Platelets induce free and phospholipid-esterified 12-hydroxyeicosatetraenoic acid generation in colon cancer cells by delivering 12-lipoxygenase

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Author Contributions

Conceptualization: PP, VBOD, MD, SS, AC

Data curation: PB

Formal analysis and Investigation: MD, SS, CH, RF, ST, RG, AL, AC

Funding acquisition: PP, VBOD, MD, AL

Methodology: PL, MM, VJT, MZ

Writing-original draft and Writing-review and editing: PP, VBOD

Introduction

Arachidonic acid (AA) can be oxygenated by platelet-type 12S-lipoxygenase (12-LOX)[1] to 12S-HpETE (hydroperoxyeicosatetraenoic acid), which is quickly reduced by glutathione peroxidase in the cell to form 12S-HETE (hydroxyeicosatetranoic acid)[2, 3]. 12-LOX is expressed principally in platelets [1], keratinocytes [4], and tumor cells (such as prostate cancer, breast cancer, colorectal

EMT confers on cancer cells traits associated with high-grade malignancy [42]. In esophageal cancer cell lines, the knockdown of the expression of 12-LOX or the use of baicalein, an inhibitor of the 12-LOX pathway [43, 44], was associated with reduced expression of EMT markers and cell migration [18].

Here, we have tested the hypothesis that the cancer cell uptake of mEVs expressing 12-LOX released from platelets induces the biosynthesis of 12-HETE and its esterification into membrane PLs. Also, we have addressed whether the pharmacological inhibition of 12-LOX restrains platelet

ar2X restrains platelet

extensively washed with PBS, harvested by trypsin and resuspended in culture medium (McCoy containing 0.5 % FBS, 1 % P/S and 2 mM L-glutamine) at the density of $1.5x10^6$ cells/ml; then 200 µl aliquots of the cell suspension were placed in 96-multiwell and pre-incubated with vehicle (DMSO) or CDC, 30 M, for 15 min; then arachidonate (AA, Sigma-Aldrich) 30 µM was added and incubated at 37 °C for 30 min. Supernatants were collected centrifuged at 276 g for 5 min at 4 °C (to eliminate possible HT29 cells). All supernatants were re-spun at 16000 g for 40 min at 4 °C; then, the supernatants were collected, frozen in liquid nitrogen, and stored at -80 °C until the assessment of 12-HETE by LC-MS/MS.

Isolation of mEVs from thrombin-stimulated washed platelets and characterization of 12-LOX activity

Washed platelets were stimulated with 1 U/ml thrombin for 30 min, and mEVs were pelleted by centrifugation for 40 min at 16000 g at 4 °C. mEV pellets were analyzed for the expression of 12-LOX by Western Blot. In some experiments, 250000 mEVs were resuspended in 250 l of HEPES Buffer and incubated with arachidonate (30 M for 30 min at 37 °C): the reaction was stopped immediately, keeping the samples at 4°C and by centrifuging them for 40 min at 16000 x g at 4°C. The supernatant was then collected, frozen in liquid nitrogen, and stored at -80°C until the assessment of 12-HETE by LC-MS/MS.

Isolation of platelets and mEVs from plasma of individuals with colorectal adenomas/adenocarcinomas and assessment of 12-LOX levels

Whole blood samples were collected from eight patients with colorectal adenomas or adenocarcinomas enrolled in a clinical study performed at Hospital Clinico Universitario Lozano Blesa (Za7(c6)-3(e)S.

(protein name: platelet 12-LOX), RHOA (protein name: RhoA), TWIST1 (protein name: Twist-related protein 1), GPR31 (protein name: GPR31) and GAPDH, was performed using TaqMan gene expression assays (Hs01023894, Hs00232783, Hs00365052, Hs00185584, Hs00167524, Hs00357608, Hs01675818, Hs00271094 and Hs99999905, respectively) (Applied Biosystems, Foster, City, CA) according to the manufacturer's instructions using a 7900HT Real-Time PCR system (Applied Biosystems). Gene expression assays were performed by relative quantification with comparative cycle threshold (Ct) using ABI Prism, SDS 2.4 software (Applied Biosystems).

V, CXP: -11, EP: -10. For eicosanoid analysis, lipid extracts were separated on a C18 Spherisorb ODS2, 5>

Effect of extracellular 12-HETE on phospholipid-esterified-12-HETE generation in HT29 cells

In some experiments, HT29 cells ($1x10^6$) cultured alone or with platelets ($1x10^8$) were incubated with 12S-HETE-d_8 (Cayman Chemical, Ann Arbor, USA; 12S-HETE-d_8 contains eight deuterium atoms at the 5, 6, 8, 9, 11, 12, 14 and 15 positions) (50 ng/ml, corresponding to total 100 ng) up to 20 hrs. HT29 cells and the conditioned media were collected, as described above, frozen in liquid nitrogen and stored at -80 °C until the assessment of free 12-HETE-d_8 and 12-HETE-d_8-PLs.

mEV characterization by flow cytometry

mEV pellets isolated from the conditioned medium of HT29 cells, platelets, and platelet-HT29 cell cocultures, as reported above, were resuspended in 100 µl of Annexin buffer (BD Biosciences, Milan, Italy) and labeled with MitoStatus-APC (Thermo-Fisher)/Phalloidin (Sigma-Aldrich)/ anti-CD41a-PerCP-Cy5.5(BD Biosciences) (a platelet marker: platelet GPIIb; integrin αIIb)/AnnexV-V500(BD Biosciences, Annexin V binds in a calcium-dependent manner to phosphatidylserine, PS)/anti-CD66PE (BD Biosciences) (i.e., carcinoembryonic antigen, CEA, the most commonly used tumor marker in a variety of cancers including colorectal cancer) [50-52] as reported in the manufacturer's instructions and counted by using flow cytometry. In some experiments, before the coculture, platelets were loaded with 5 g /ml of 5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester (CFSE, Sigma-Aldrich) for 15 min at 37 °C, then, they were washed and added to cancer cells as previously described [53]. The number of mEVs positive to CFSE53

and 12-HETE-PLs were assessed in all compartments of the cell cultures [i.e., conditioned medium (2 ml) and cellular lysates (1 ml)] and reported as the total amount (ng) detected in each compartment or the sum of all compartments. Statistical analysis was performed using GraphPad Prism Software (version 9.0 for Windows, GraphPad, San Diego, California). The statistical tests used to calculate P-values are reported in the Figure legends. $P < 0.05$ values were considered statistically significant.

Results

12-HETE was generated by HT29 cells following the transfer of 12-LOX from platelets

In cancer cells cultured alone, 12-HETE in the conditioned medium was undetectable. In HT29 cells cultured with platelets for 20 hrs, free 12-HETE was detected in the medium (Figure 1A). 12-HETE levels were not significantly different from those found in the supernatant of platelets cultured alone (Figure 1A). Thus, released 12-HETE detected in platelet-cancer cell coculture was mainly of platelet origin.

12-HETE was also assessed in the intracellular compartment of HT29 cells cultured alone or with platelets. As shown in Figure 1B, the intracellular levels of 12-HETE in HT29 cells cultured alone were undetectable, while 12-HETE was found in cancer cells after the co-incubation with platelets.

We also assessed 15-HETE levels in the conditioned medium of platelet-HT29 cell cocultures and

M) also significantly reduced 12-HETE generated by HT29 cells exposed to platelets and, after extensive washing, incubated with AA (30 M) for 30 min (Figure 1E).

12-LOX protein was undetectable in HT29 cells cultured alone, while it was highly expressed in platelets (Figure 1F). After the culture of HT29 cells with platelets for 20 hrs, the 12-LOX protein was detected by Western blot analysis (Figure 1F). In contrast, 12-LOX mRNA levels were undetectable by qPCR (not shown). This excludes the possibility that platelets induce ALOX12 in HT-29 cells, instead indicating that 12-LOX was delivered to cancer cells by platelets.

12-LOX inhibitors mitigated the induction of EMT marker genes in HT29 cells induced by platelets

Incubation of platelets with HT29 cells enhanced the expression of the EMT marker genes [58,59] ZEB1 (a transcription factor promoting EMT and loss of cell polarity), VIM (involved in changes of shape, adhesion, and motility), TWIST1 (another transcription factor promoting EMT and metastasis), FN1 (an extracellular matrix protein involved in migration and invasion) and RHOA (a GTPases implicated in migration and proliferation) (Figure 2A). In contrast, E-cadherin mRNA levels were down-regulated (E-cadherin loss is associated with EMT) (Figure 2A). Treatment of tumor cells with CDC interfered with the platelet-dependent gene induction (Figure 2A and B); differently, CDC did not significantly affect E-cadherin reduction (Figure 2A and B). CDC 30 M (causing a maximal inhibition of 12-HETE generation, as shown in Figure 1D) significantly reduced the enhanced expression of all genes analyzed (Figure 2A and B). At lower concentrations, the response was heterogeneous. VIM expression was very susceptible, while TWIST1 was less sensitive to CDC treatment (Figure 2A and B). However, the CDC's 50% reduction of 12-LOX activity was sufficient to significantly mitigate gene induction except for TWIST1 (Figure 2A and B). A significant linear relationship between % reduction of 12-HETE and gene expression of ZEB1, VIM, and RHOA by CDC was found (Supplementary Table 2). We tested the effect of additional inhibitors (chemically distinct) to support the contribution of 12-LOX to EMT marker gene expression induced by platelet-

Phospholipid-esterified-12-HETE was generated

and 12-HETE was released into the medium when mEVs were incubated with AA (30 M) (Figure 6F). These data show that 12-LOX expressed in platelet-derived mEVs is catalytically active.

Circulating mEVs from patients adenomas/adenocarcinomas expressed 12-LOX

We then verified whether 12-LOX is also expressed in vivo in circulating mEVs of eight patients with colorectal adenomas/adenocarcinomas (Supplementary Tabl

Dm-amiloride affected platelet mEV internalization and reduced 12-LOX levels in HT29 cells cultured with platelets

In HT29 cells cultured with platelets for 20 hrs, dm-amiloride, an inhibitor of the Na+/H+ exchanger reported to affect macropinocytosis [55], significantly ($P<0.05$) reduced the number of CFSE-labeled HT29 cells (Figure 8D).

The reduction of platelet mEV internalization by dm-amiloride was associated with a significant decrease of 12-LOX levels in HT-29 cells exposed to platelets for 20 hrs (Figure 8E and F). These data suggest that mEVs from platelets are internalized by cancer cells, at least in part, via the macropinocytosis pathway.

Discussion

Platelets trigger various phenotypic changes in tumor cells, recapitulating a pro-metastatic phenotype [29-34].

of EMT genes further strengthened the role of 12-LOX activity in platelet-dependent induction of EMT in cancer cells.

We have previously found [33, 34] that platelets induce downregulation of E-cadherin (a key cell-to-cell adhesion molecule whose expression reduction potentiate tumor cell invasion and metastasis) [42],

released mEVs containing 12-LOX were incorporated into HT29 cells by performing experiments with fluorescence labeling platelets. This phenomenon was mitigated by dm-amiloride, which inhibits the Na^+/H^+ exchanger involved in macropinocytosis [55]. Interestingly, in the presence of dm-amiloride, reduced levels of 12-LOX were detected in HT29 cells cocultured with platelets. The formation of macropinosomes is believed to arise from deformations of the plasma membrane known as ruffles [71], which are highly dynamic structures and are rich in actin and actin-associated proteins [72]. Amiloride is not a direct inhibitor of macropinocytosis. However, it acts via the induction of submembranous acidification caused by metabolic H^+ generation, unopposed by the regulatory extrusion across the membrane by Na^+/H^+ antiporter [55]. Decreased cytosolic pH by amiloride may affect the activation of the small GTPases Rac1 known to stimulate actin filament accumulation at the plasma membrane, forming membrane ruffles [55]. We have previously shown that RAC1 expression is enhanced by the interaction of HT29 cells with platelets [34]. This pathway might promote platelet-derived mEV internalization and remodeling of cancer cell phospholipids with 12-HETE. Further studies are needed to address this hypothesis. The finding that mEV internalization was prevented, at least partly by amiloride, which affects macropinocytosis, seems to exclude the possibility that platelet uptake is involved in the transfer of 12-LOX into cancer cells. Studies involving the use of dynamic transmission ele1 09(ah] TPhol)-2(ip0p7TQq0.000008871 0 595.32 841.92 reW*

pattern of products is dictated by the substrates available in the plasma membrane. In platelets, 12-HETE was found esterified mainly in PC species with acyl-linked 16:0 or 18:0 at Sn1 and PE 18:0a.

It has been reported that 12-LOX is regulated by the interaction with cellular proteins such as human type II keratin K5, nuclear envelope protein lamin A, integrin beta4 cytoplasmic domain, and human C8FW phosphoprotein [74]. Further studies are requested to investigate the contribution of 12-LOX interacting proteins to platelet-induced EMT in cancer cells shown here.

It was previously reported that 12-LOX expressed in PRP is a promising diagnostic and prognostic biomarker of prostate cancer [61, 62]. Here, we have shown that circulating mEVs collected from patients with colorectal adenomas/adenocarcinomas contain 12-LOX protein. It is noteworthy that an enhanced number of platelet-derived mEVs is detected in the bloodstream of patients with CRC and other cancer types [75,76]. Thus, the crosstalk between mEV expressing 12-LOX with circulating cancer could enhance their prometastatic potential in vivo. This issue should be verified in an appropriate clinical study. If confirmed, our findings may open the way to novel therapeutic strategies to dampen tumor metastasis: (i) affecting 12-

Data availability statement

All individual data are reported in the Figures. There is no restriction on the availability of any data.

Conflict of Interest statement

The authors declare no conflict of interest.

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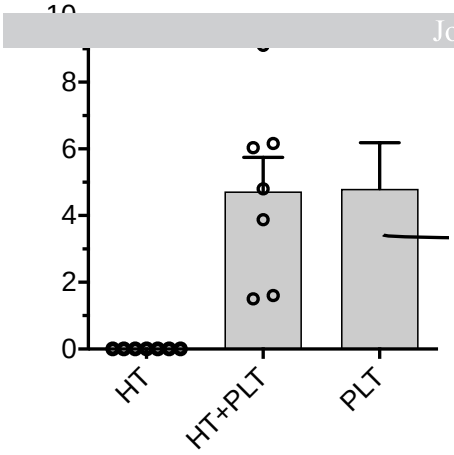
scatter dot plots with mean+SEM, n=3; **P<0.01 *vs.* $CD41^+$ mEVs. **(B)** The count of $CD41^+$ mEVs in the medium of coculture at 2 and 20 hrs was assessed; results are reported as $CD41^+$ mEV/ l, all data are shown as scatter dot plots with mean+SEM, n=5-9; **P<0.01 *vs.* HT alone. **(C)** The count of $CD41^+$ $AnnexinV^+$ MPs in the medium of coculture at 2 and 20 hrs was assessed; results are reported as $CD41^+$ $AnnexinV^+$ mEVs/ l, all data are shown as scatter dot plots with mean+SEM, n=6-13; **P<0.01 *vs.* HT alone. **(D, E)** Western Blot analysis of 12-LOX and -actin in mEVs isolated from the conditioned medium of HT+PLT **(D)** or from the releasate of thrombin-stimulated platelets**(E)**; platelets (PLT) were loaded into the gels as a positive control for the 12-LOX. **(F)** mEVs isolated from the releasate of thrombin-stimulated platelets (250000 mEVs in 250

of PBS and analyzed for the CFSE fluorescence signal by flow-cytometry **(A, D)** or Amnis imaging flow-cytometry **(B, C). (A)** Flow cytometry dot plots represent CFSE negative (red) and positive (purple) HT29 cells cultured alone or with CFSE-loaded platelets for 20 hr, respectively. **(B, C)** HT29 cell suspension was analyzed by Amnis imaging flow-cytometry for CFSE fluorescence; the representative images of cells which internalized CFSE+ mEVs are reported **(B),** and the percentage of cells with internalized CFSE+ mEVs to total CFSE+ cells are shown **(C)**, all data are shown as scatter dot plots with mean+SEM, n=4**;** **P<0.01 *vs.* HT alone. **(D)** HT29 cells were cocultured with CFSE-loaded platelets in the presence of vehicle (DMSO) or dm-amiloride (250 M) for 20 hrs; at the end of the incubation, cancer cells were harvested and analyzed for CFSE signal by flow-cytometry after trypan blue quenching; data are reported as % of cells with internalized CFSE+ mEVs *vs.* control (HT+PLT, vehicle), all data are shown as scatter dot plots with mean+SEM, n=4, **P<0.01 *vs.* vehicle. **(E, F)** Western blot analysis of 12-LOX in HT29 cells cultured with platelets in the presence of vehicle (DMSO) or dm-amiloride (250 M) for 20 hrs; the optical density of 12-LOX bands was normalized with those of -actin, and results are reported as % of control (vehicle), all data are shown as scatter dot plots with mean+SEM, n=3, *P<0. 05 *vs.* vehicle. (C) Two-way ANOVA with Tukey's multiple comparisons test and (D and F) unpaired t-test (two-tailed) were used.

A



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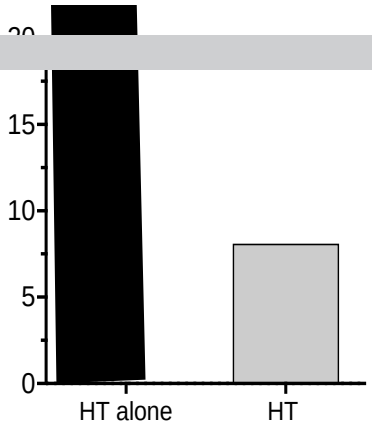
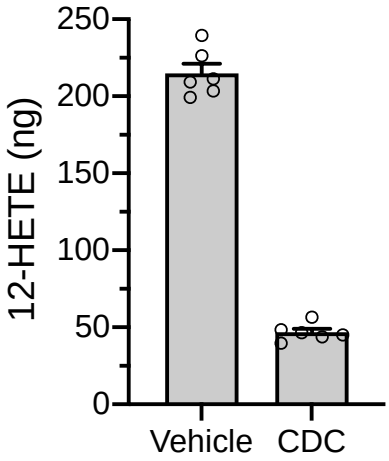


C

Thrombin-an



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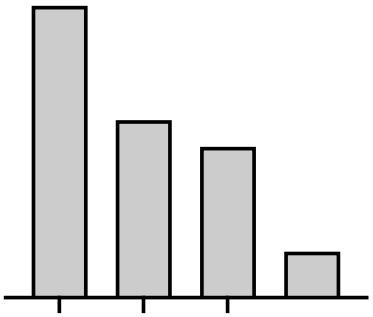
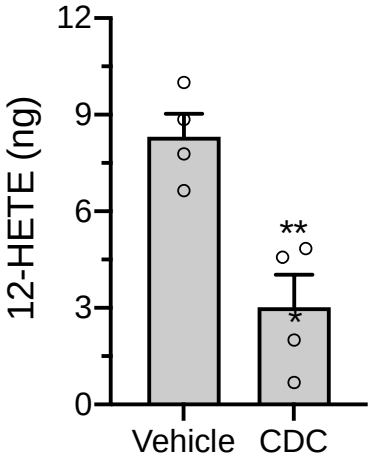


E

HT29 pre-exposed to platelets



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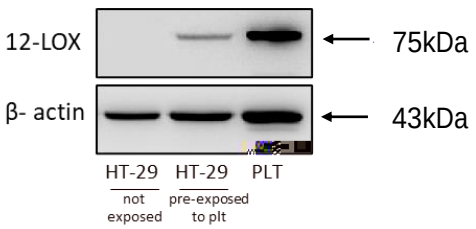

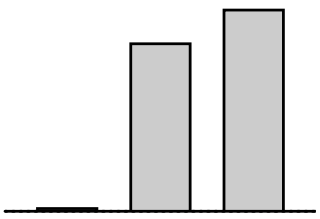

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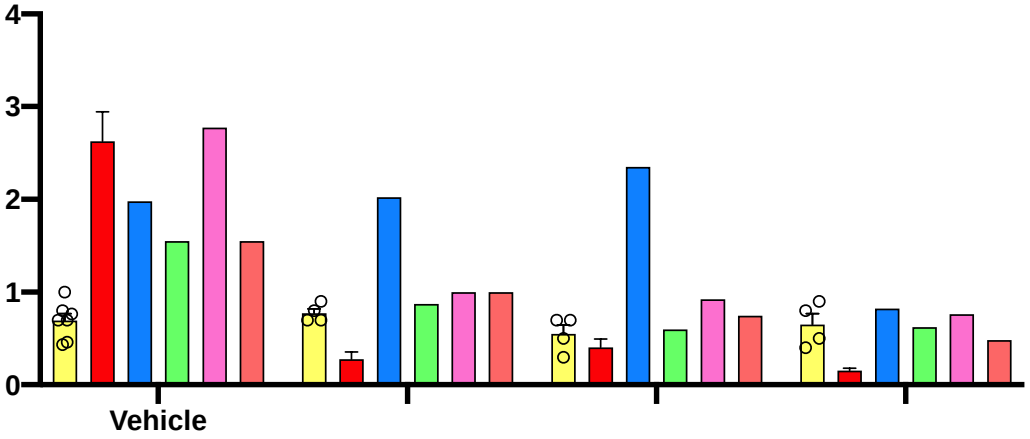

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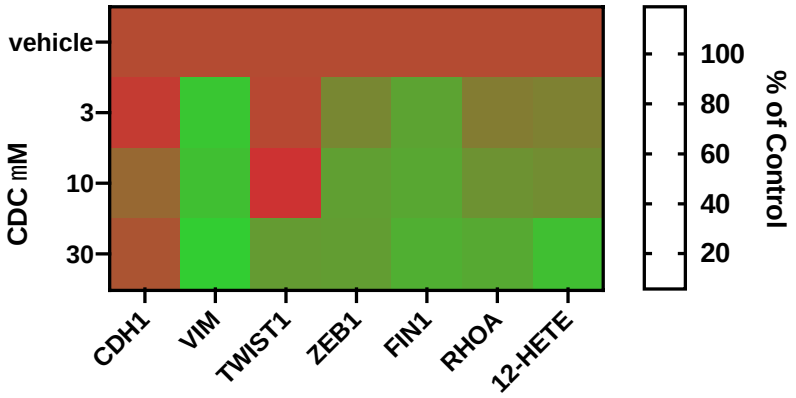


C

D

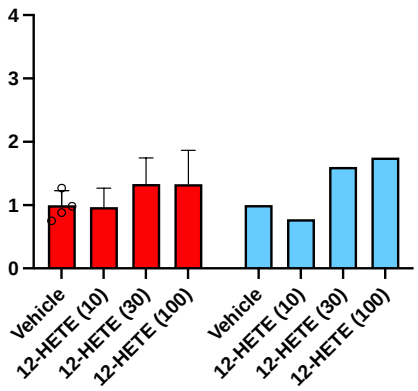
Figure 2

A

B

C

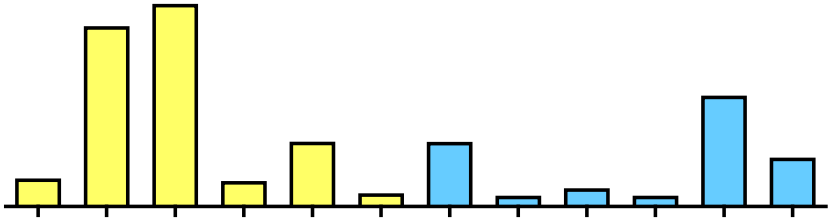
D

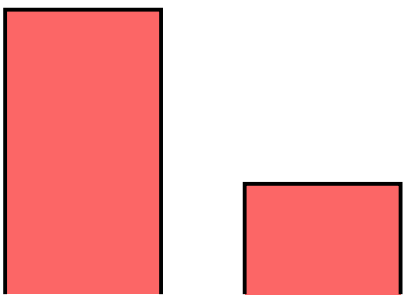

4
3
2
1
0
Vehicle
12-HETE (10)
12-HETE (30)
12-HETE (100)
Vehicle
12-HETE (10)
12-HETE (30)
12-HETE (100)

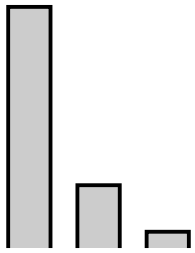
E

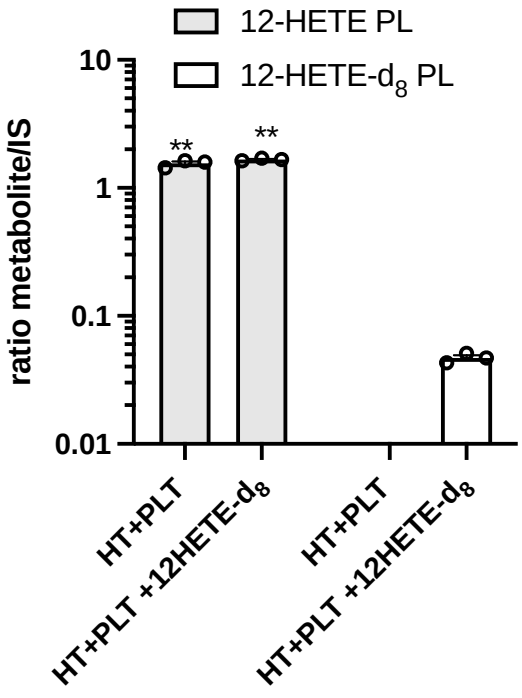
F

G








12-HETE PL
12-HETE-d8 PL
ratio metabolite/IS
10
1
0.1
0.01
**
**
HT+PLT
HT+PLT +12HETE-d8
HT+PLT
HT+PLT +12HETE-d8

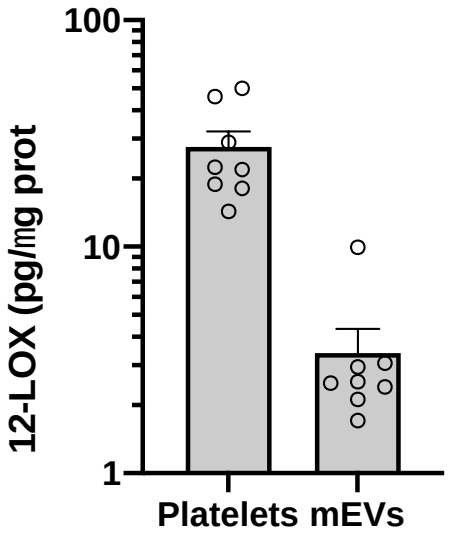
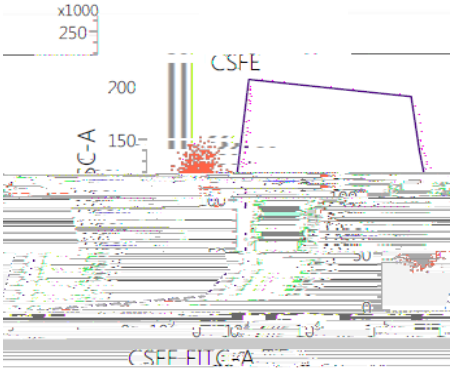
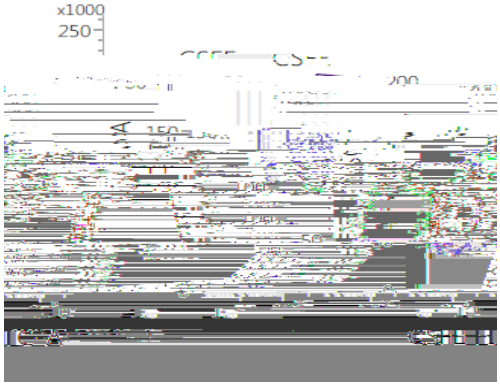
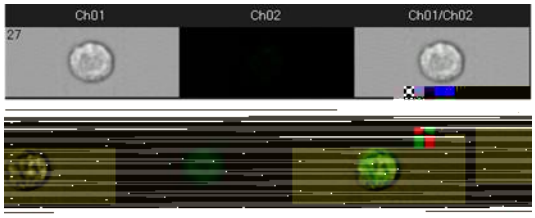
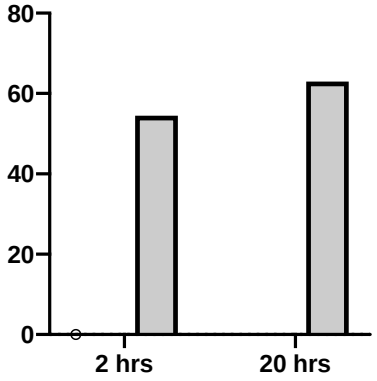


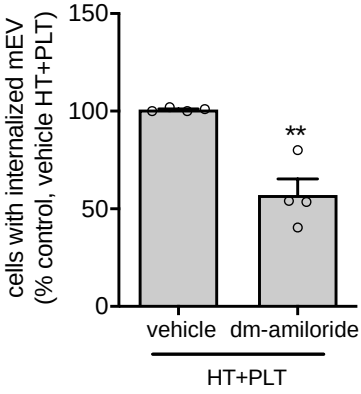
Figure 7

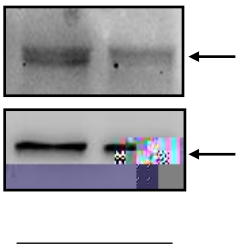

x1000
250
200
150
CSFE
CSFE-FITC-A


x1000
250
200
150


Ch01
Ch02
Ch01/Ch02


80
60
40
20
0
2 hrs
20 hrs


cells with internalized mEV
(% control, vehicle HT+PLT)
150
100
50
0
**
vehicle
dm-amiloride
HT+PLT



Declaration of interests

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