

activation and IL-1 β release (14, 15), besides being involved in T lymphocyte proliferation and differentiation (16). Gorecki and co-workers showed that P2X7R activation triggers release of matrix metallo-proteinase-2 (MMP-2), which in turn cleaves and releases a truncated form of P2X7R itself (17). However, it is not known whether the P2X7R is released into circulation. In addition, a technically easy and affordable laboratory procedure for the measurement of shed P2X7R (sP2X7R) has not been validated yet.

It has been previously reported that full length P2X7 subunits are associated to MVs/MPs released by immune cells (18), or that a C-terminal cleaved form can be shed following MMP-2 activation (17). However, there are no measurement of P2X7R levels in blood. Increased circulating levels of the P2X7R might be associated to inflammation, and possibly be a useful diagnostic or prognostic marker. In the present study, we have used a recently developed commercial ELISA kit to detect sP2X7R in the circulation of healthy controls and of subjects admitted to the S. Anna Hospital, University of Ferrara, with various diagnosis. Blood sP2X7R levels were correlated to C reactive protein (CRP) concentration. Based on previous evidence on the pathophysiological role of the P2X7R (10, 19), patients affected by infections, cancer, ischemia and traumatic and autoimmune diseases were included. Our data also show that the full-length P2X7R was released into circulation in association to plasma membrane-derived MVs/MPs. sP2X7R blood levels were comprised within the 16.74 to 82.17 ng/L, and 33.10 to 1092.30 ng/L range in healthy and diseased subjects, respectively. Determination of sP2X7R blood levels might provide a novel diagnostic procedure to monitor inflammation.

MATERIALS AND METHODS

Sample Collection

Control Blood Samples

Blood samples were obtained from 30 anonymous healthy controls (age range 25–65 years) from the Ferrara blood donor bank. Twenty-six samples were directly collected into EDTA-coated tubes. Plasma was obtained by centrifugation at 4°C for 15 min at 1000xg, and stored at –80°C until use. Freezing/thawing cycles were carefully avoided. Four samples were split into EDTA-coated or EDTA-free tubes to compare sP2X7R levels in plasma or serum from the same subjects. Serum was prepared by allowing blood coagulation for 15 min at room temperature, followed by clot removal at 4°C for 15 min at 1500xg. Each plasma or serum sample was then split into two aliquots, which were stored overnight either at 4° or at –80°C, to check the effect of storage temperature on sP2X7R measurement. The protocol was approved by the Ethical Committee of Ferrara district (Study n. 170891). All subjects gave written informed consent in accordance with the Declaration of Helsinki.

Serum Samples

Eighty-seven anonymous serum samples (39 females and 48 males, mean age 68 years, range 27–94 years), from patients admitted at the S. Anna Hospital (Ferrara) were stratified according to the diagnosis at admission into four groups:

infectious diseases ($n = 42$), cancer ($n = 16$), ischemic heart or brain disease ($n = 10$) and others (trauma and autoimmune diseases) ($n = 19$). The protocol was approved by the Ethical Committee of Ferrara district (Study n. 040408). Sera, kept at 4°C until sP2X7R measurement, were tested within few hours from CRP determination.

Isolation of Microvesicles/Microparticles (MVs/MPs) From Plasma

Eight freshly prepared plasma samples were used to isolate MVs/MPs by centrifugation for 15 min at 2200xg (4°C) to get rid of platelets, followed by centrifugation for 60 min at 14000xg (4°C). In addition, MVs/MPs were also isolated from four plasma and four serum samples by centrifugation for 90 min at 100000xg (4°C) after the initial centrifugation at 2200xg. MVs/MPs enriched pellets and MVs/MPs-deprived plasma and sera were collected and stored at –80°C until analysis by SDS-PAGE and Western blot, or ELISA.

Separation of Platelets and Peripheral Blood Mononuclear Cells (PBMCs)

Platelets were isolated by centrifugation of plasma at 2200xg (4°C) for 15 min. Peripheral blood mononuclear cells (PBMCs) were isolated as previously described (20). Non-adherent cells were removed and the adherent mononuclear cells (monocytes/macrophages) were incubated in 10% FBS-supplemented RPMI under the different conditions described in the figure legend.

Cell Cultures

To validate sP2X7R measurement in serum and plasma, three human cell lines were used: wild type HEK293 cells (wt-HEK293), HEK293 cells stably transfected with the human P2X7R (HEK293-P2X7R), and ACN (human neuroblastoma) cells. wt-HEK293 cells that do not express the endogenous P2X7R were used as negative control, while ACN cells, that over-express the endogenous P2X7R, and HEK293-P2X7R were used as positive controls. wt-HEK293, HEK293-P2X7R and ACN cells were cultured as previously described (21). For ELISA analysis, cell monolayers were scraped off the plate, suspended in PBS at a concentration of 10⁸ cells/ml, and lysed by two freeze-thaw cycles. Cell lysates were centrifuged for 5 min at 5000xg at 4°C and supernatants collected and immediately assayed.

Measurement of sP2X7R by ELISA

sP2X7R was measured in plasma, sera, cell lysates and supernatants from monocyte cultures with the Human P2X purinoceptor 7 (P2RX7) ELISA kit (Cusabio, Houston, TX, USA) following manufacturer's instructions. Optical density was determined at 450 nm by a Multiskan FC spectrophotometer (Thermo Scientific).

SDS-PAGE and Western Blot

For SDS-PAGE/Western Blot analysis, cell lysates, MVs/MPs and platelets were solubilized in RIPA-buffer (150 mM NaCl/0.1% SDS/0.5% Na-deoxycholate/1% Triton X-100/50 mM Tris, pH 7.2). Protein concentration was measured with the Bradford

assay. Ten μg of protein were loaded onto a 4–12% Bis-Tris Nu-PAGE gel (Invitrogen, Life Technologies, Carlsbad, CA, USA). Proteins were blotted for 3 h onto a nitrocellulose membrane (Amersham, GE Healthcare, Chicago, IL, USA). Non-specific binding sites were blocked with 5% skim milk in TBS buffer (10 mM Tris-HCl, 150 mM NaCl, pH 8.0). Nitrocellulose strips were then incubated overnight at 4°C either with a rabbit anti-human P2X7R antibody (Millipore, Burlington, MA, USA, cat n AB5246) diluted 1:600, or with a rabbit anti-human actin antibody (Sigma-Aldrich), diluted 1:1000 in TBS-t buffer (TBS plus 0.1% Tween 20 and 1% BSA). Secondary antibody was a HRP-conjugated goat anti-rabbit IgG (H+L) antibody (BioRad, Hercules, CA, USA) at a 1:3000 dilution in TBS-t buffer. Finally, ECL reagent (Invitrogen, Life Technologies) was used for detection with a LI-COR blot scanner (LI-COR Biosciences, Lincoln, NE, USA).

C Reactive Protein (CRP) Measurement

CRP was measured at the Laboratory Division of the S. Anna Hospital with the immune-turbidimetric kit CRP OSR6147 (Beckman Coulter, Brea, CA, USA).

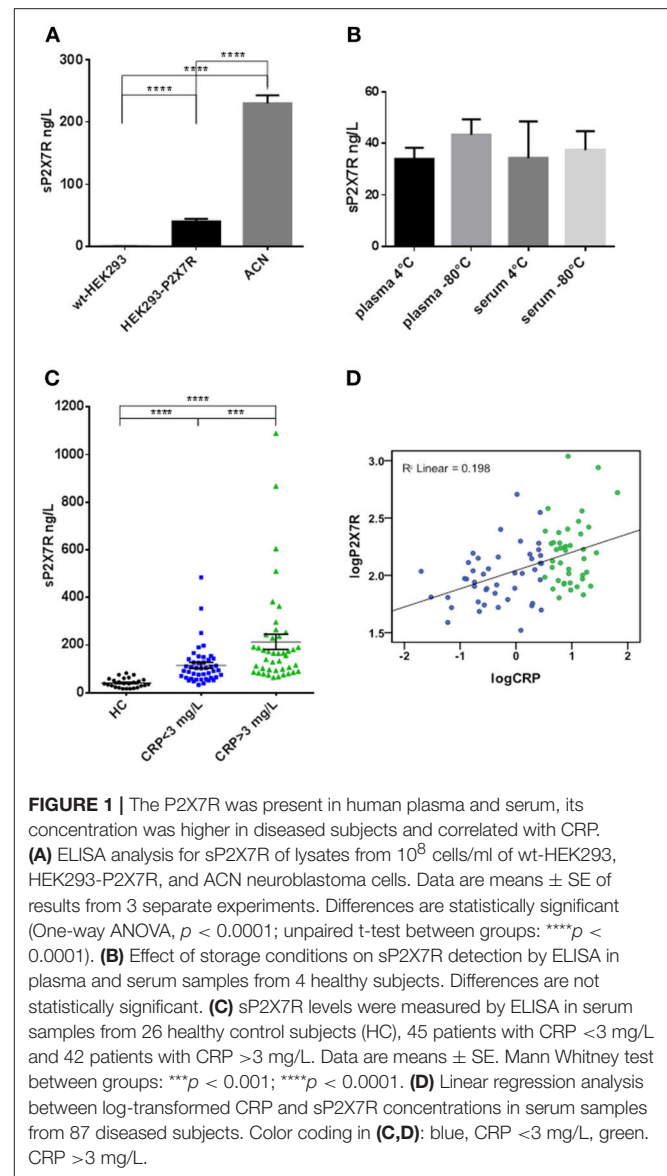
Statistics

Data were expressed as means \pm SE. Variables that did not follow normal distribution were log transformed before statistical analyses. One way ANOVA or Kruskal-Wallis tests were used for comparisons among groups and unpaired *t*-test or Mann-Whitney test, paired *t*-test or Wilcoxon matched-pairs signed rank test were performed for comparisons between groups. Spearman's correlation coefficient was used to test the association between P2X7R, age and CRP. This test was followed by multiple regression analysis in order to check the independence of the observed simple associations. All tests were considered significant for a *p*-value < 0.05 . Statistical analysis was performed using SPSS 22.0 software (SPSS, Chicago, IL, USA).

RESULTS

Validation of the Test: Detection of Shed P2X7R (sP2X7R) in Plasma, Serum and Cell Lysates

Plasma, serum and cell lysate samples were tested for the presence of sP2X7R by ELISA. sP2X7R concentration in plasma from healthy subjects ($n = 26$) ranged from 16.7 to 82.17 ng/L (mean \pm SE 40.97 ± 3.82). Due to high protein content of plasma, sP2X7R detection by Western blot produced unreliable results since most proteins run in the 60 to 80 kDa range, thus overshadowing the P2X7 band. Therefore, specificity of the ELISA assay was validated with cell lysates from wt-HEK293 (P2X7R-null), HEK293-P2X7R, and ACN cells. The assay showed virtually no P2X7R immunoreactivity in wt-HEK293 cells (< 0.2 ng/L), and a robust signal in HEK293-P2X7R and ACN cells, 38 and 220 ng/L, respectively (Figure 1A). No significant difference was observed in sP2X7R levels in plasma or serum samples prepared from the same individuals, whether stored at 4°C or -80°C (Figure 1B).



Blood sP2X7R Levels and Correlation With CRP

In healthy control subjects (HC), sP2X7R was comprised in the 16.7 to 82.17 ng/L range (mean \pm SE = 40.97 ± 3.82 , Figure 1C). We then investigated the correlation with CRP. Eighty-seven anonymous serum samples (39 females and 48 males, mean age 68 years, range 27–94 years) were randomly and anonymously received from the Laboratory Division of the S. Anna Hospital. In these samples, sP2X7R levels ranged from 33.10 to 1092.30 (mean \pm SE = 157.9 ± 16.82), and correlated with age (Spearman correlation Coefficient 0.212, $p = 0.048$) and CRP concentration (Spearman correlation Coefficient 0.412, $p = 0.000$). To further assess the strength of sP2X7R/CRP correlation, we performed a linear regression analysis (Figure 1D) (R^2 linear = 0.198) as well as a multivariate regression analysis, adjusting for sex, age, and

diagnosis sub-group (β -coefficient = 0.431, $p = 0.000$). After this adjustment, R^2 linear slightly increased from 0.198 to 0.213.

Samples were split into 2 groups on the basis of CRP values: 45 samples with CRP < 3 mg/L, and 42 samples with CRP > 3 mg/L. The 3 mg/L cut-off value was chosen as this is the CRP threshold suggestive of systemic inflammation (22). Serum sP2X7R levels in the population identified by CRP > 3 mg/L were about twice higher than those in the < 3 mg/L CRP group, mean \pm SE = 204.2 ± 30.94 ($n=42$), vs. 114.8 ± 12.22 , ($n = 45$), respectively, and significantly different (Mann Whitney test between groups: $p < 0.001$) (Figure 1C).

Samples were then stratified into 4 sub-groups according to diagnosis at admission to hospital: infectious diseases ($n = 42$), cancer ($n = 16$), heart or brain ischemia ($n = 10$), and others (trauma, autoimmune diseases) ($n = 19$), and CRP-sP2X7R correlation in the 4 diagnosis sub-groups analyzed (Figure 2). sP2X7R levels in each of the 4 sub-groups were significantly higher than in healthy controls (Mann Whitney test: $p < 0.0001$). Both CRP and sP2X7R levels were significantly higher in the infectious disease patients vs. the other three subgroups (Figures 2A,B), with the exception of sP2X7R in the ischemia patients (Mann Whitney test: $p = 0.268$). Linear regression

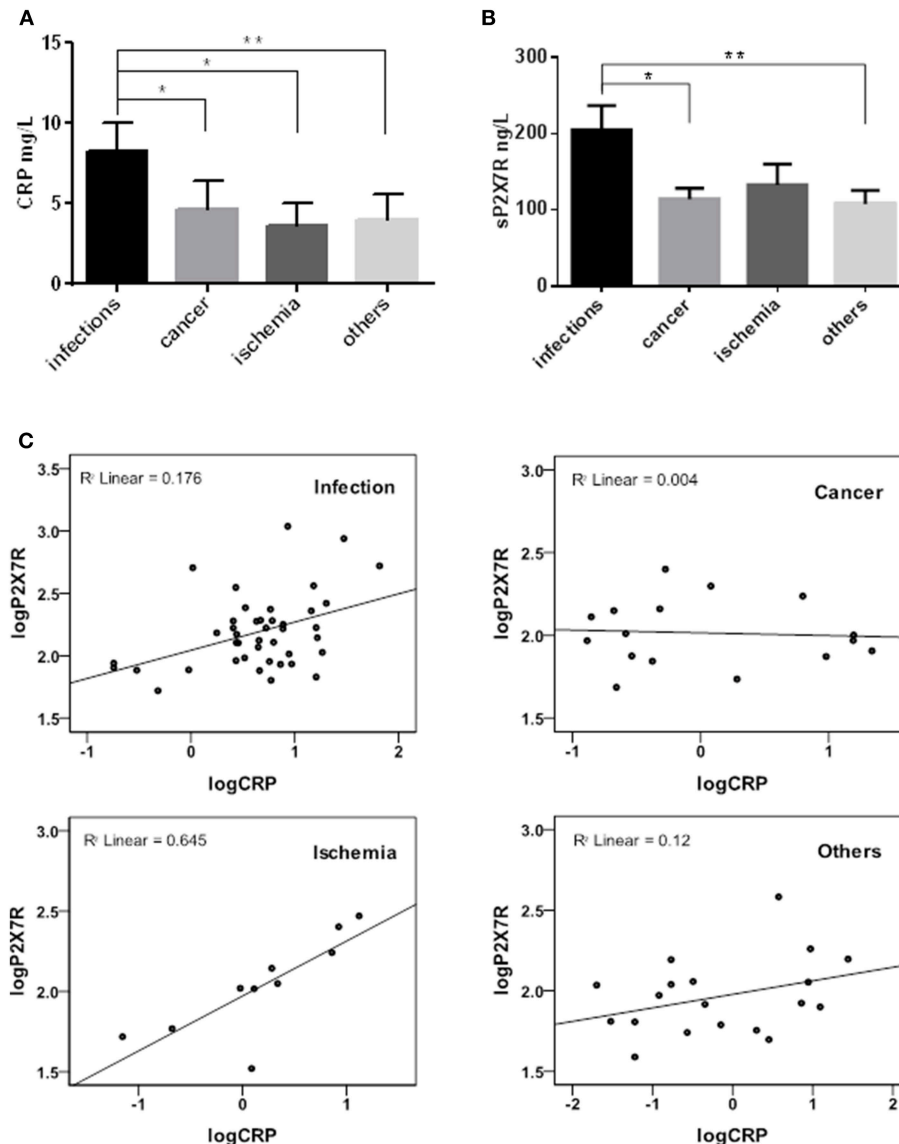


FIGURE 2 | sP2X7R was differentially concentrated in different diagnosis sub-groups. CRP and sP2X7R were measured in serum samples stratified into four sub-groups according to diagnosis at admission to the hospital: infectious diseases ($n = 42$), cancer ($n = 16$), ischemia ($n = 10$) and others (trauma, autoimmune diseases) ($n = 19$). Data are means \pm SE. Only significant differences are shown in the graphs. **(A)** Kruskal-Wallis test $p = 0.0057$; Mann Whitney test between groups: $*p < 0.05$; $**p < 0.01$. **(B)** Kruskal-Wallis test $p = 0.0205$; Mann Whitney test between groups: $*p < 0.05$; $**p < 0.01$. **(C)** Linear regression analysis between log-transformed CRP and sP2X7R in the different diagnosis sub-groups: ischemia, R^2 linear = 0.645; infectious diseases, R^2 linear = 0.176; cancer, R^2 linear = 0.004; others, R^2 linear = 0.12.

analysis showed a strong correlation between log-transformed CRP and sP2X7R in the ischemia subgroup (R^2 linear = 0.645), and a weaker correlation in the infectious disease subgroup (R^2 linear = 0.176), while there was basically no correlation in the cancer (R^2 = 0.004) and “others” (R^2 = 0.12) sub-groups (Figure 2C).

Sources of sP2X7R

A substantial level of sP2X7R was released from blood derived monocytes isolated from healthy subjects under basal conditions (Figure 3A). Challenge with LPS (1 μ g/ml) did not further increase sP2X7R release. On the contrary, BzATP (300 μ M), whether alone or added to LPS-primed cells, triggered a large sP2X7R release. Stimulation of plasma membrane P2X7R by ATP or BzATP is a potent trigger for shedding of MVs/MPs carrying the P2X7R itself (18). To verify the association of sP2X7R with extracellular micro-particulated material, MVs/MPs were isolated according to established protocols from plasma samples of 8 healthy controls, two of which are shown in Figure 3B. Western blot analysis suggests that the sP2X7R was associated to MVs/MPs. MVs/MPs are a heterogeneous population of particles of different sizes and cellular origin, which might also contain platelets or platelet fragments. Therefore, we performed Western blot analysis of MVs/MPs isolated from plasma or serum by an alternative protocol (final centrifugation at 100,000xg). P2X7R immunoreactivity was tested in parallel in purified platelet preparations. As shown in Figure 3C, the P2X7 band was clearly visible in the MVs/MPs fraction, whether from plasma or serum, while only a faint band was visible in platelet fractions. In the MVs/MPs fraction we detected a lower molecular mass band (about 50 kDa) which was also present in HEK293-P2X7 cells, but it is not clear whether this might be a lower molecular mass variant. Two major higher molecular mass bands are also visible in the MVs/MPs preparation and in the platelet lysates, that might however be non-specific. Plasma analysis by ELISA after MVs/MPs deprivation showed a significant reduction of sP2X7R levels (58.03 \pm 5.75 vs. 42.69 \pm 6.82, in whole vs. MVs/MPs-depleted plasma, respectively, Wilcoxon matched pairs test p = 0.0078) (Figure 3D).

DISCUSSION

Extracellular nucleotides, first of all ATP, have relevant roles in a great number of physiological and pathological phenomena (23). The P2X7R is an extracellular ATP-activated key inflammatory receptor expressed at high levels by immune and tumor cells (10, 19). In the immune system, the P2X7R has been shown to drive NLRP3 inflammasome activation, T lymphocyte proliferation and differentiation, generation of reactive oxygen species, release of cell-derived microvesicles/microparticles (MVs/MPs) and precipitation of pyroptotic cell death (10). The P2X7R is a homotrimer made by the assembly of identical P2X7 subunits. P2X7 subunits are integral membrane proteins characterized by a large ecto-domain, and N- and C-terminal residues both on the cytoplasmic side of the plasma membrane. P2X7 subunits could in principle be shed by proteolytic cleavage, as shown by Gorecki and co-workers (17). However, P2X7 subunits might also

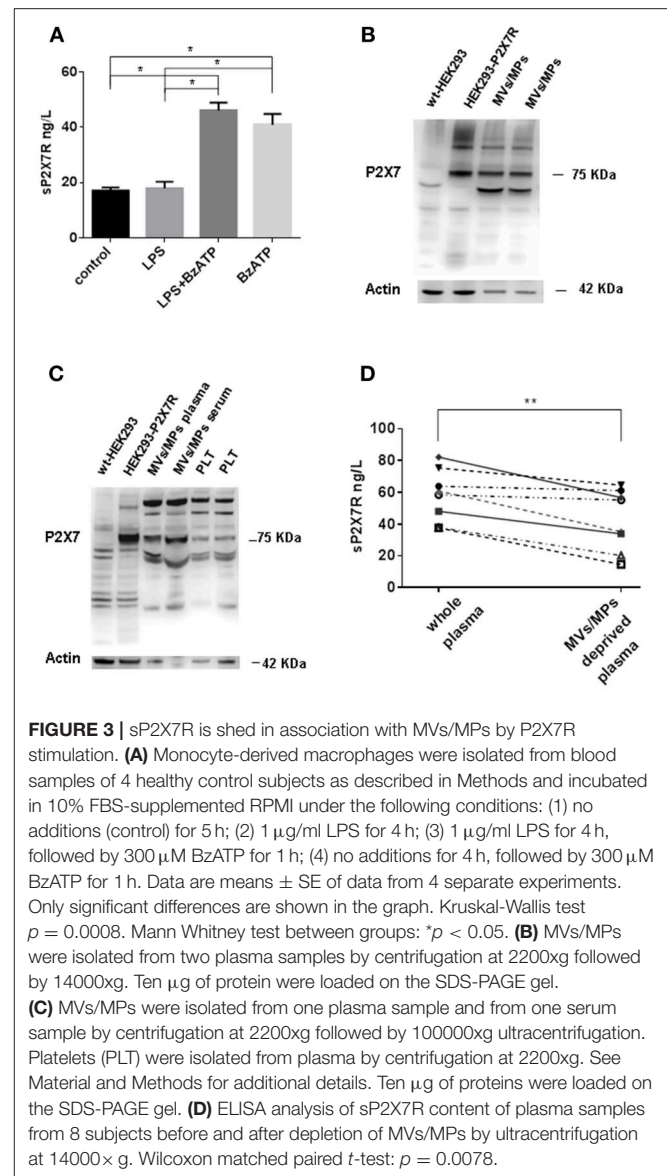


FIGURE 3 | sP2X7R is shed in association with MVs/MPs by P2X7R stimulation. **(A)** Monocyte-derived macrophages were isolated from blood samples of 4 healthy control subjects as described in Methods and incubated in 10% FBS-supplemented RPMI under the following conditions: (1) no additions (control) for 5 h; (2) 1 μ g/ml LPS for 4 h; (3) 1 μ g/ml LPS for 4 h, followed by 300 μ M BzATP for 1 h; (4) no additions for 4 h, followed by 300 μ M BzATP for 1 h. Data are means \pm SE of data from 4 separate experiments. Only significant differences are shown in the graph. Kruskal-Wallis test p = 0.0008. Mann-Whitney test between groups: * p < 0.05. **(B)** MVs/MPs were isolated from two plasma samples by centrifugation at 2200xg followed by 14000xg. Ten μ g of protein were loaded on the SDS-PAGE gel. **(C)** MVs/MPs were isolated from one plasma sample and from one serum sample by centrifugation at 2200xg followed by 100000xg ultracentrifugation. Platelets (PLT) were isolated from plasma by centrifugation at 2200xg. See Material and Methods for additional details. Ten μ g of proteins were loaded on the SDS-PAGE gel. **(D)** ELISA analysis of sP2X7R content of plasma samples from 8 subjects before and after depletion of MVs/MPs by ultracentrifugation at 14000xg. Wilcoxon matched paired t -test: p = 0.0078.

be shed in association with plasma membrane-derived MVs/MPs. Sometime ago, we showed that human dendritic cells stimulated *in vitro* via the P2X7R, release plasma membrane-derived MVs/MPs loaded with various plasma membrane molecules, among which P2X7 subunits (18). Based on immunoreactivity with an antibody raised against the C-terminal tail of the P2X7 subunit, we concluded that this subunit was released as the full-length variant. In the present study, we show that also MVs/MPs isolated from peripheral blood carry the P2X7 subunit as the full-length variant. We don't know if in association with MVs/MPs the P2X7R is present as the trimeric receptor (i.e., P2X7R) or as individual subunits (i.e., P2X7), although previous data showing that dendritic cell-released MVs/MPs are lysed by added ATP (18) suggest that it might be present as P2X7R. Since Western Blots are obviously impractical for routine identification of plasma/serum

components, we verified whether a recently made available commercial ELISA kit was suitable for measurement of P2X7R as a shed (sP2X7R) molecule. Our data support the validity of ELISA measurement of sP2X7R in blood as concentrations in plasma and serum were almost perfectly superimposable, and did not differ whether measured in samples kept at 4°C or frozen at -80°C. P2X7R concentration was comprised in the 16.7 to 82.17 ng/L and 33.1 to 1090.00 ng/L range in healthy and diseased subjects, respectively. The ELISA kit also provided an accurate measurement of sP2X7R in cell lysates. Although limited information was available on the epitope specificity of the antibodies in the P2X7-ELISA kit, ability to recognize MVs/MPs-associated P2X7R suggest that they should be directed against epitopes exposed on the outer domain of the receptor, as per manufacturer indication.

The full length molecule was detected in blood, but we cannot exclude that some P2X7R was shed following proteolytic cleavage (17). The extracellular ATP concentration can reach the hundred micromolar level at inflammatory sites (24), i.e., sufficient to activate even the low affinity P2X7R and trigger sP2X7R release. sP2X7R levels were significantly lower in healthy vs. diseased subjects, especially in the patient cohort with CRP >3 mg/L. In this cohort, only 6 out of 42 subjects showed sP2X7R serum levels overlapping with the range of healthy subjects. In the cohort with CRP <3 mg/L, 19 out of 45 patients showed P2X7R levels within the healthy subject concentration range. All diagnosis cohorts screened in this study showed P2X7R serum levels significantly higher ($p < 0.0001$) than healthy subjects, suggesting that P2X7R might be a non-specific disease marker. We found that correlation of sP2X7R blood levels with CRP was widely different depending on the disease. Best correlation was found in patients suffering of ischemia (R^2 linear = 0.645), while the lowest correlation was found in patients with cancer (R^2 linear = 0.004). Infectious patients showed an intermediate correlation (R^2 linear = 0.176). Since all these diseases are accompanied by local or systemic inflammation, and therefore in principle by CRP release and P2X7R activation, these data suggest that depending on the etiology, different inflammatory conditions may promote a differential level of both CRP and sP2X7R release, and ischemia is the disease condition where release of both biomarkers is matched. P2X7R measurement might complement that of CRP in the differential diagnosis of inflammatory conditions of different etiology (i.e., infectious vs. non-infectious). Most circulating cells express the P2X7R and release MVs/MPs, thus it is likely

that there are multiple sources of sP2X7R in blood and vessels, endothelial cells included.

Although not routinely used in laboratory practice, blood IL-1 β levels are of potential usefulness in the diagnosis of inflammatory diseases (25). sP2X7R determinations might complement and strengthen the diagnostic significance of IL-1 β measurements.

At this early stage, the pathophysiological significance of P2X7R shedding is unknown. It might be a mere epiphenomenon of inflammation, but we cannot exclude that fusion of MVs/MPs might transfer the P2X7R to other immune cells at sites distal to the original inflammatory site.

In conclusion, we have shown that the P2X7R is released into the blood in association to MVs/MPs, and that its levels are significantly higher in diseased subjects.

ETHICS STATEMENT

The protocols were approved by the Ethical Committee of Ferrara district (Studies n. 170891 and 040408). All subjects gave written informed consent in accordance with the Declaration of Helsinki.

AUTHOR CONTRIBUTIONS

FDV projected this work, planned experiments and wrote the manuscript. ALG was responsible for most of the experiments and for drafting and editing the manuscript. MB collected samples, stratified patients' samples according to diagnosis and performed some of the experiments. JMS was responsible for statistical analysis. AP helped in projecting this work and in the statistical analysis. SP collected samples and stratified patients' samples according to diagnosis. VV-P and ACS performed some of the experiments.

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Conflict of Interest Statement: FDV is a member of the Scientific Advisory Board of Biosceptre Ltd., a UK-based biotech Company involved in the development of P2X7R-targeted therapeutics.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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