 61.1, $P(H_0: \text{Mean} = 0) = 0.803$], PltMPs: -2.42 [95% CI, -18.1 to 13.3, $p = 0.379$], ActEndoMPs: 20.5 [95% CI, -15.1 to 56.2, $p = 0.873$], EoMPs: 13.9 [95% CI, -34.7 to 62.5, $P = 0.716$], MCMPs: 12.9 [95% CI, -40.1 to 65.8, $P = 0.686$], BasoMPs: -16.9 [95% CI, -50.1 to 16.3, $P = 0.156$] and ActBasoMPs: -3.26 [95% CI, -33.6 to 27.1, $P = 0.415$]). These data suggest good repeatability of the MP

246 measurements.

247

248 **Table E1. Antigens and antibodies used for MP identification**

Differentiation cluster	Antigen	Fluorescence	Clone	Manufacture
CD31	PECAM-1	FITC	WM59	BD Biosciences
CD41	GPIIb	PE	P2	Beckman Coulter
CD62E	E-selectin	PE	68-5H11	BD Biosciences
CD69	VEA	APC	FN50	eBioscience
CD117	c-kit	APC	104D2	BioLegend
CD137	4-1BB	FITC	4B4-1	eBioscience
CD203c	HLDA7	FITC	NP4D6	BioLegend
CD235a	Glycophorin A	PE	GA-R2	BD Biosciences
CD324	E-cadherin	PE	67A4	BioLegend
CD326	EpCAM	PE	1B7	eBioscience
Not applicable	FcεRIα	PE	AER-37	BioLegend
Not applicable	EMR1	PE	A10	AbD Serotec
Not applicable	Annexin V	FITC		eBioscience
Not applicable	Annexin V	PE		BD Biosciences

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Table E2. Univariable and multiple logistic regression analyses for independent values that distinguish AERD subjects from CRSwNP subjects

Univariable analysis

Variables	Risk value (95% CI)	p value
Asthma	1.0X10 ⁹ (15.1-)	0.99
Oral steroid	3.57 (1.01- 14.6)	< 0.05
MP types		
ActEndoMPs $\geq$ cut-off value	4.27 (1.58- 12.21)	< 0.006
PltMPs $\geq$ cut-off value	11.4 (3.97- 36.7)	< 0.0001
EpCAM+EpiMPs $\geq$ cut-off value	10.9 (3.40- 43.13)	< 0.0003
E-cad+EpiMPs $\geq$ cut-off value	5.21 (1.96- 15.00)	< 0.002
MCMPs $\geq$ cut-off value	5.21 (1.96- 15.01)	< 0.002
ActMCMPs $\geq$ cut-off value	5.54 (2.05- 16.02)	< 0.002
AV+MCMPs $\geq$ cut-off value	6.25 (2.25- 19.66)	< 0.001
BasoMPs $\geq$ cut-off value	4.88 (1.45- 19.55)	< 0.02
ApoBasoMPs $\geq$ cut-off value	4.35 (1.67- 11.96)	< 0.004

Multivariable analysis

ActEndoMPs

Variables	Risk value (95% CI)	p value
Asthma	8.5X10 ⁷ (11.5-)	0.99
Oral steroid	3.82 (0.80- 28.4)	0.12
ActEndoMPs $\geq$ cut-off value	3.04 (0.94- 10.8)	0.07

PltMPs

Variables	Risk value (95% CI)	p value
Asthma	1.5X10 ⁸ (19.2 -)	0.99
Oral steroid	4.41 (0.72- 37.4)	0.13
PltMPs $\geq$ cut-off value	22.7 (5.10- 166)	< 0.0004

EpCAM+EpiMPs

Variables	Risk value (95% CI)	p value
Asthma	1.6X10 ⁸ (19.2-)	0.99
Oral steroid	6.45 (1.24- 50.1)	< 0.04
EpCAM+EpiMPs $\geq$ cut-off value	30.4 (5.03- 592)	< 0.004

E-cad+EpiMPs

Variables	Risk value (95% CI)	p value
Asthma	7.9X10 ⁷ (11.2-)	0.99
Oral steroid	5.30 (1.01- 43.3)	0.07

E-cad+EpiMPs $\geq$ cut-off value	5.31 (1.57- 20.6)	< 0.02
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MCMPs

Variables	Risk value (95% CI)	p value
Asthma	1.0×10^8 (14.3 -)	0.99
Oral steroid	3.09 (0.59- 24.3)	0.21
MCMPs $\geq$ cut-off value	5.90 (1.80- 21.4)	< 0.005

ActMCMPs

Variables	Risk value (95% CI)	p value
Asthma	1.0×10^8 (13.9-)	0.99
Oral steroid	2.76 (0.52- 21.3)	0.26
ActMCMPs $\geq$ cut-off value	5.74 (1.65- 23.9)	< 0.01

AV+MCMPs

Variables	Risk value (95% CI)	p value
Asthma	8.8×10^7 (12.4-)	0.99
Oral steroid	2.83 (0.55- 22.0)	0.25
AV+MCMPs $\geq$ cut-off value	5.59 (1.67- 20.8)	< 0.007

BasoMPs

Variables	Risk value (95% CI)	p value
Asthma	78.7×10^7 (11.3-)	0.99

Oral steroid	4.30 (0.91- 31.6)	0.09
BasoMPs $\geq$ cut-off value	2.69 (0.73- 11.6)	0.15

ApoBasoMPs

Variables	Risk value (95% CI)	p value
Asthma	1.1X10 ⁸ (15.1-)	0.99
Oral steroid	4.76 (0.92- 37.2)	0.09
ApoBaso MPs $\geq$ cut-off value	6.31 (1.85- 25.0)	< 0.006

Table E3. Correlations between eosinophil MPs and platelet MPs in CRS and AERD**subjects****CRSsNP**

	EoMPs	ActEoMPs	ApoEoMPs
PltMPs	$r_s = 0.284, p=0.110$	$r_s=0.337, p=0.055$	$r_s=0.166, p=0.360$

CSRwNP

	EoMPs	ActEoMPs	ApoEoMPs
PltMPs	$r_s=0.220, p=0.147$	$r_s=0.388, p< 0.009$	$r_s=0.037, p=0.808$

AERD

	EoMPs	ActEoMPs	ApoEoMPs
PltMPs	$r_s=0.467, p< 0.009$	$r_s=0.731, p<0.0001$	$r_s=0.474, p< 0.008$

Table E4. Circulating PltMPs, EndoMPs in control subjects (n=9)

Circulating MP types	Levels in plasma
BasoMPs (/uL PFP)	3269 $\pm$ 885
PltMPs (/uL PFP)	7199 $\pm$ 4843
EndoMPs (/uL PFP)	8329 $\pm$ 2301

PFP; platelet free plasma

Data are presented as means $\pm$ standard deviation.

291 **Table E5. Comparisons of demographics between CRSwNP without asthma and CRSwNP**
 292 **with aspirin intolerant asthma (ATA)**

	CRSwNP without asthma (n=23)	CRSwNP with aspirin intolerant asthma (n=22)
Gender, male/female	19/4	11/11*
Ethnicity, White/ non-White	20/3	14/8
Age (years), mean $\pm$ standard deviation	49.7 $\pm$ 10.8	43.9 $\pm$ 11.2
Smoking status, never/ex- smokers ¹⁾	15/8	19/3
Comorbid Atopy (%)	12 (52%)	16 (73%)
Steroid use		
Oral (%)	2 (9%)	2 (9%)
Dose (prednisone mg/day), median [Range]	5.0 [5-5]	10.0 [5-20]
Intranasal (%)	4 (17%)	5 (23%)

Fluticasone propionate	2	4
Mometasone furoate	2	1
Dexamethasone	0	0

293 *; p< 0.05, vs. CRSwNP without asthma

294

Table E6. Comparisons of MP levels in nasal lavage between CRSwNP without asthma and CRSwNP with aspirin tolerant asthma (ATA)

MP types	CRSwNP without asthma	CRSwNP with ATA	P values
ActEndoMPs	1103 [337-7181]	1172 [233-4892]	0.510
EndoMPs	2740 [1165-7452]	3715 [1146-7924]	0.364
EpCAM+EpiMPs	1702 [343-6680]	1346 [484-5911]	0.586
E-cad+EpiMPs	937 [146-2795]	876 [310-3252]	0.540
PltMPs	70 [0-487]	55 [0-874]	0.242
EoMPs	3071 [1609-7879]	2913 [1105-8100]	0.634
ActEoMPs	257 [20-1787]	345 [41-1662]	0.555
ApoEoMPs	1284 [260-2490]	1257 [420-3527]	0.892
MCMPs	414 [73-4280]	663 [78-5275]	0.716
ActMCMPs	115 [0-1619]	287 [47-703]	0.286
AV+MCMPs	152 [0-1446]	132 [10-1321]	0.602
BasoMPs	2519 [1019-6107]	3001 [873-9578]	0.666
CD203cMFI	1.95 [1.10-6.91]	15.9 [0.94-8.74]	0.461
	X10 ³	X10 ³	
ApoBasoMPs	235 [11-1708]	128 [0-1180]	0.256
AV+MPs	5981 [2437-15629]	6315 [3611-15218]	0.700
ErythroMPs	1732 [336-4185]	1797 [453-4198]	0.617
FM+CFSE+MPs	25736 [12000-48245]	30966 [15112-68764]	0.329

All data of MP levels are presented as median [range].

*, p<0.05, **, p<0.01, ***, p<0.001

Figure Legends

Fig.E1: Gating strategy of MP FACS analysis

(A) MP gate setting using Megamix plus beads.

Because the BD LSRII flow cytometer is a SSC optimized cytometer, we used Megamix plus SSC calibration beads to establish the MP gate according to the manual provided with the Megamix beads. Pink dots: 0.16 μ m beads, Blue dots: 0.2 μ m beads, Green dots: 0.24 μ m beads, Red dots: 0.5 μ m beads. Separation Index⁷ of the Megamix beads is 5-6 in our LSRII flow cytometer, and we configured the MP gate on the FSC-SSC scattergram to include both small MP and large MP as described in the Megamix bead manual. This gate allows standardization of a SSC-based MP analysis region comparable in platelet MP counts to that defined in FSC as 0.3 - 1 μ m-bead-equivalents.

(B) Nonspecific fluorescence background (tested with filtered 0.9% NaCl).

Due to the limitations of flow cytometry technology with PM tubes set at high gain, noise appeared in the MP gate in the FSC vs SSC scatter-gram and in the low FITC and PE intensity area (nonspecific fluorescence background) even when a filtered water sample was analyzed at the lowest flow rate^{7, 10}. Thus, we detected MP based upon fluorescent labelling and quantified MP numbers as absolute MP numbers per one microliter nasal lavage fluid (NLF) using flowcount beads according to the manual of the Megamix beads.

(C) Unstained control and (D) fluorescence minus one (FMO) control.

Unstained and FMO controls were used to distinguish true positive events from the noise referred to above, and to increase the specificity of MP detection.

(E) PltMPs (Nasal lavage of control subjects stained with CD31 FITC and CD41 PE).

(F) PltMPs (Nasal lavage of AERD subject stained with CD31 FITC and CD41 PE).

Fig.E2. Comparison of AV+MPs, ErythroMPs, and membrane dye positive MPs.

*, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$, N.S.; Not significant

Fig.E3. Correlations between eosinophil MPs and platelet MPs in AERD subjects.

Although there was a weak but significant correlation between PltMPs and EoMPs ($r_s = 0.467$, $p < 0.009$) and between PltMPs and ApoEoMPs ($r_s = 0.474$, $p < 0.008$) in AERD, the PltMP levels showed a strong and significant correlation with ActEoMPs in AERD ($r_s = 0.731$, $p < 0.0001$).

Fig.E4. Correlations between ActEoMPs and ActMCMPs in CRS and AERD subjects.

ActEoMPs were significantly correlated with ActMCMPs in control ($r_s = 0.797$, $p < 0.0002$), CRSsNP ($r_s = 0.662$, $p < 0.0001$), CRSwNP ($r_s = 0.627$, $p < 0.0001$) and AERD ($r_s = 0.734$, $p < 0.0001$).

Fig.E5. Comparison of ECP levels among CRSsNP (n=33), CRSwNP (n=34), AERD (n=20) and control (n=21) subjects.

ECP levels were significantly increased in CRSwNP and AERD (CRSwNP: $p < 0.001$, AERD: $p < 0.001$) and were not increased in CRSsNP ($p = 0.380$) when compared with control. ECP levels were also significantly higher in CRSwNP and AERD than CRSsNP (CRSwNP: $p < 0.001$, AERD: $p < 0.001$). ***, $p < 0.001$

Fig.E6. Principal component analysis (PCA) of MPs in NLF.

The PCA plot summarizes variation and covariation patterns in nasal lavage levels of MPs across different groups of subjects with chronic sinus disease. Dots represent individual subjects, and distinct clusters of control, CRSsNP, CRSwNP and AERD subjects are outlined and shaded in different colors: green (healthy control), blue (CRSsNP), magenta (CRSwNP) and red (AERD).

Fig.E7. Bland-Altman plot representation between two tests of EpCAM+EpiMPs, PltMPs, ActEndoMPs, EoMPs, MCMPs, BasoMPs, and CD203cMFI (Preliminary study of 10 control and 40 CRS subjects)

One dot stands for one subject and the thin lines indicate the mean $\pm$ 1.96 Standard deviation (SD) interval. The regression line shows no significant decreases or increases in all 6 MP types and CD203cMFI. In addition, because the same method was used in Test 1 and Test 2, the means of differences [Test 1 – Test 2] of 6 MP types and CD203cMFI were nearly zero as expected.

Fig.E8. Gating strategy of new MP types, EpiMPs, EoMPs, MCMPs, and BasoMPs

(A) EpCAM+EpiMPs, (B) E-cad+EpiMPs, (C) EoMPs, (D) MCMPs and (E) BasoMPs are shown. Plasma was analyzed for 60 seconds. Blue dots indicate Target MPs and Red dots indicate other MPs or background noise (Nonspecific fluorescence background)^{7, 9, 10}. MCMP scattergram showing a typical double staining pattern of MP analysis as shown in the manual of Megamix calibration beads⁴⁹ and previously reported^{50, 51}. Each new MP type (EpCAM+EpiMPs, E-cad+EpiMPs, EoMPs, MCMPs, BasoMPs) is shown in nasal lavage samples and the correspondent *in vitro* samples were evaluated using the same gating strategy without inconsistencies.

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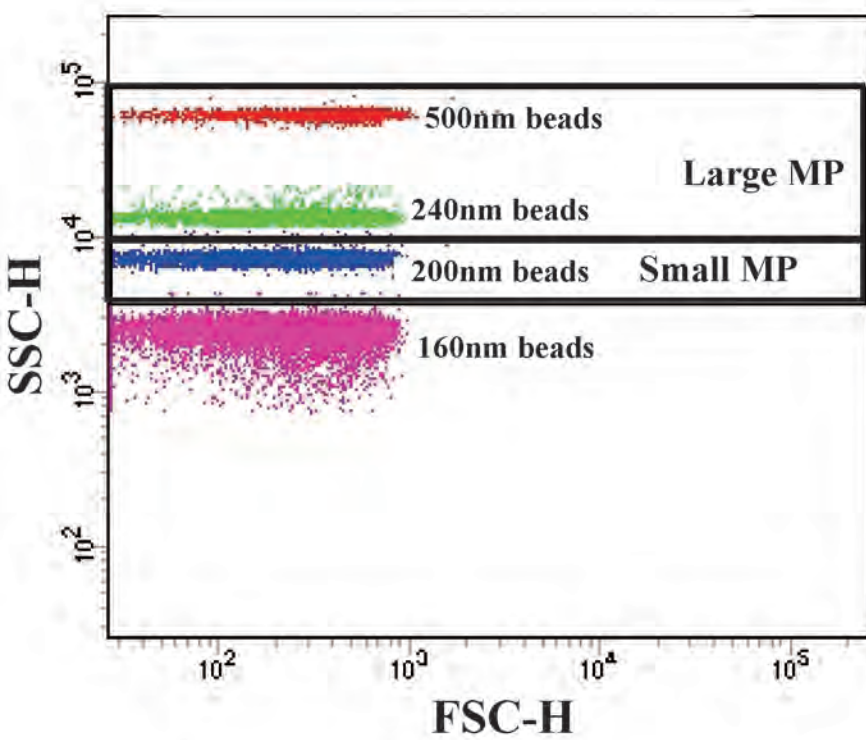
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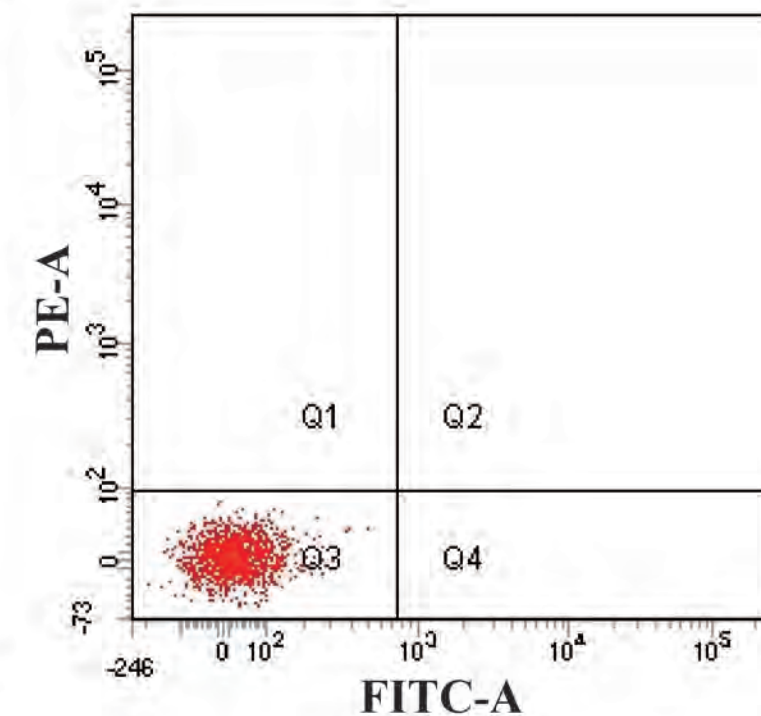
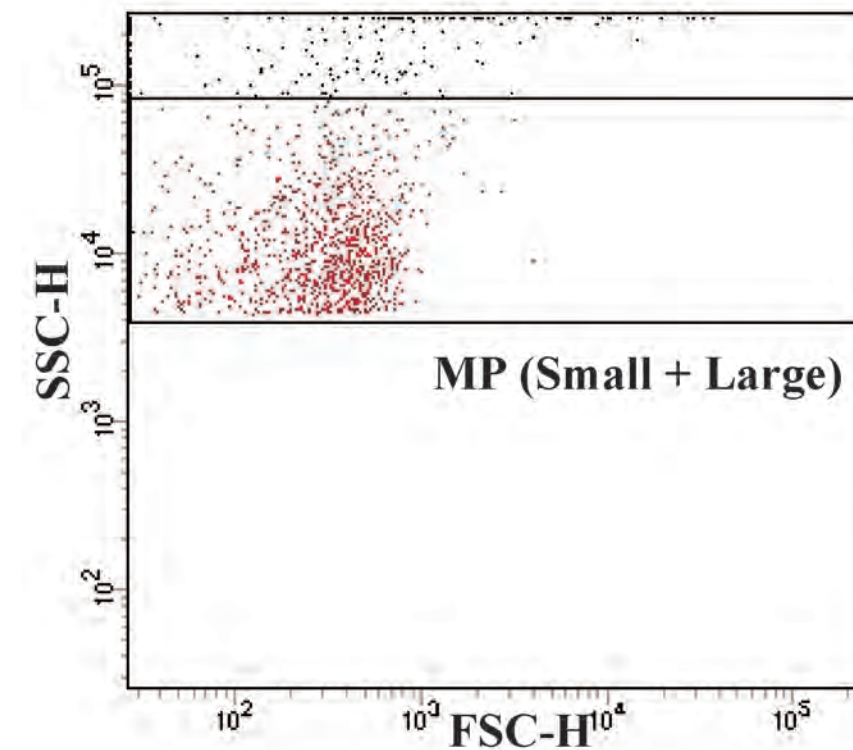
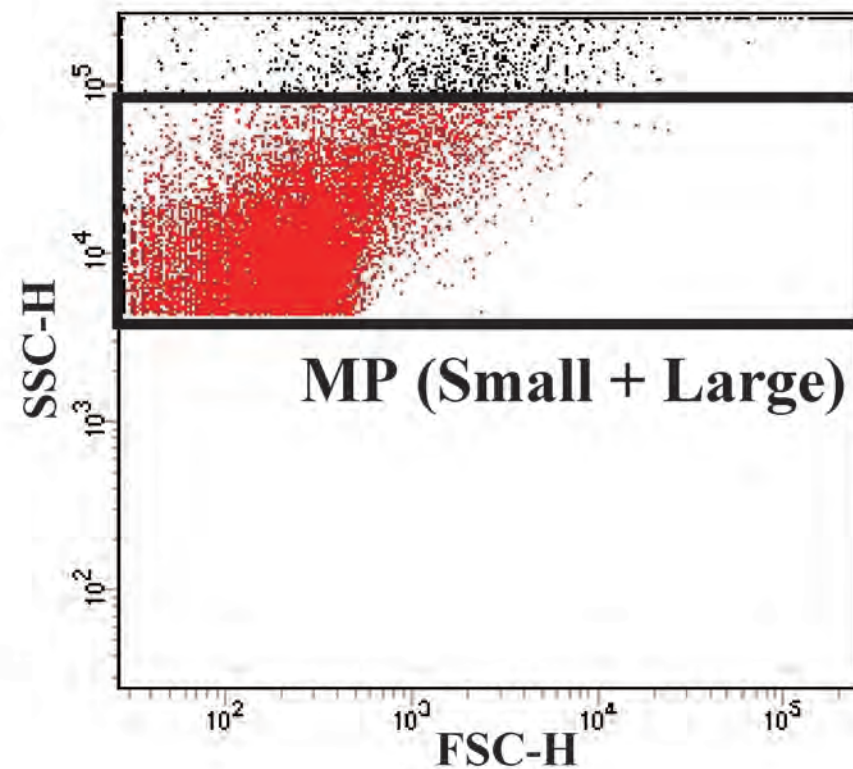
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(A) Megamix SSC

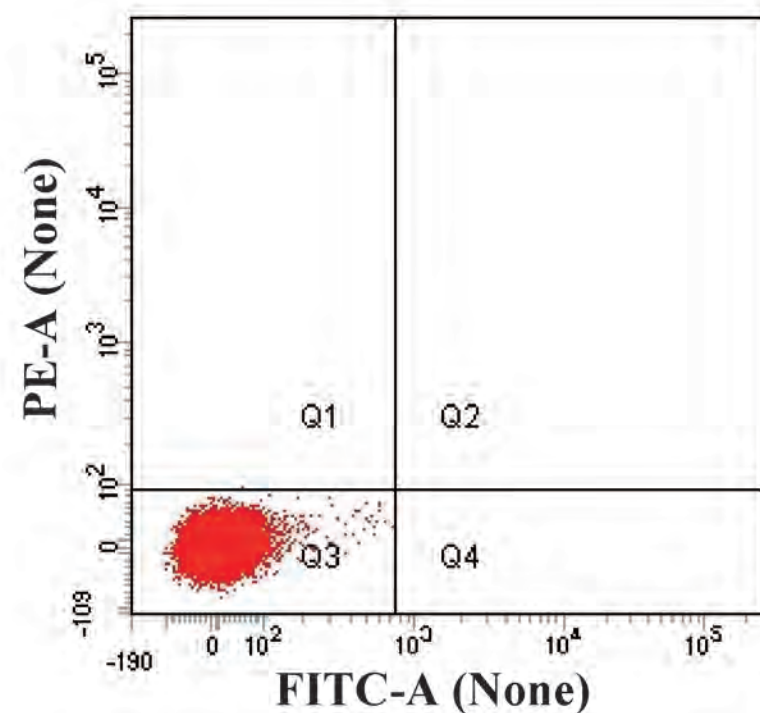


(B) Filtered 0.9%NaCl



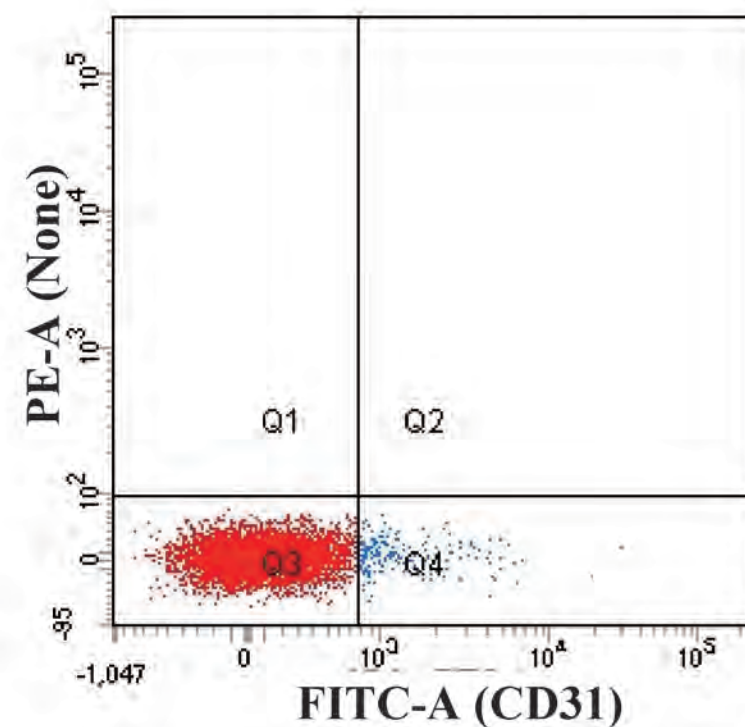
(C) Unstained control

(NLF without staining)



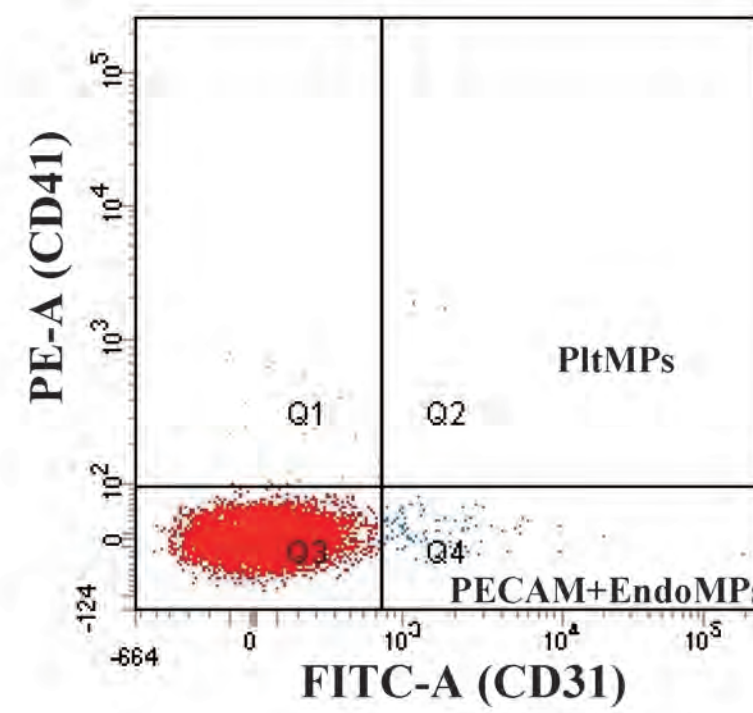
(D) FMO

(NLF stained with CD31 FITC)



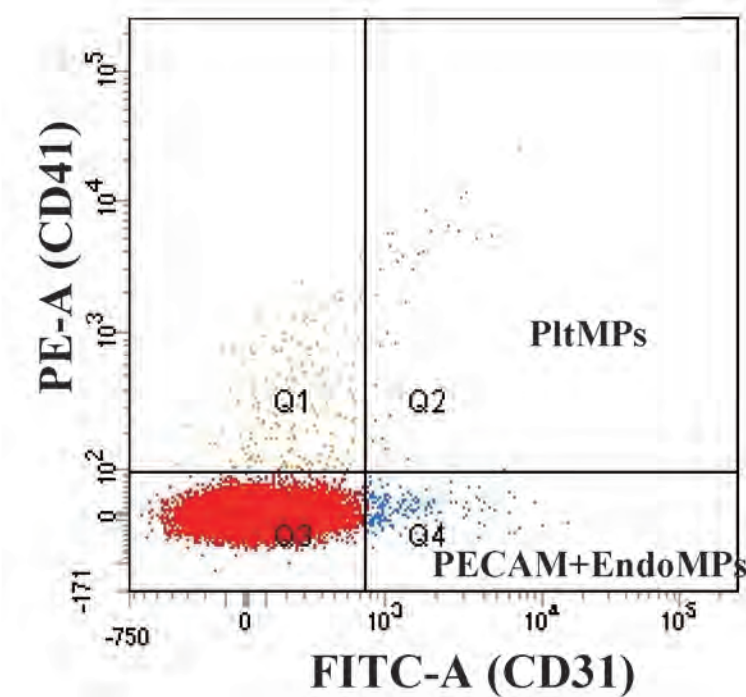
(E) PltMPs

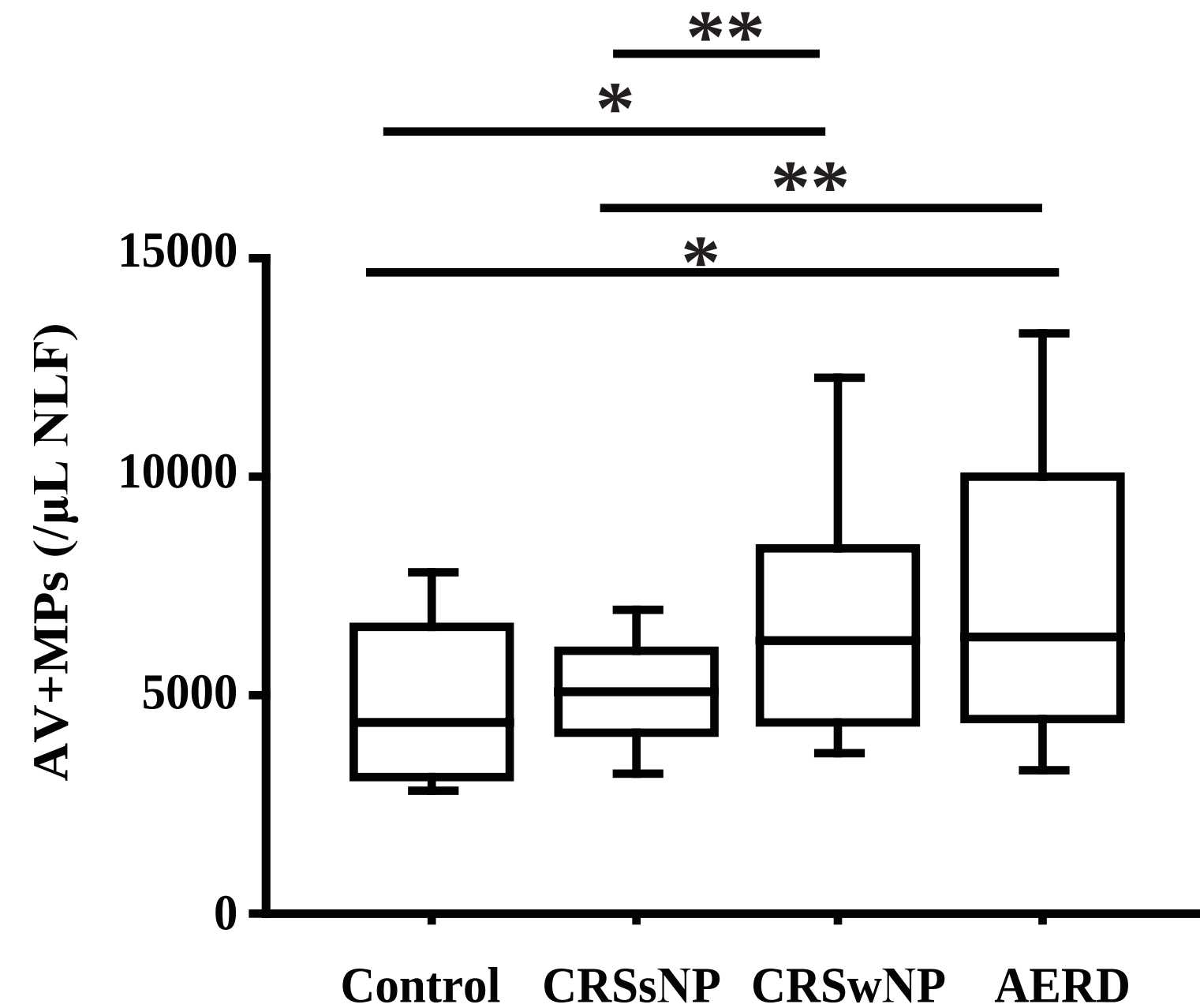
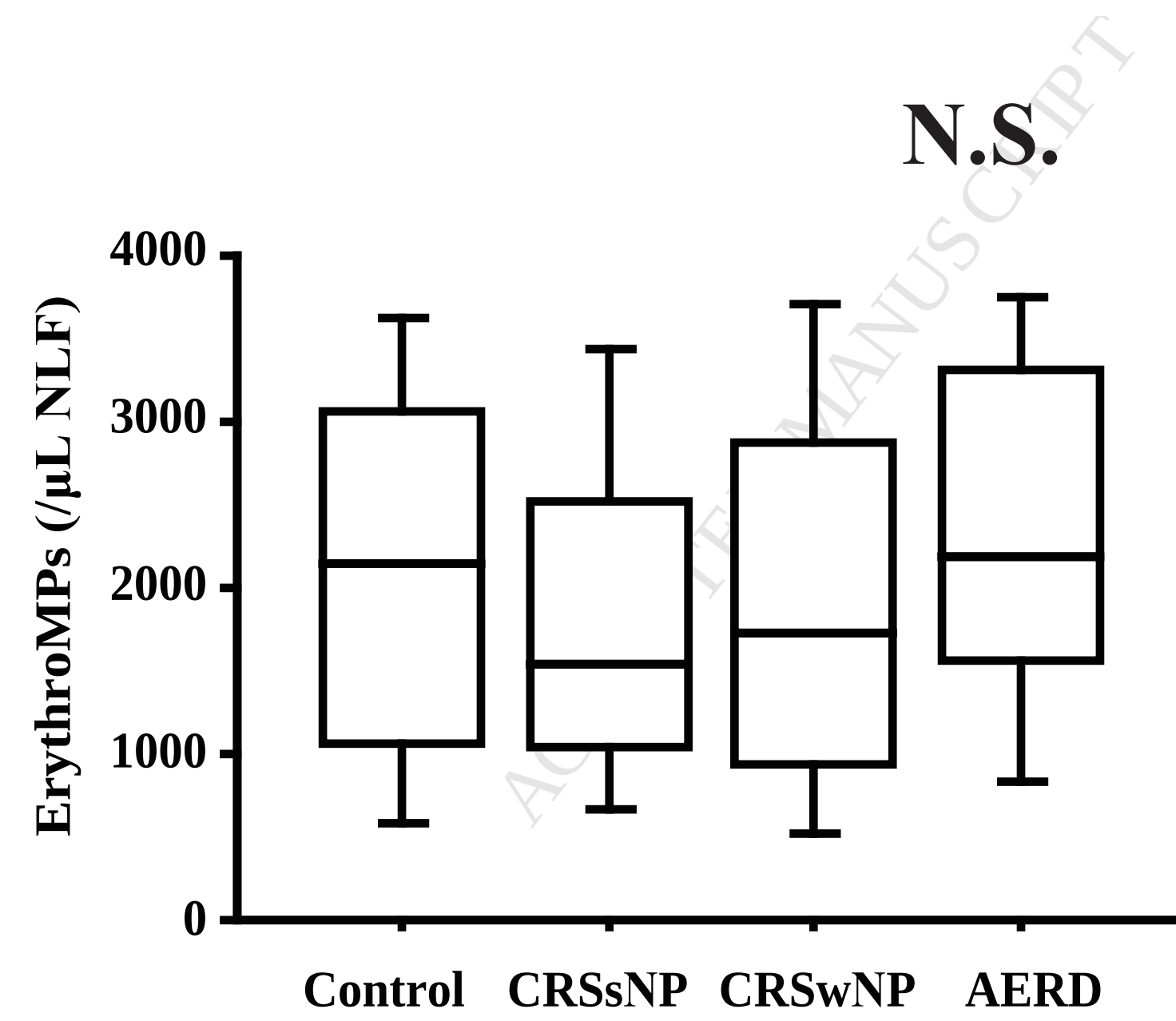
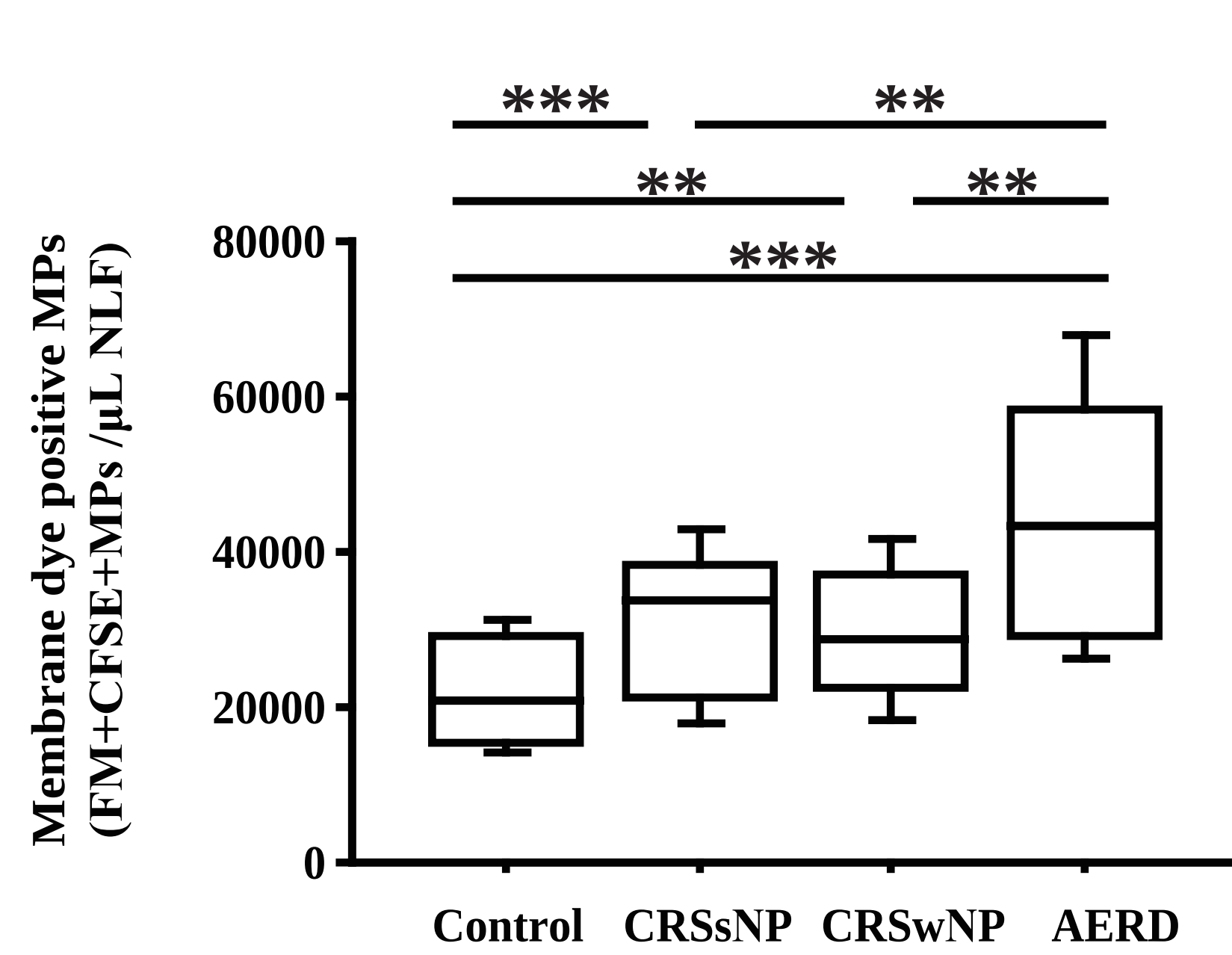
(Control subject)



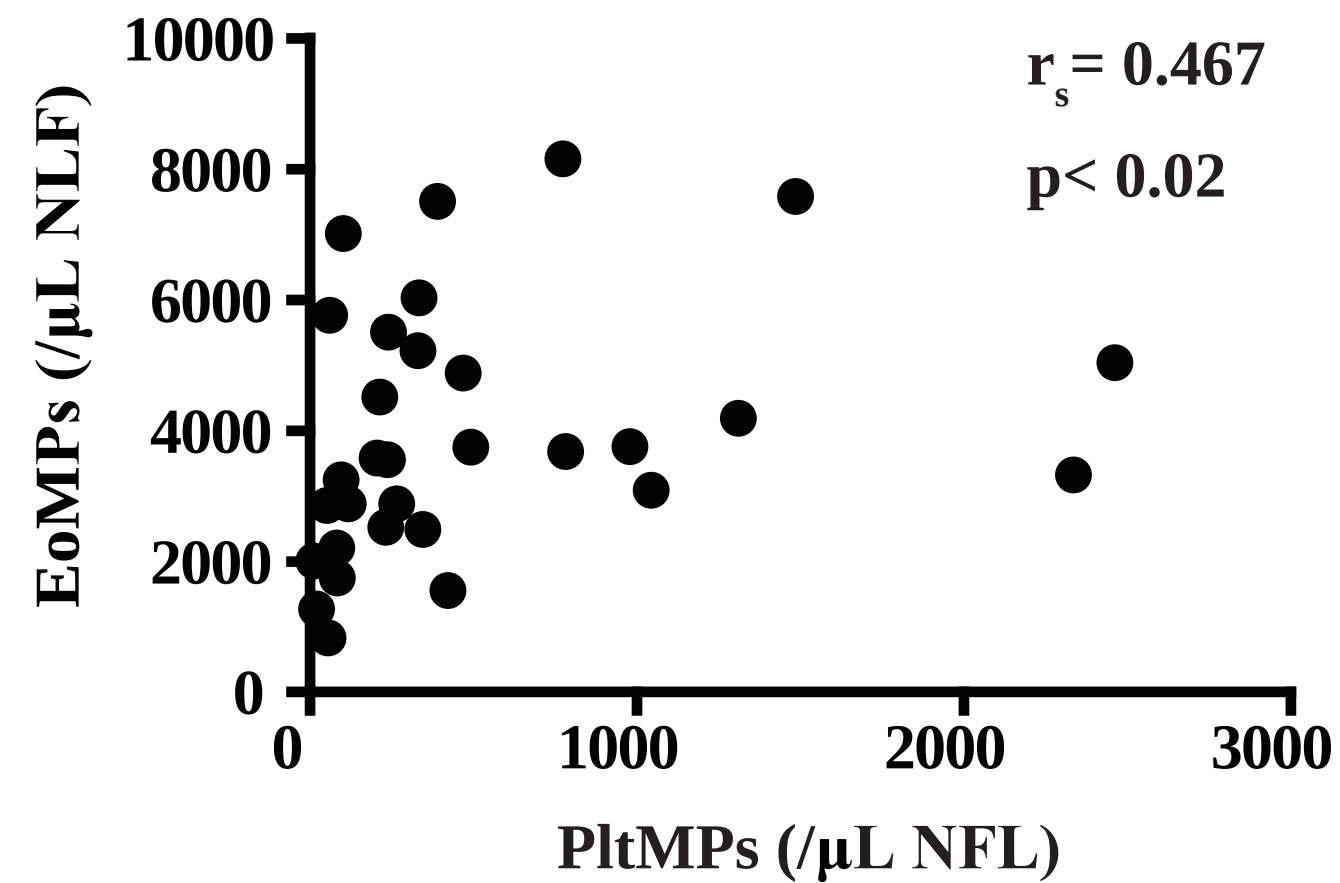
(F) PltMPs

(AERD subject)

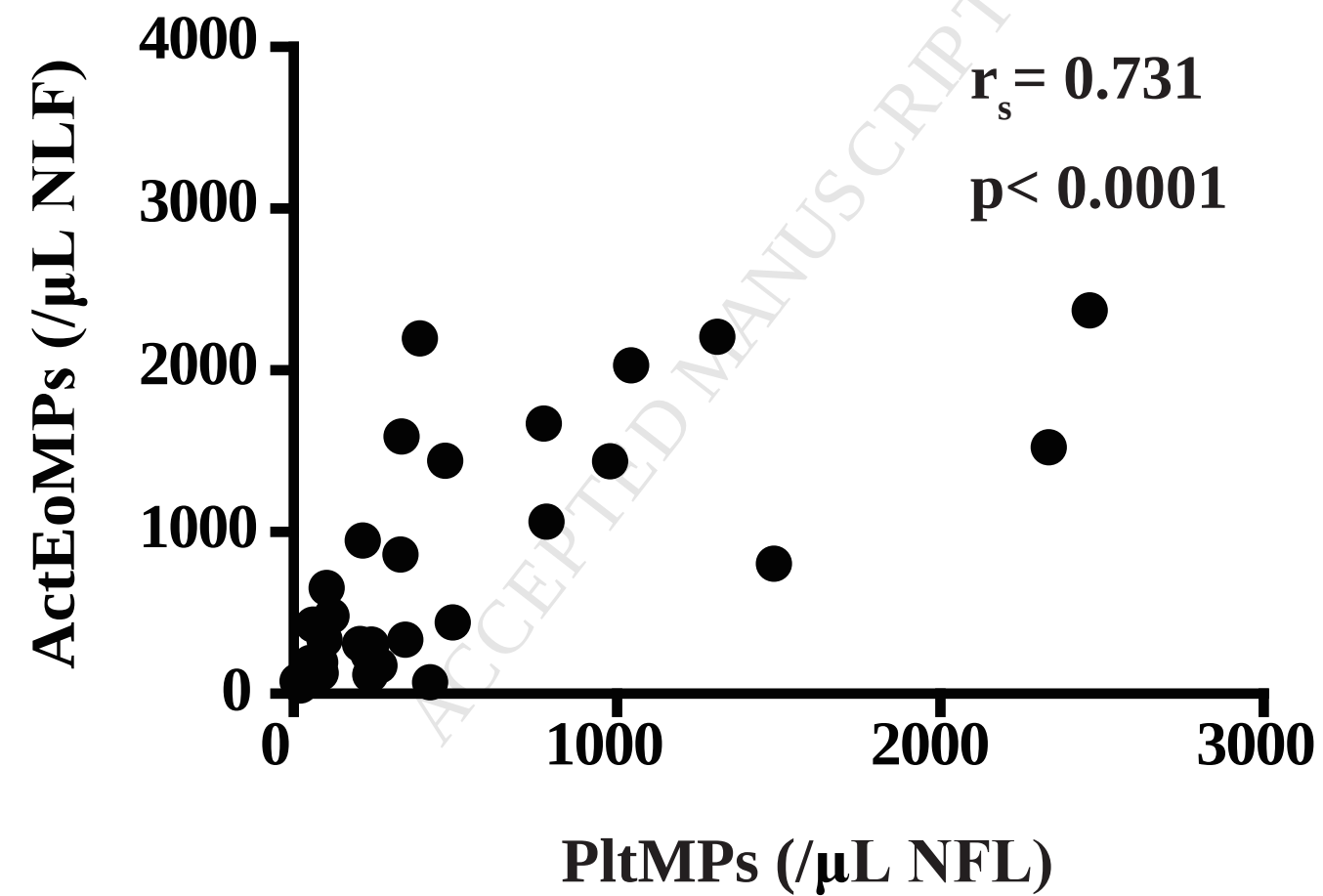


A**AV+MPs****B****ErythroMPs****C****Membrane dye positive MPs**

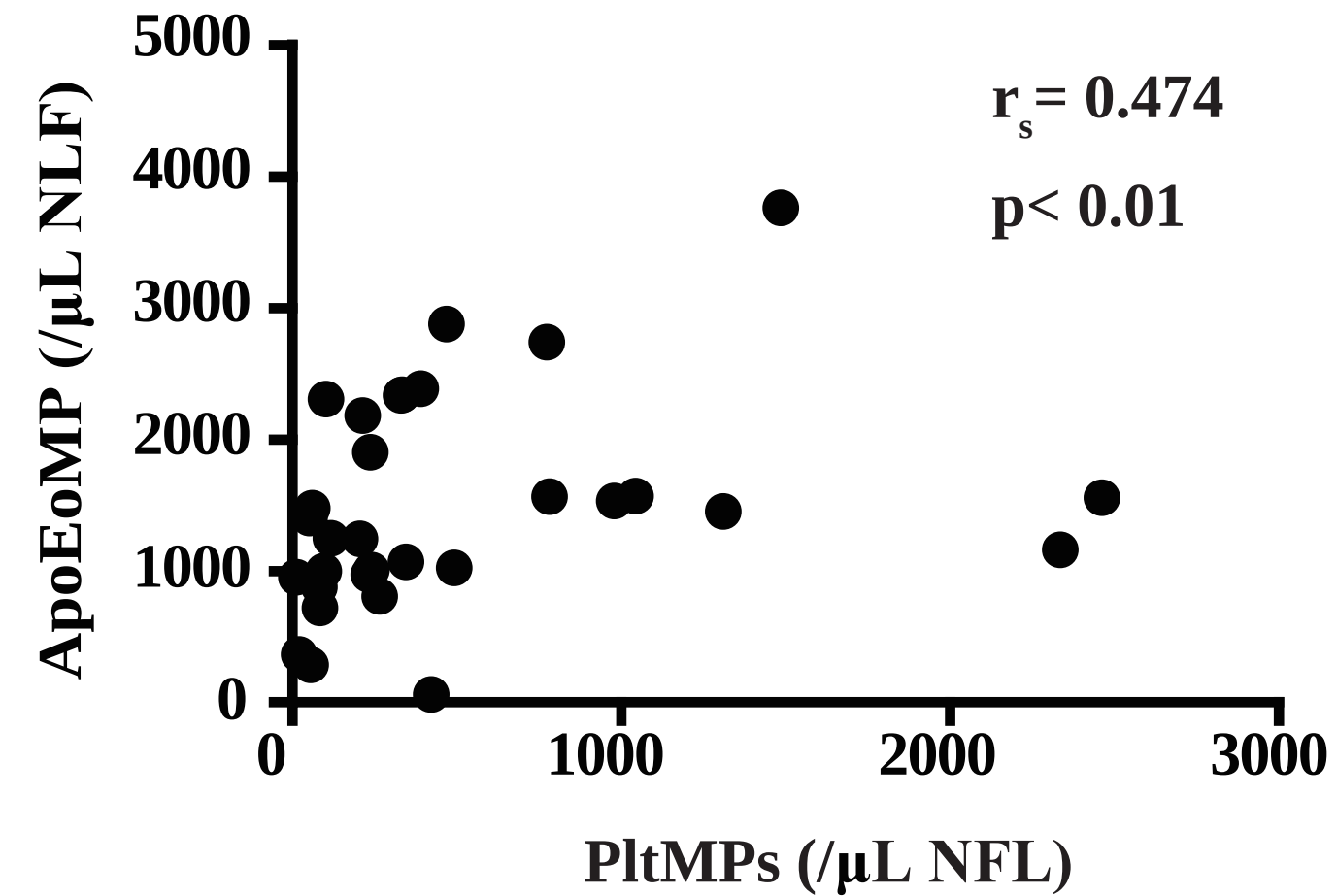
AERD (EoMPs vs. PltMPs)

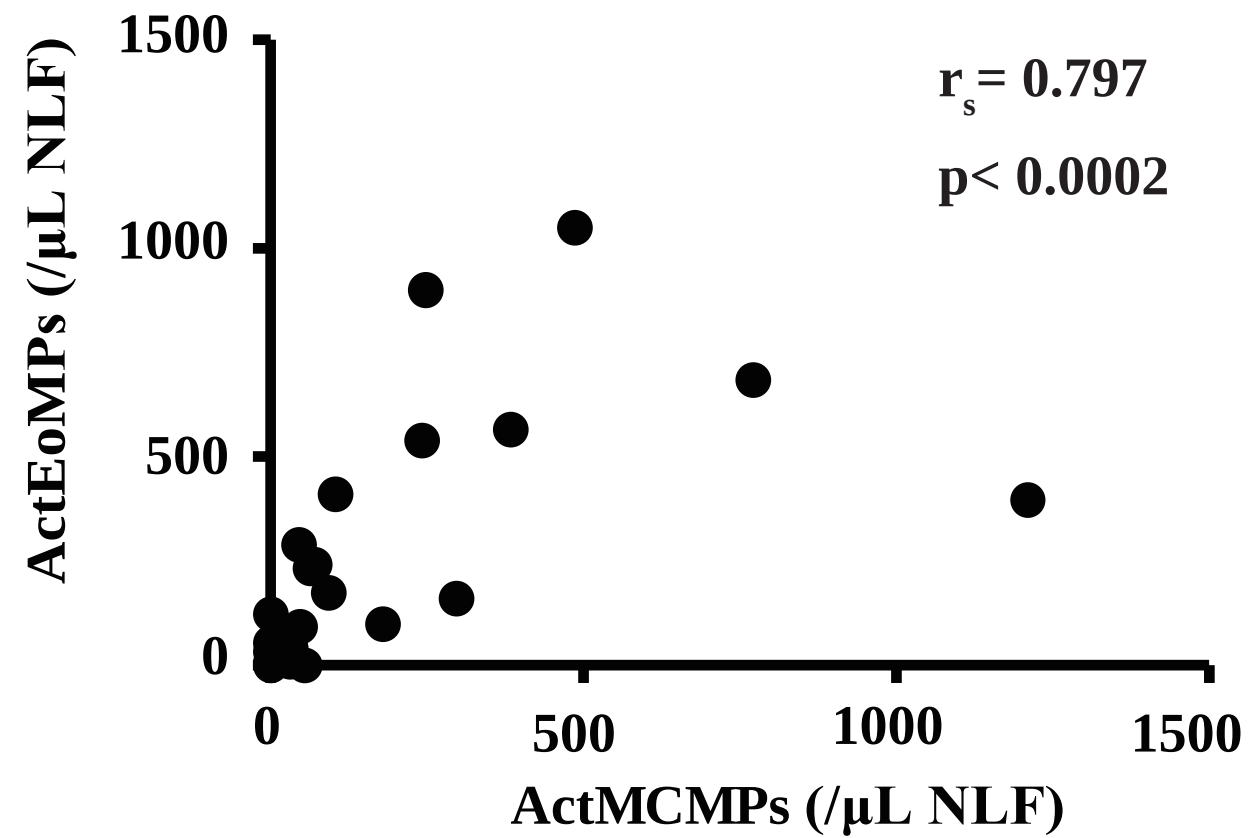
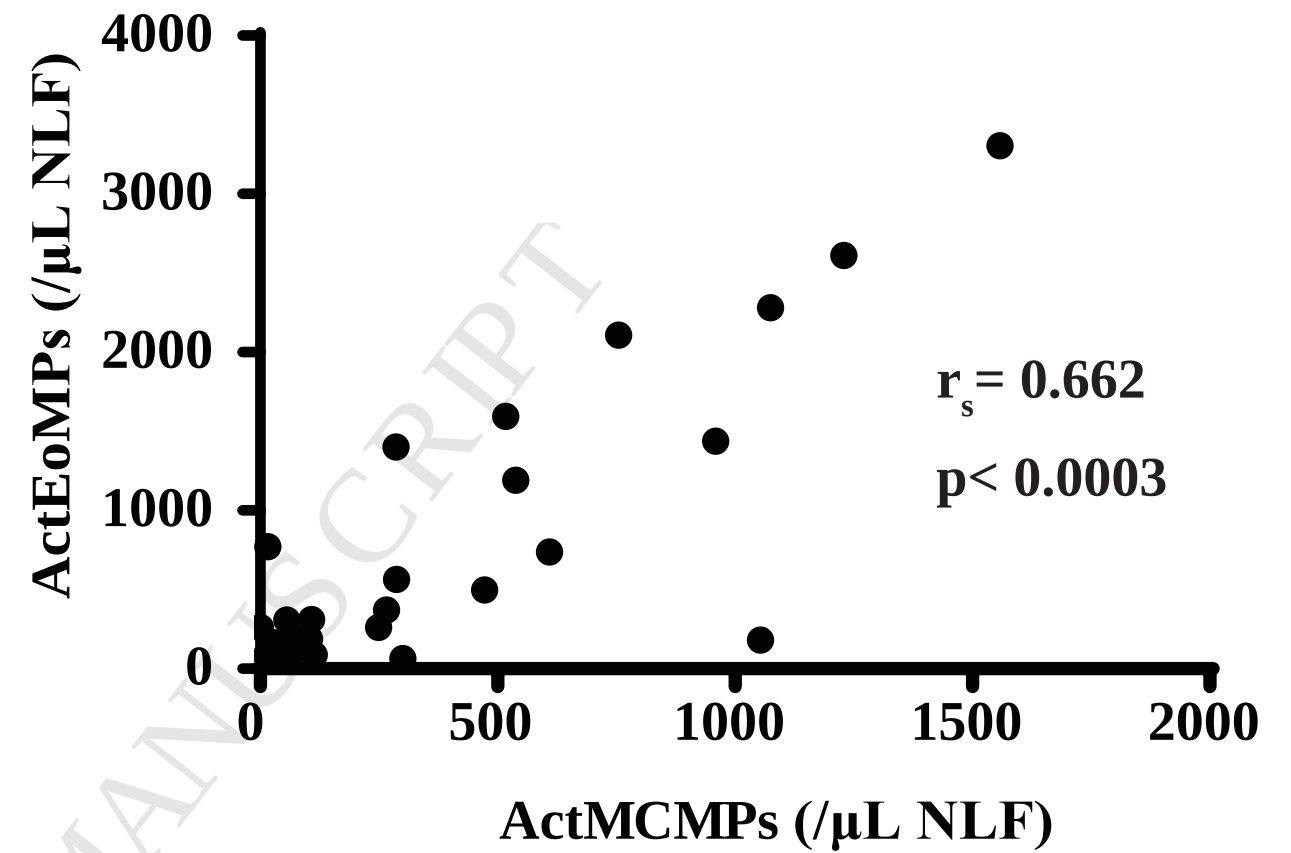
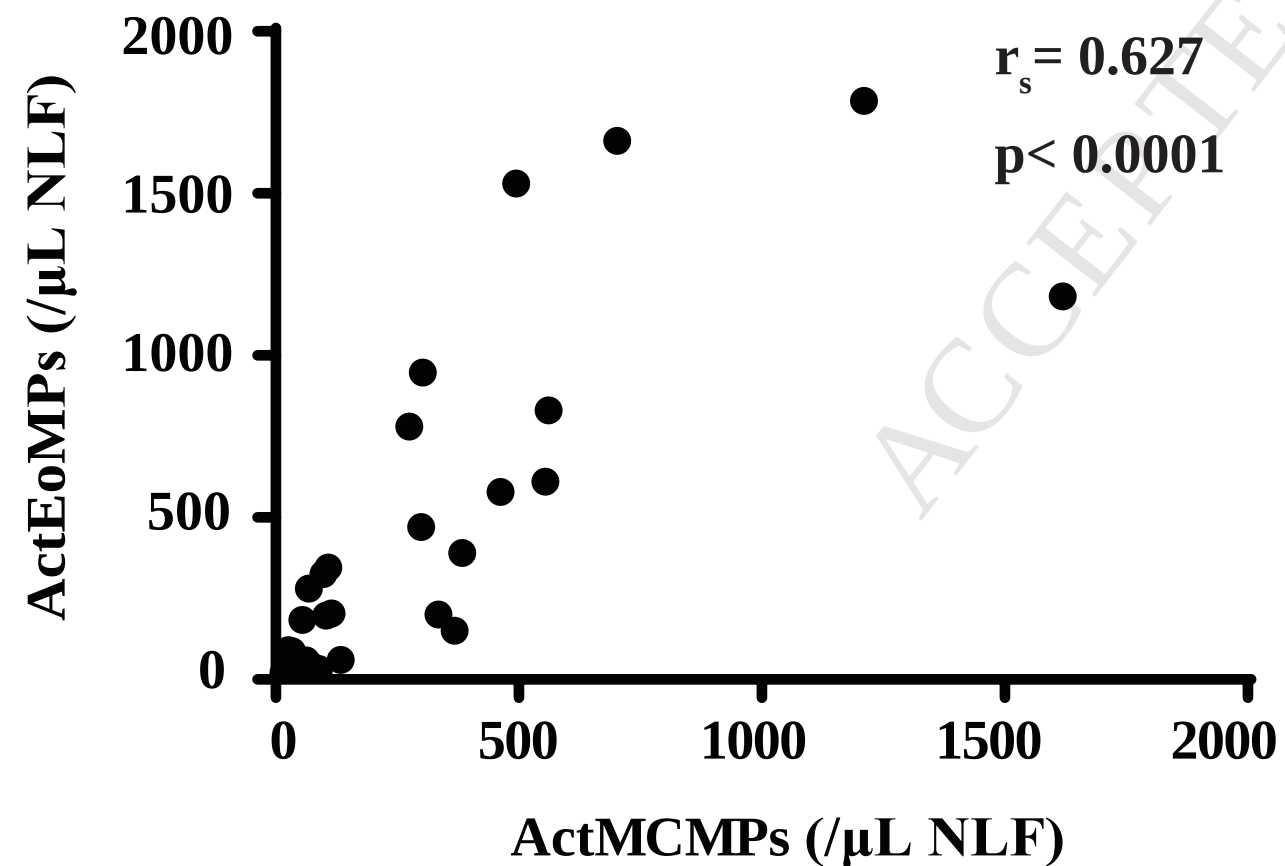
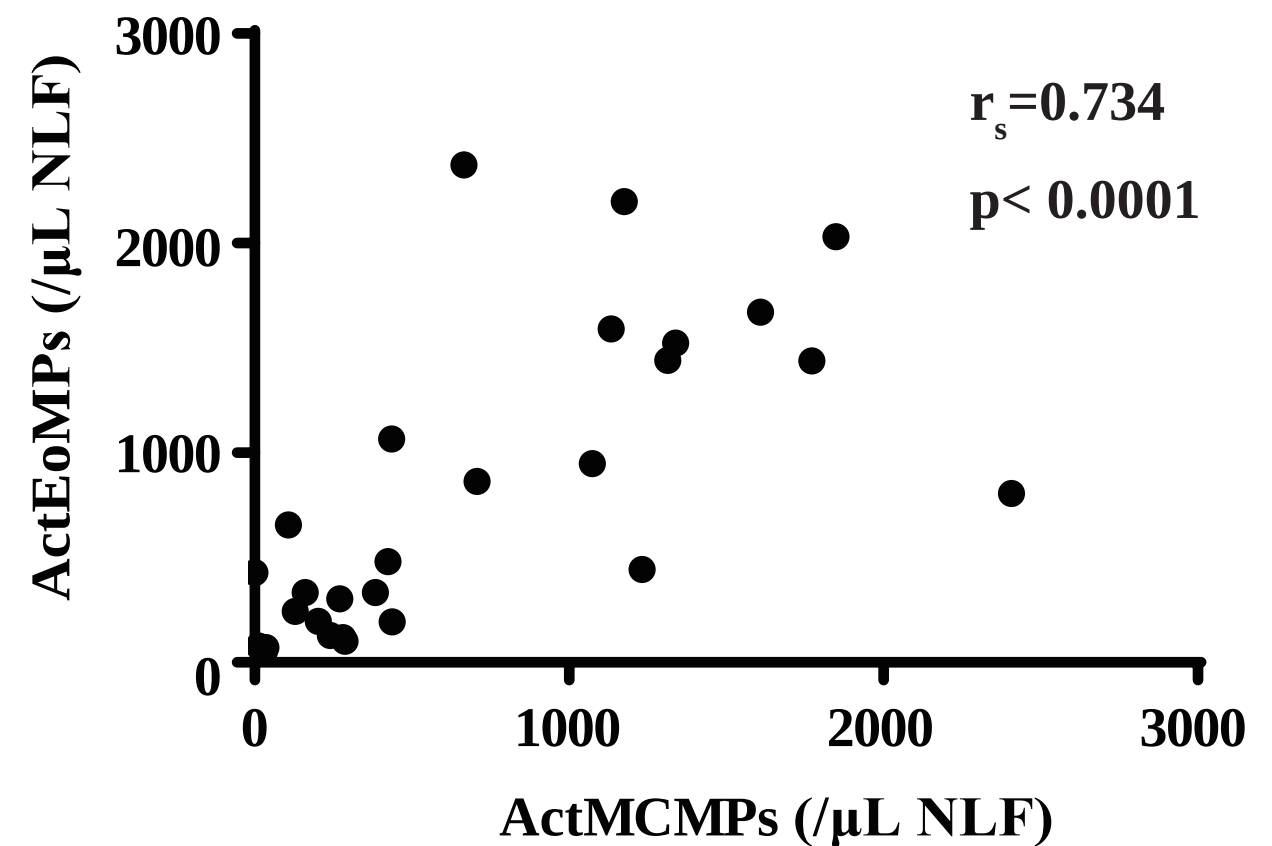


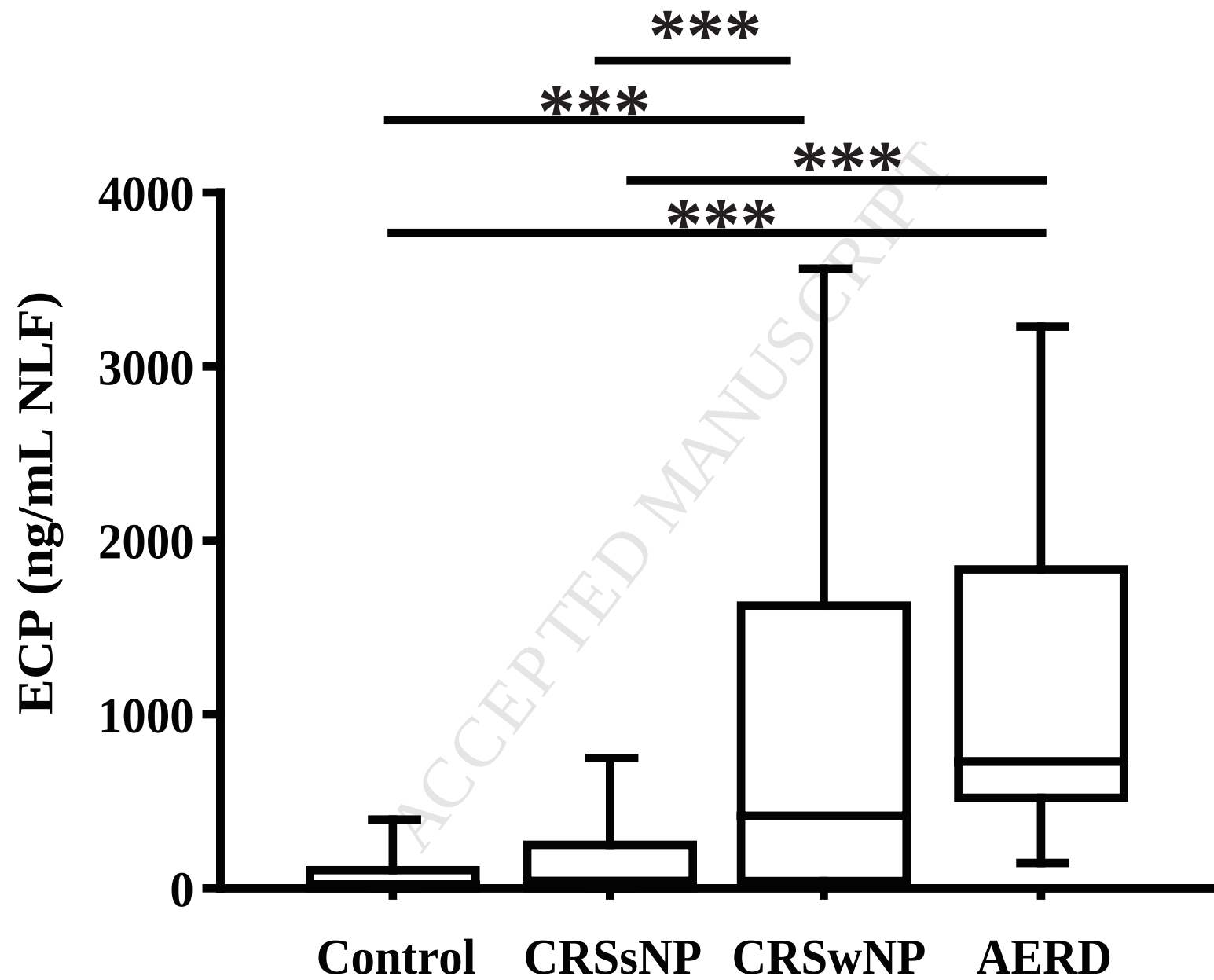
AERD (ActEoMPs vs. PltMPs)



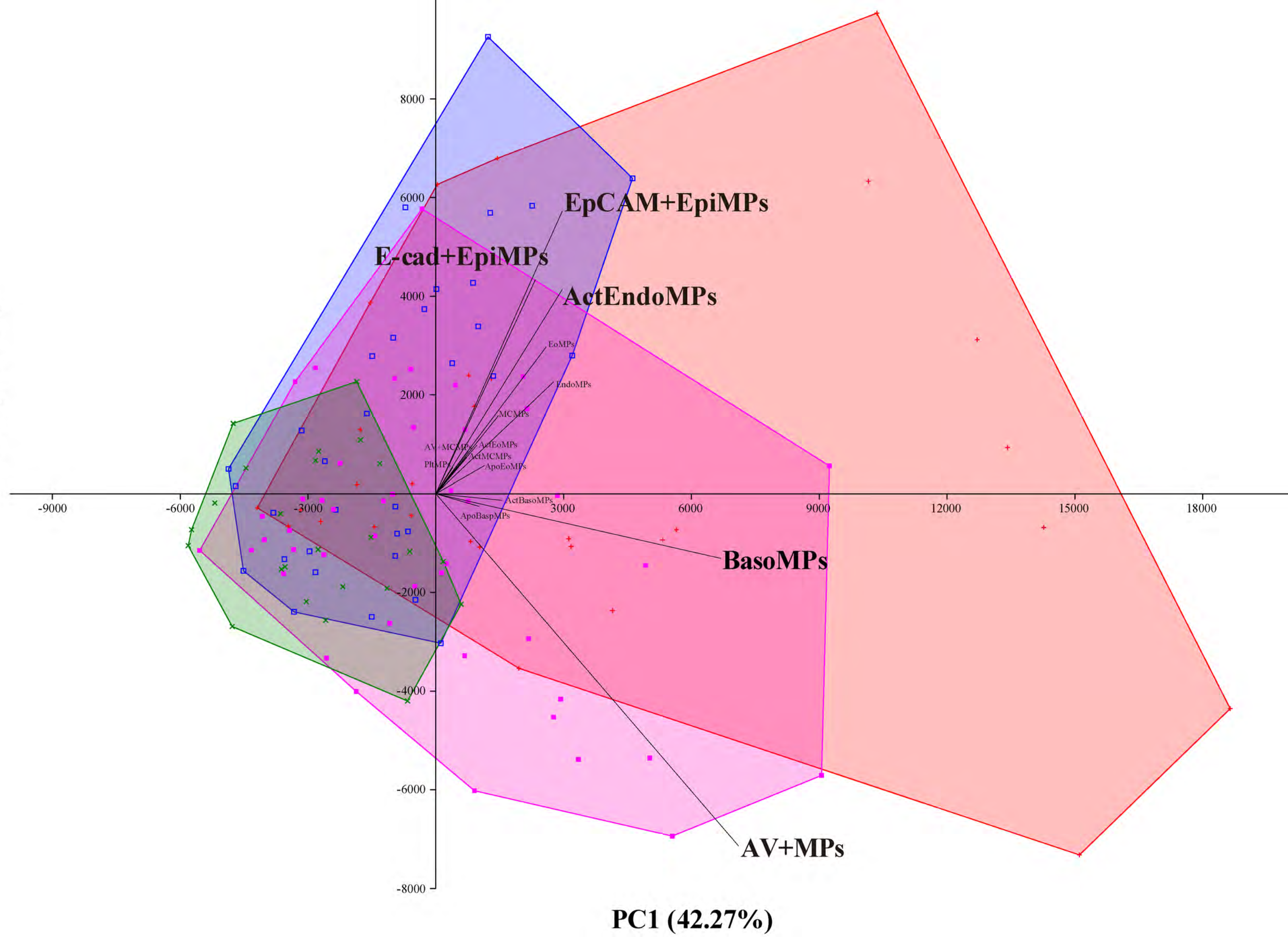
AERD (ApoEoMPs vs. PltMPs)



Control**CRSsNP****CRSwNP****AERD**

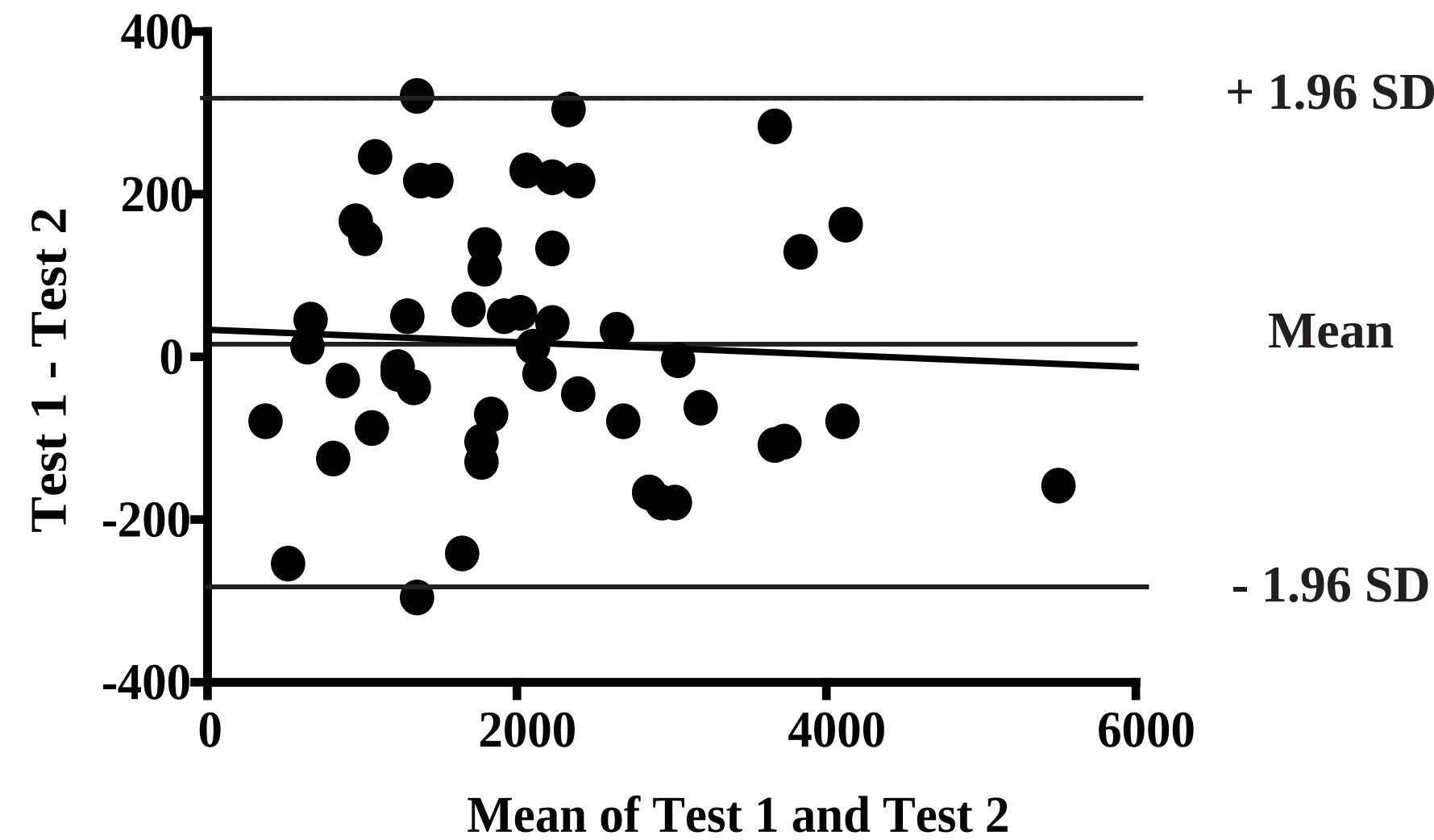


PC2 (20.06%)

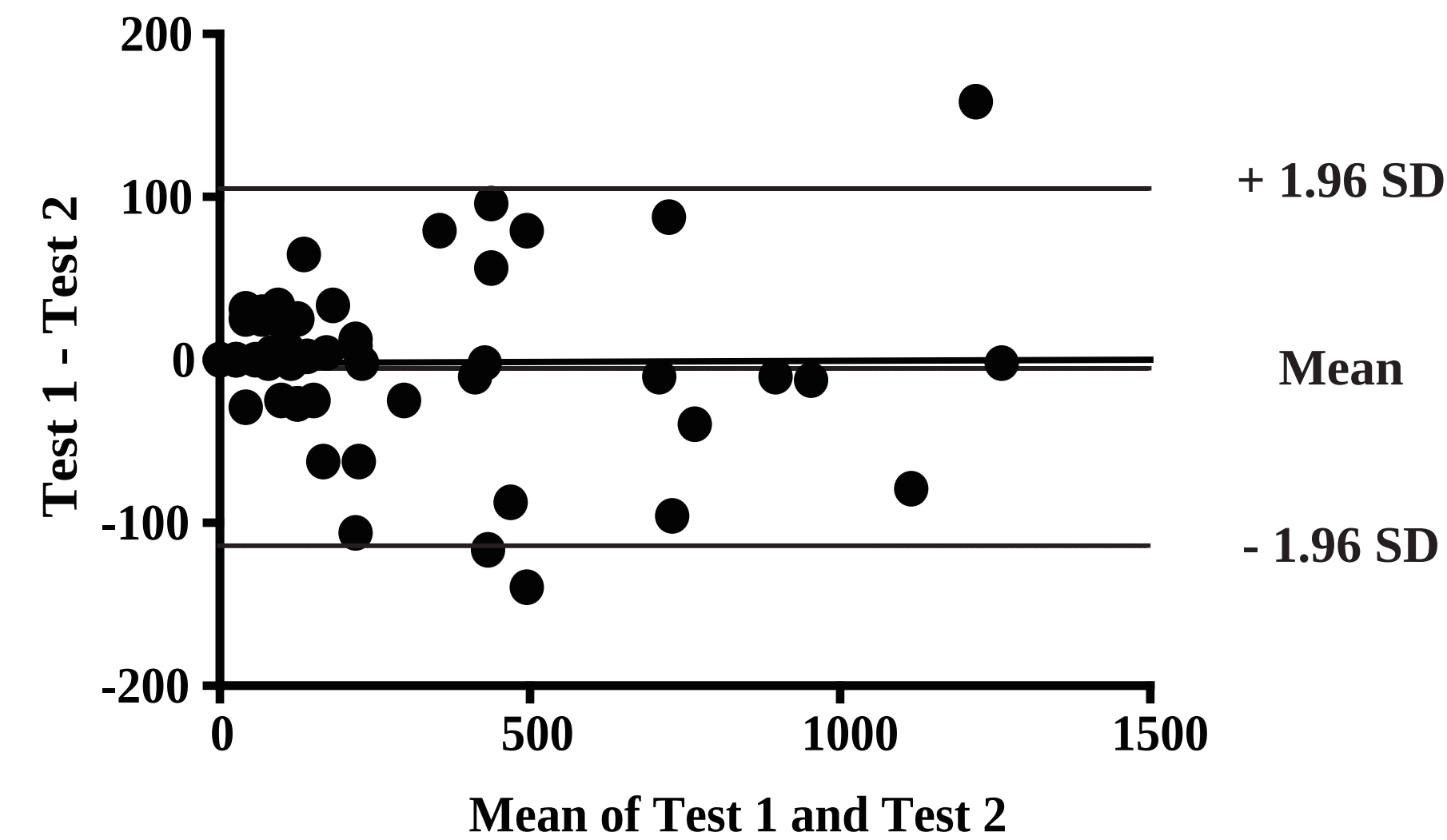


PC1 (42.27%)

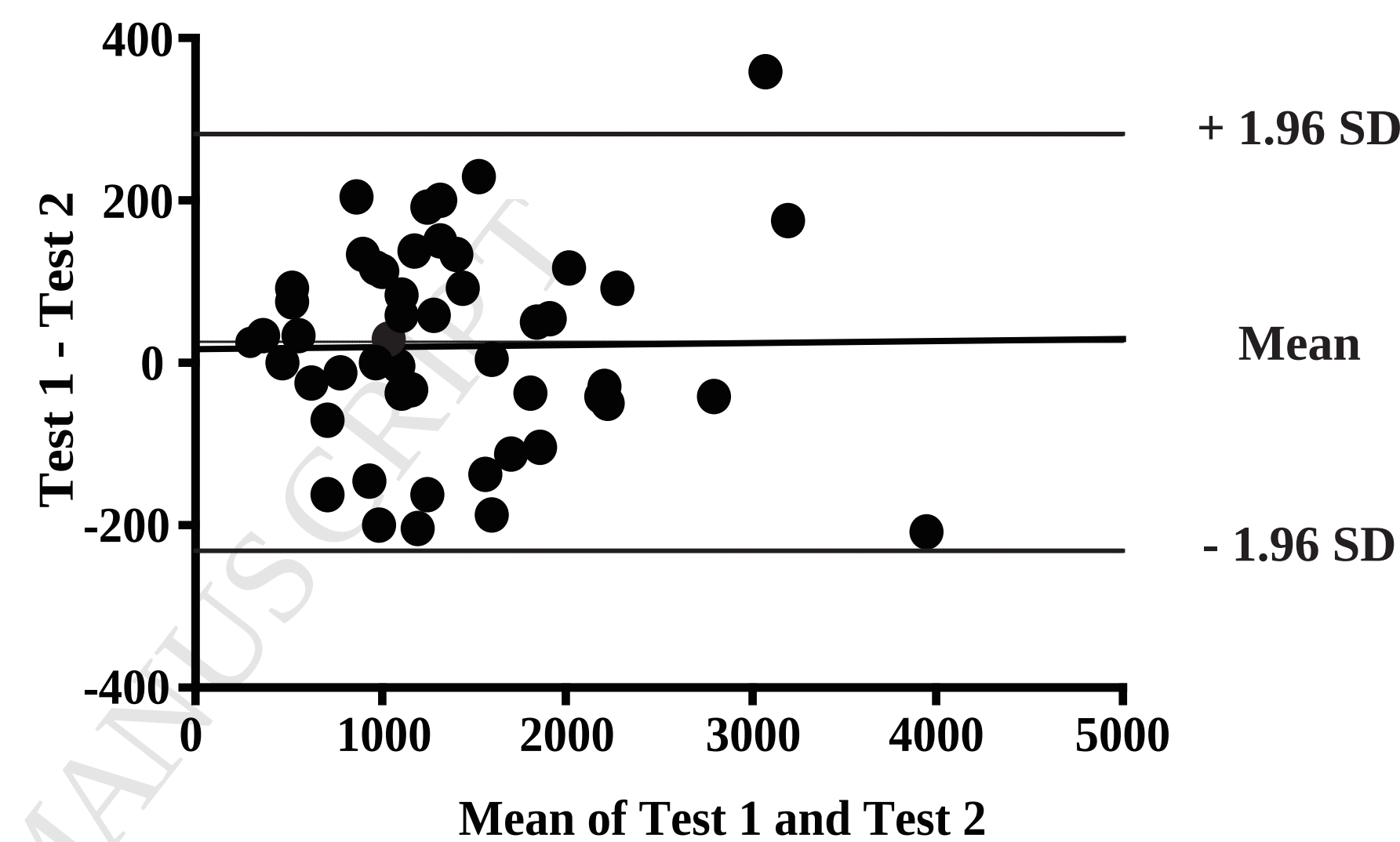
EpCAM+EpiMPs



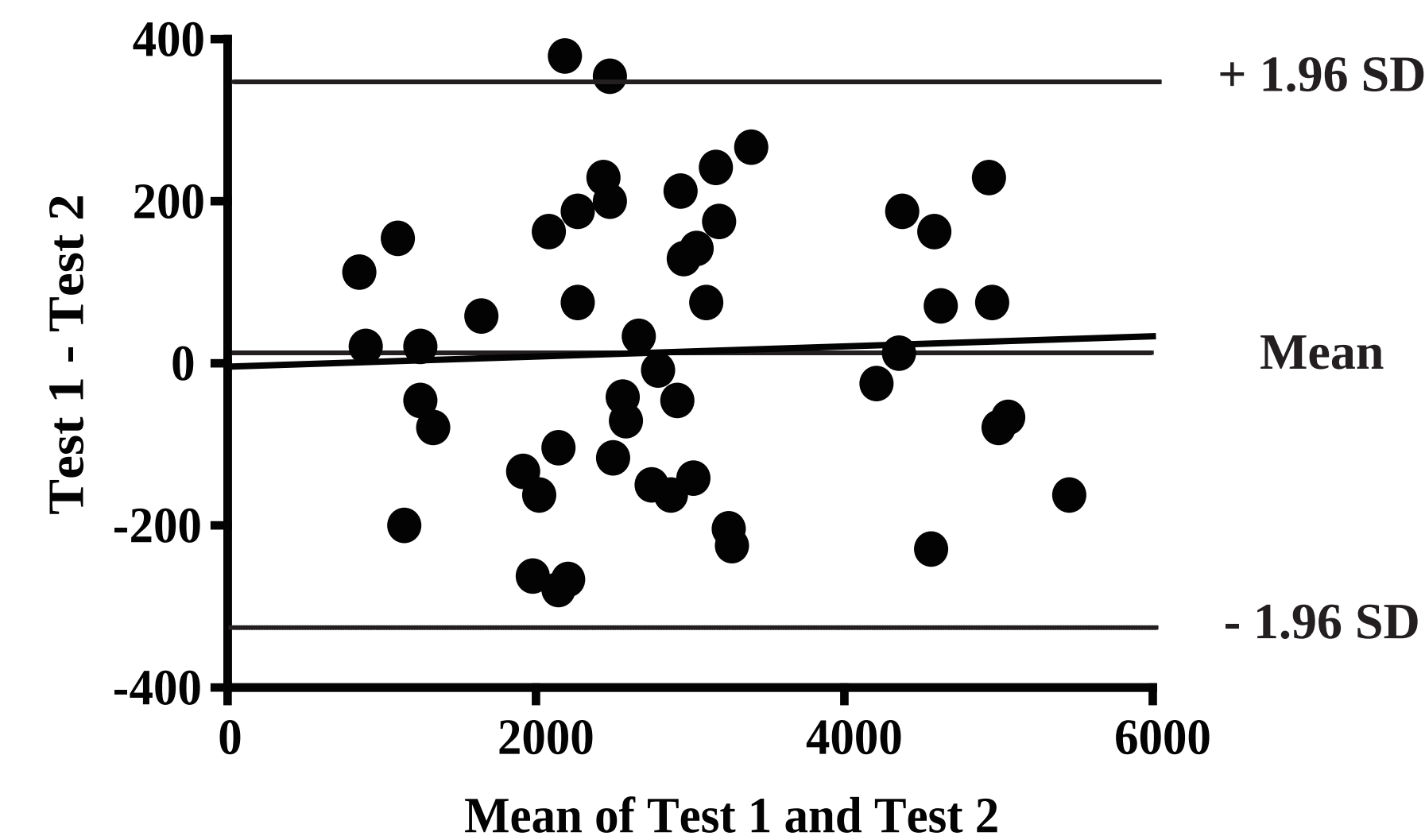
PltMPs



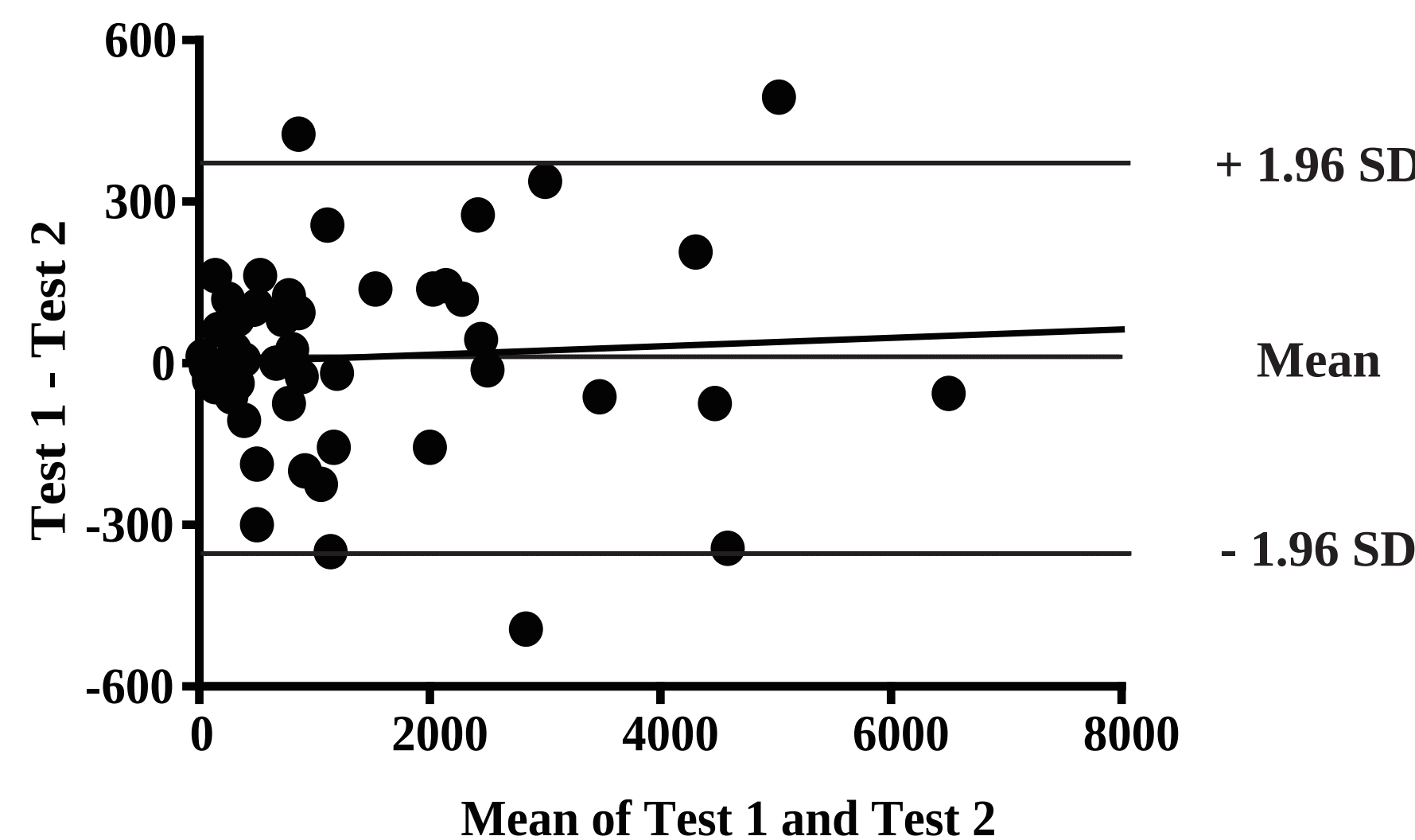
E-selectin+EndoMPs



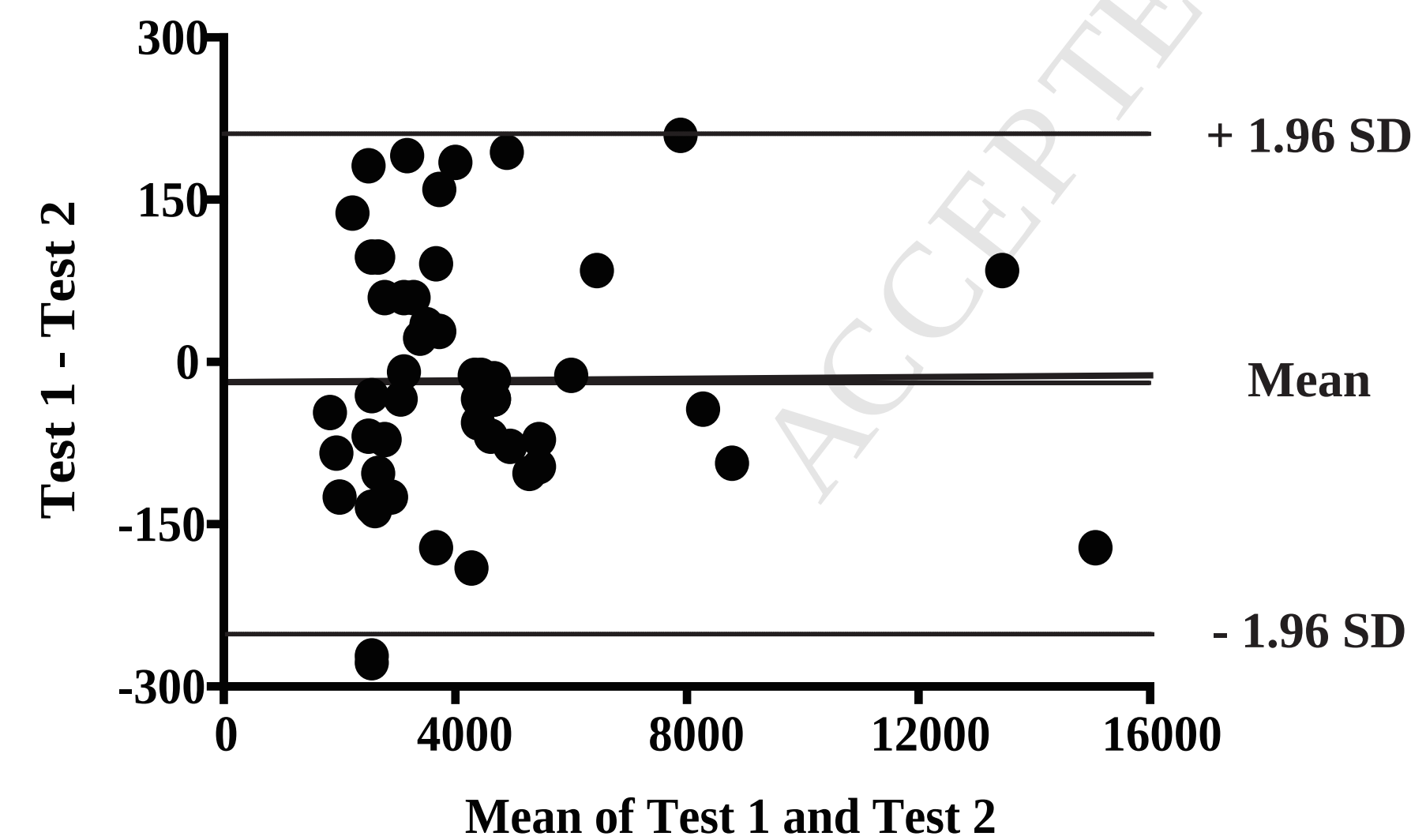
EoMPs



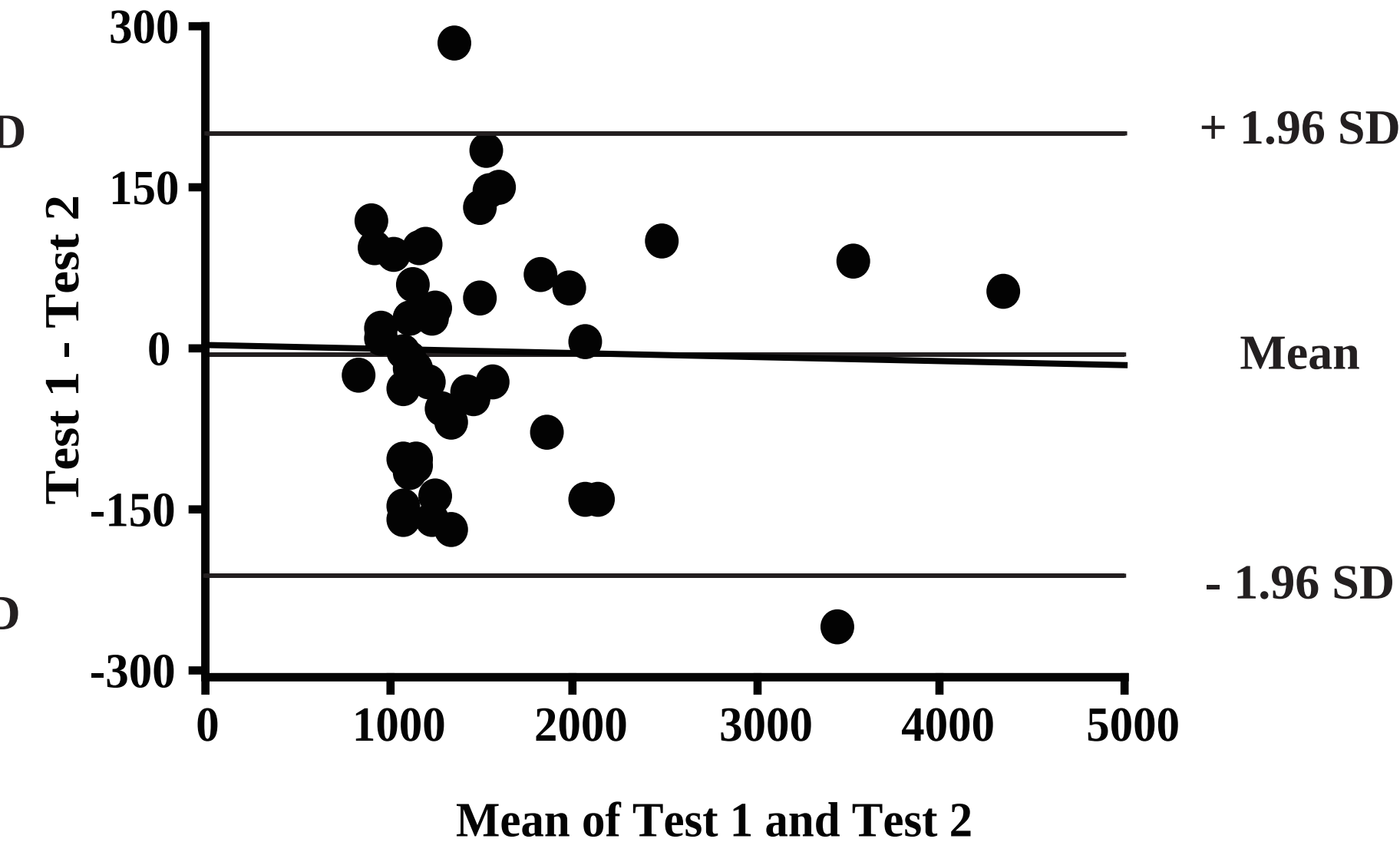
MCMPs

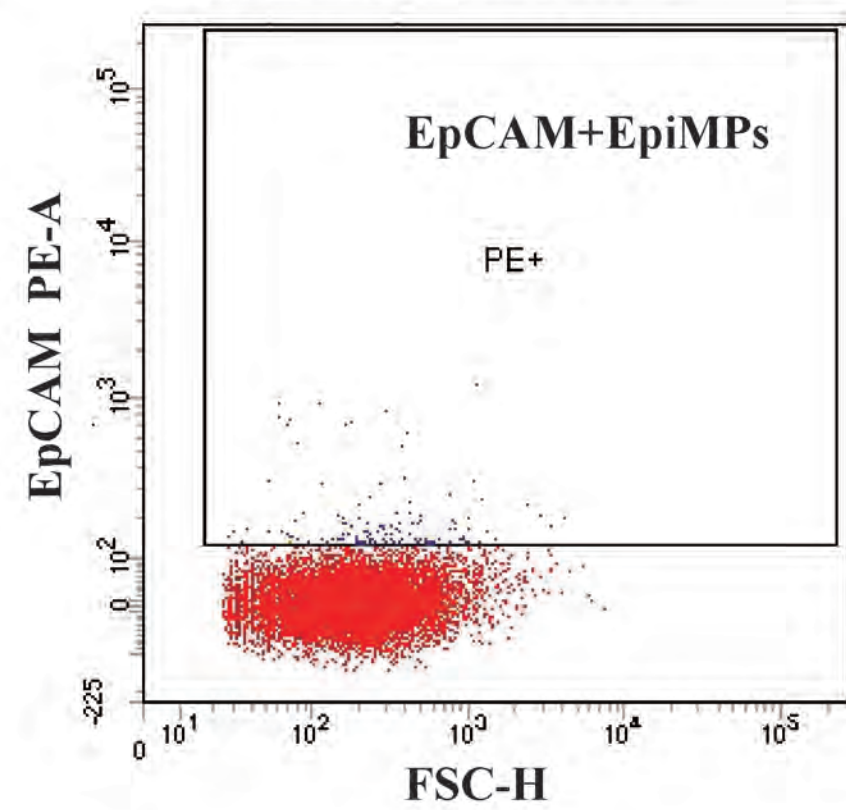
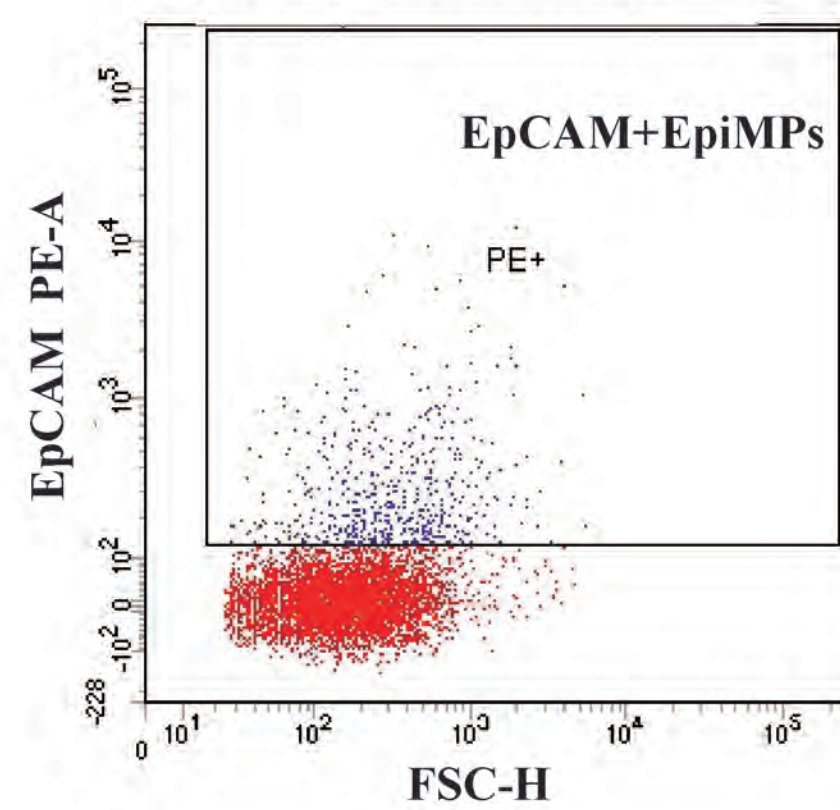
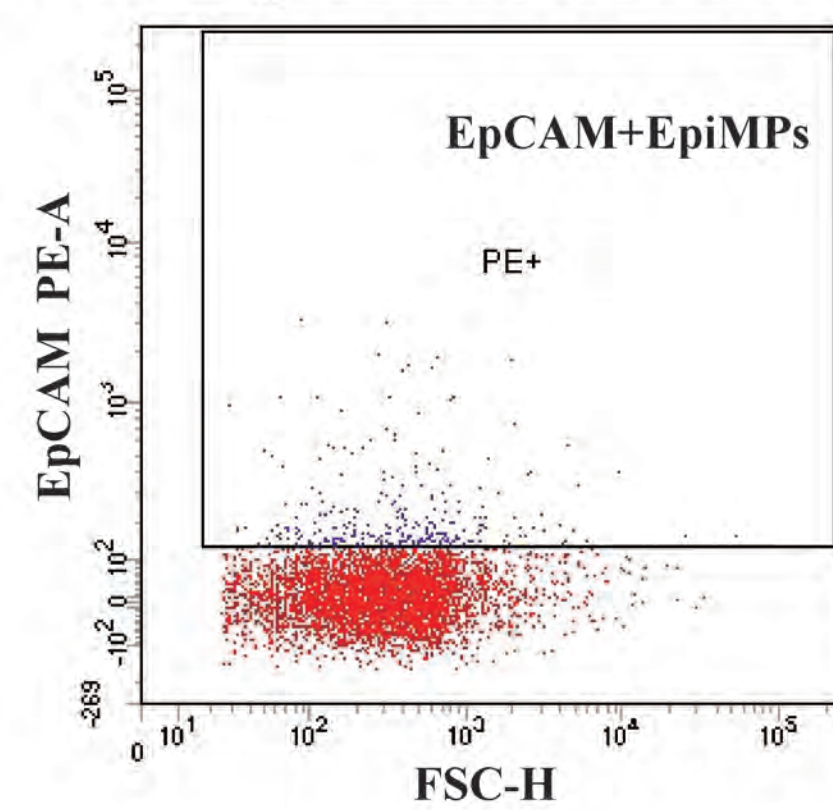
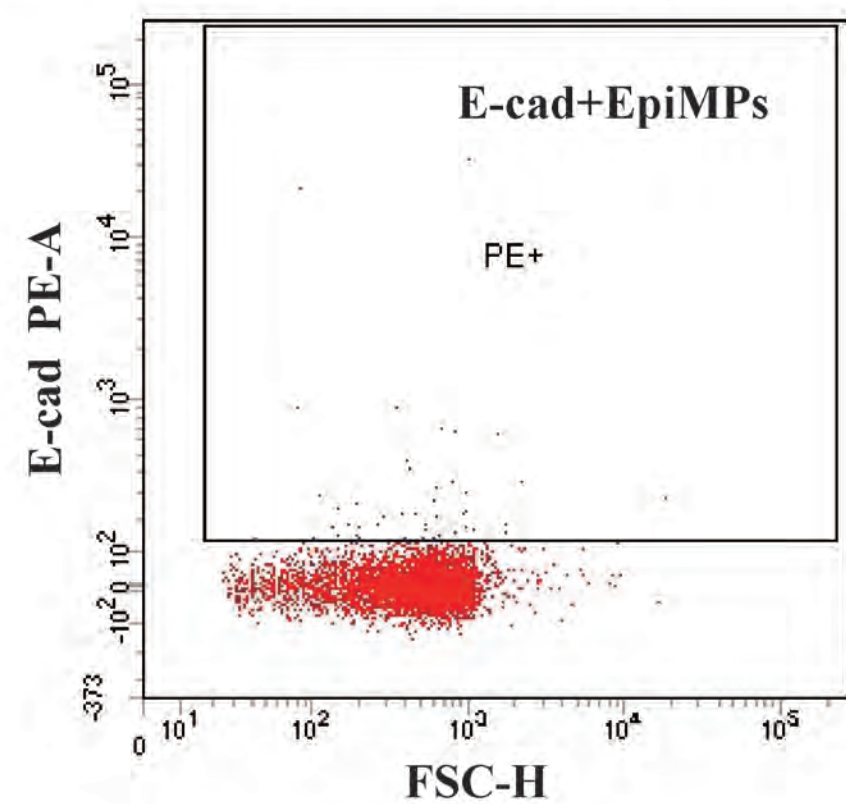
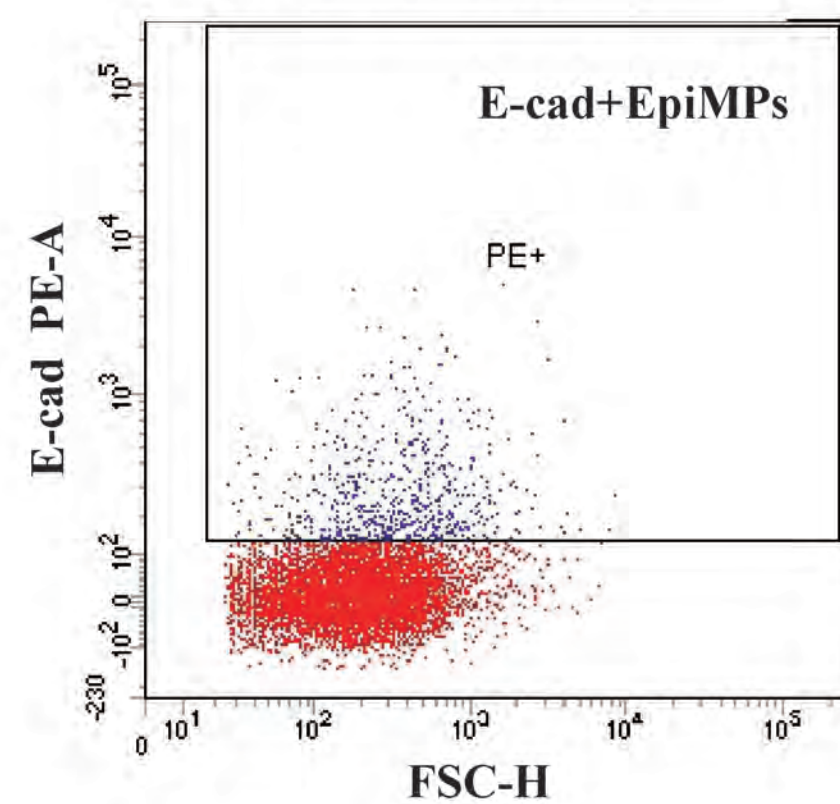
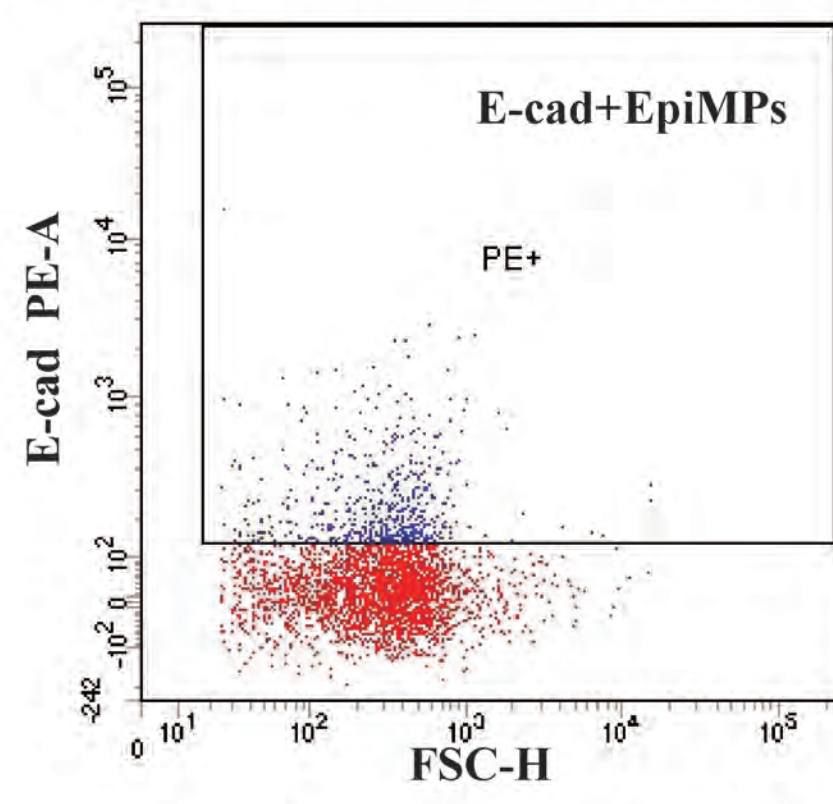
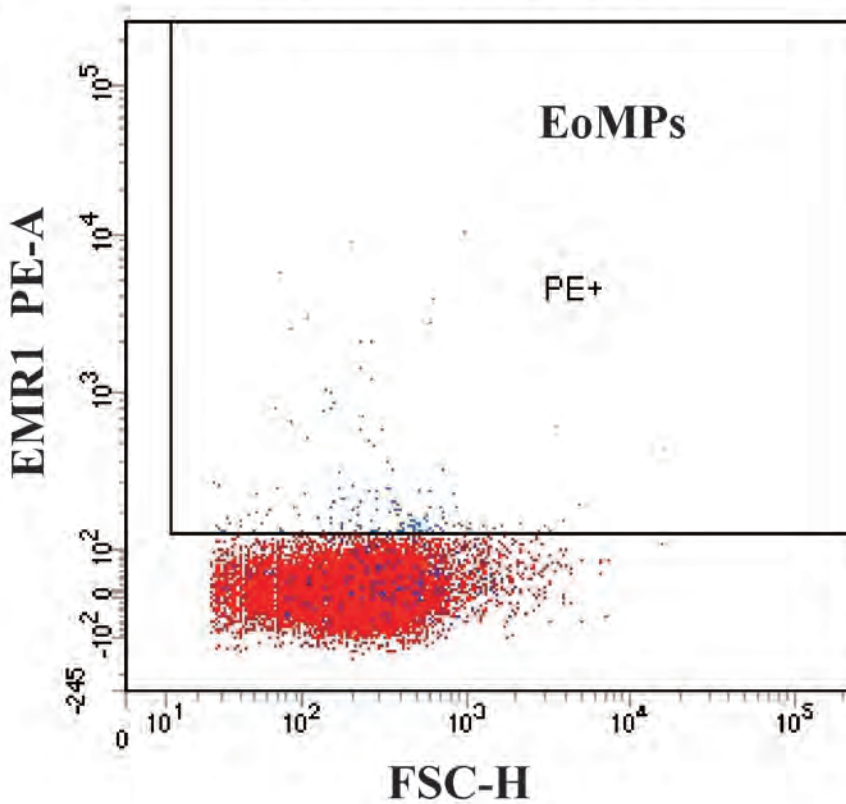
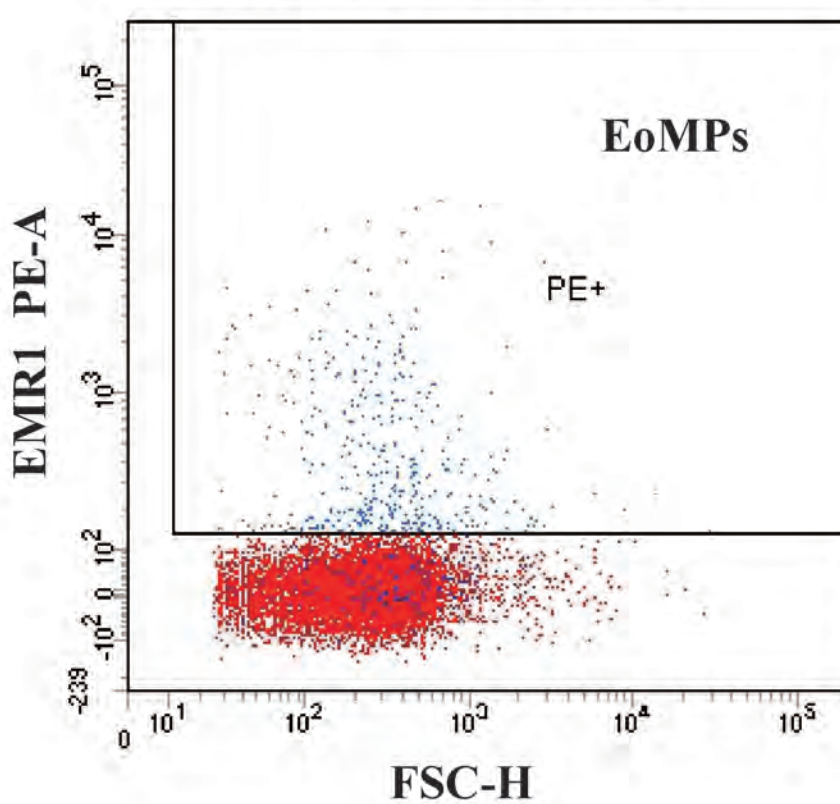
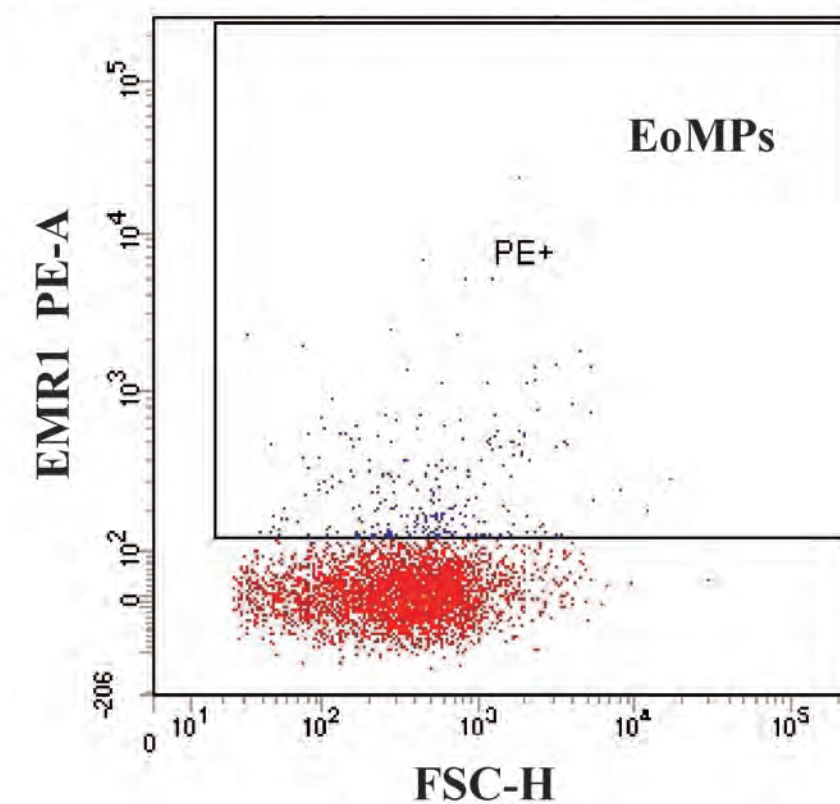
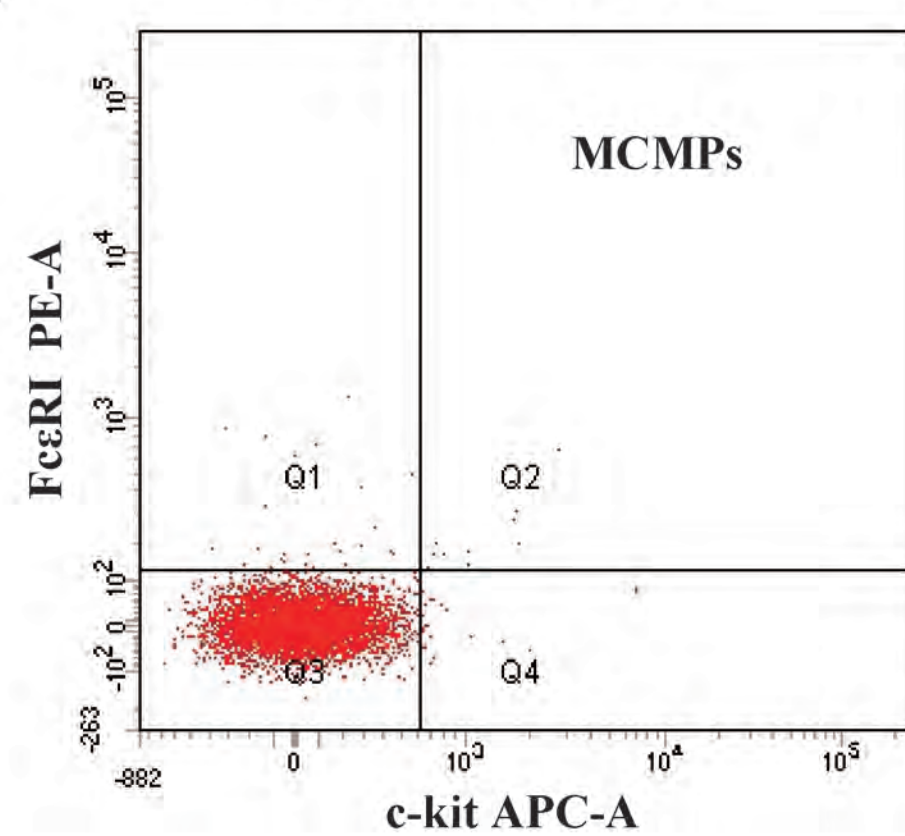
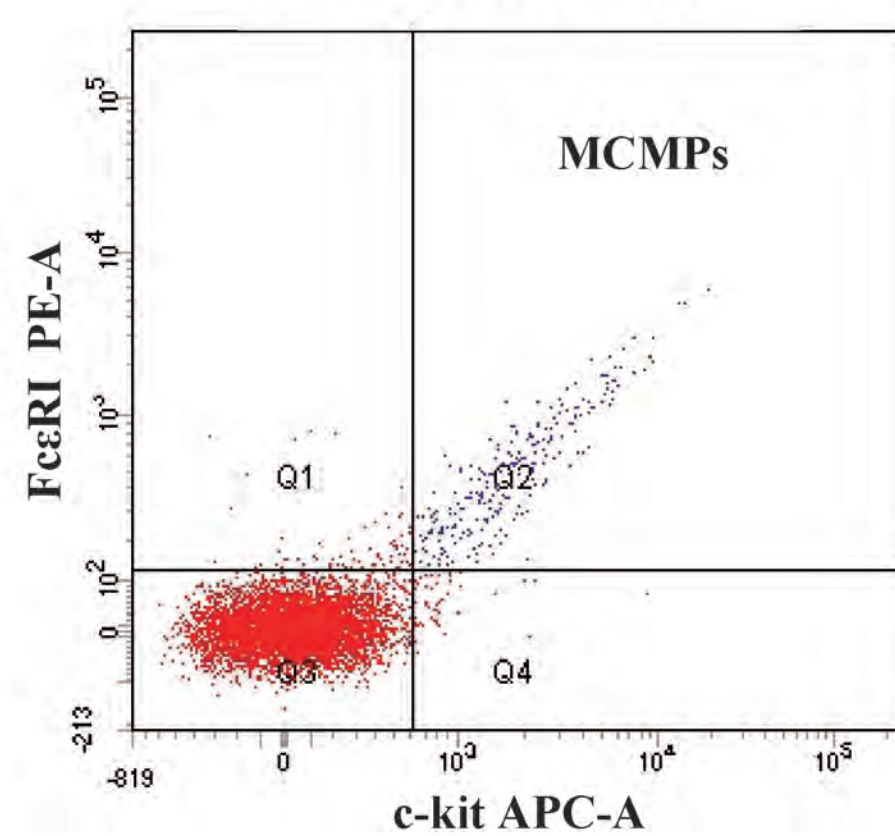
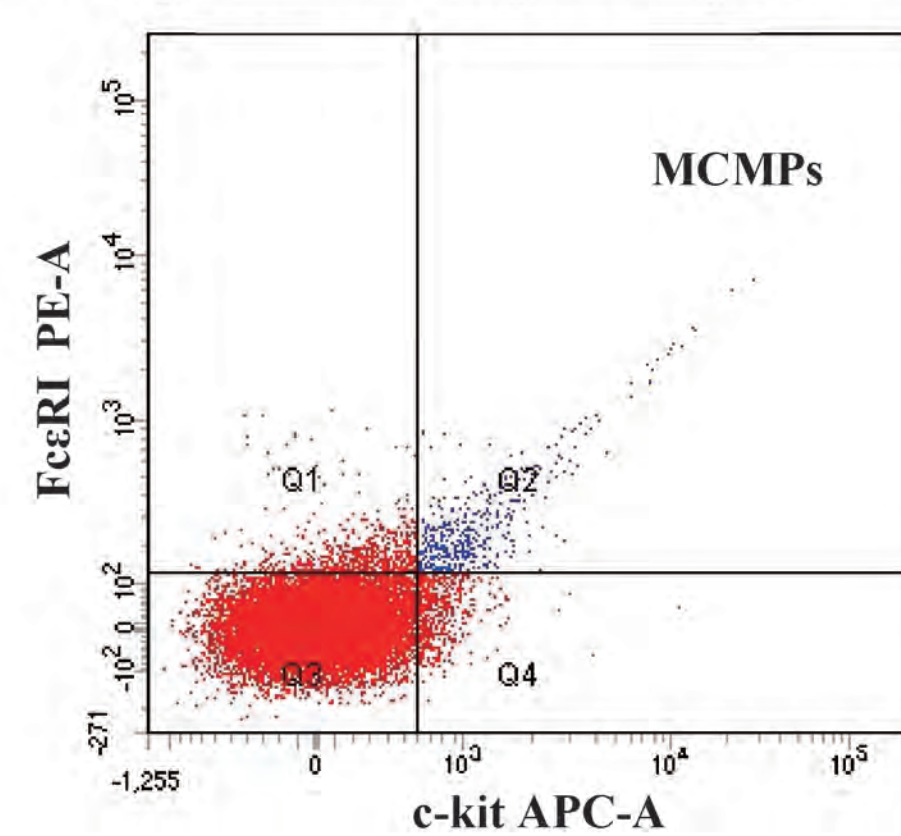
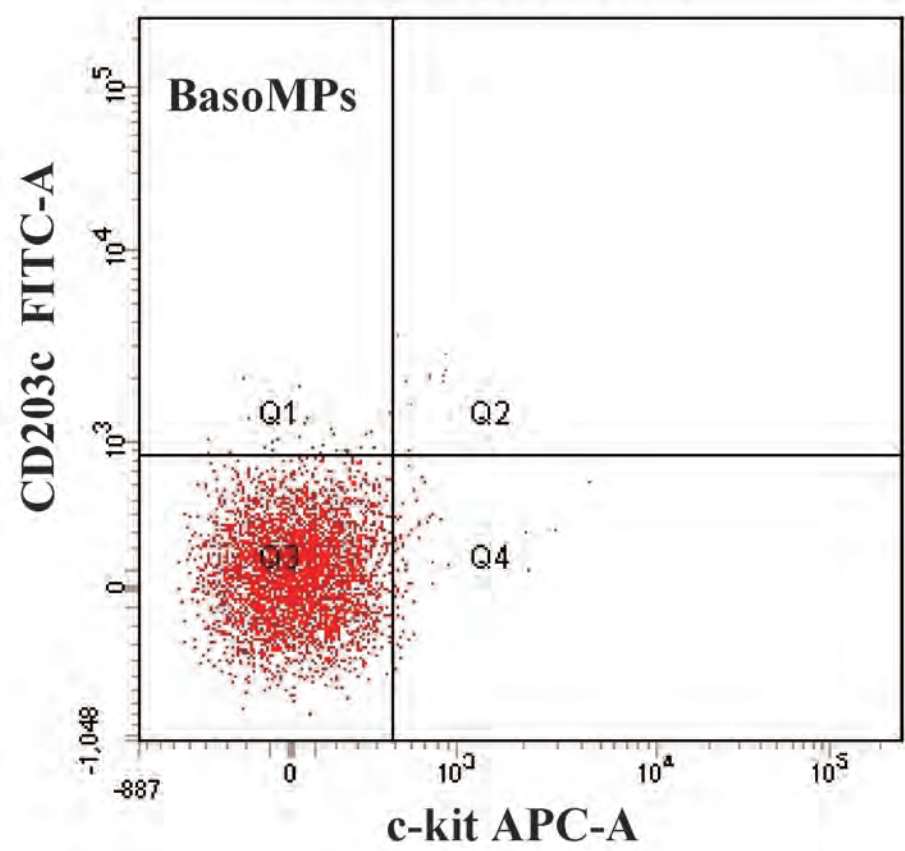
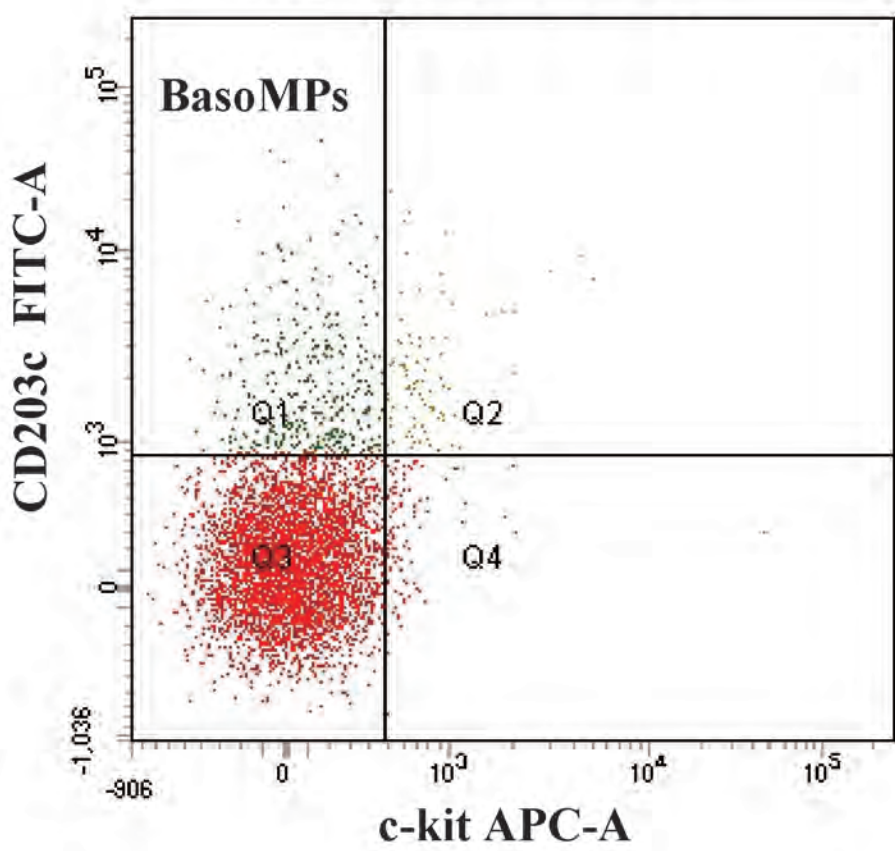
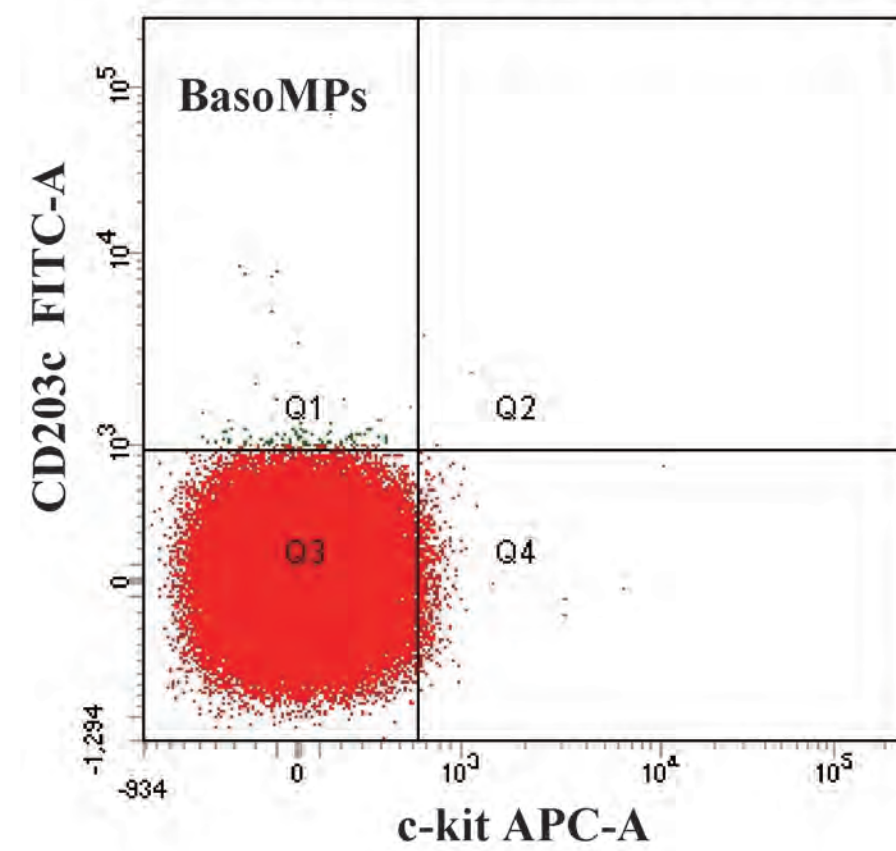


BasoMPs



CD203cMFI



(A)**NLF of CRSwNP****NLF of CRSsNP****Culture supernatant of A549 cells****(B)****NLF of Control****NLF of CRSsNP****Culture supernatant of BEAS2B cells****(C)****NLF of Control****NLF of CRSwNP****Culture supernatant of human primary eosinophils****(D)****NLF of CRSwNP****NLF of CRSsNP****Culture supernatant of primary cultured mast cells****(E)****NLF of Control****NLF of CRSwNP****Blood without stimulation****Blood stimulated with
f MLP for 30 min**