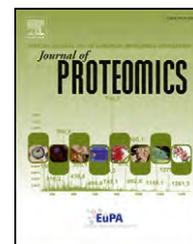


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Proteomic analysis of Intercept-treated platelets[☆]

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ABSTRACT

In the past decades, transfusion medicine has been driven by the quest for increased safety against transfusion-transmitted infections, mainly by better donor selection and by the development of improved serological and nucleic-acid-based screening assays. Recently, pathogen reduction technologies became available and started to be implemented in several countries, with the primary goal to fight against bacterial contamination of blood products, a rare but dramatic event against which there was no definitive measure. Though pathogen reduction technologies represent a quantum leap in transfusion safety, the biomedical efficacy of platelet concentrates (PCs) treated with various pathogen reduction techniques has been recently questioned by clinical studies. Here, a gel-based proteomic analysis of PCs ($n=5$), Intercept-treated or untreated, from pooled buffy-coat (10 donors per PC) at Days 1, 2 and 8, shows that the Intercept process that is the most widespread pathogen reduction technique to date, has relatively low impact on the proteome of treated platelets: the process induces modifications of DJ-1 protein, glutaredoxin 5, and G(i)alpha 2 protein. As for the impact of storage, chloride intracellular channel protein 4 (CLIC4) and actin increased independently of Intercept treatment during storage. Whereas alteration of the DJ-1 protein and glutaredoxin 5 points out an oxidative stress-associated lesion, modification of G(i)alpha2 directly connects a possible Intercept-associated lesion to haemostatic properties of Intercept-treated platelets.

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1. Introduction

For several decades, transfusion science has been driven by the increase of patient's safety in terms of compatibility and transfusion-transmitted infections (TTIs). Through years, remarkable progress has been made in preventing TTIs by better donor screening through questionnaire and exclusion rules, diversion of the first milliliters of blood to prevent bacterial contamination from the donor skin, extensive screening of all blood donations by serology and nucleic acid testing (NAT) against HIV, HBV, and HCV. The level of safety or residual risk of TTIs depends on local epidemiology, and

implementation of serological and NAT tests (either in minipools or in individual donations, for example). In Switzerland, the residual risks for HBV are estimated as 1:141,000, HCV 1:2,200,000, and HIV 1:2,600,000, which is well in line with figures from other countries [1–3].

However, substantial TTI risks still exist in transfusion: on one part, emerging pathogens such as West Nile Virus [4], Chikungunya, or *Trypanosoma cruzi* repeatedly appear in non endemic regions, such as Northern Italy for Chikungunya [5], or recently Southern France for Dengue [6,7]; additionally, new vectors are regularly imported: for example there are currently outbreaks of mosquito colonization such as *Aedes*

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japonicus in two urban regions of Switzerland [8], and three potentially problematic mosquito species in the Netherlands [6]. In parallel to an increase potential of emerging pathogens outbreak due to migration and travels, goods exchange and climate changes, there are well-known risks of bacterial contamination of blood products that can be potentially lethal to the recipient. In this respect, bacterial contamination of platelet concentrates (PCs) is of crucial importance, because platelets are stored in 30–100% plasma at room temperature, conditions that promote bacterial growth [9–16]. In Switzerland, the risk for bacterial contamination of PCs was estimated as 1:15,000 transfusions, one of the highest in developed countries [3].

Recently, the Swiss regulatory authority Swissmedic decided to introduce the Intercept technique to reduce pathogens in PCs all-over the country, with the main objective to eradicate bacterial contamination of PCs, and secondary objective to provide some protection of the blood supply chain to emerging pathogens. The Intercept process (Cerus, Concord, USA) is a photochemical process during which Amotosalen hydrochloride (CAS: 161262-45-9), an aminoethoxymethyl derivative of psoralens, docks on DNA and RNA and forms inter- or intramolecular covalent bonds upon UV-A illumination [17,18]. After UV-A illumination, residual Amotosalen is removed by adsorption to guarantee a final concentration in the final PC below 2 μM [19]. The process proved effective in reducing pathogen infectivity by 4–6 logs for a large range of bacteria, viruses and parasites. The Intercept process was chosen in Switzerland because it was the only one approved by the regulatory authorities, but there are two other pathogen reduction processes under development/validation: first the Mirasol process is based on the addition of riboflavin to the platelet suspension followed by a mixed UVA–UVB illumination to induce permanent lesions to DNA [20]; alternatively, the Theraflex–UV process uses the germicidal properties of UVC alone to reduce pathogens [21].

Intercept-treated platelets proved to be safe from a toxicological [22] and immunological points of view [23], and proved to be efficient in maintaining haemostasis *in vitro* [24] as well as in all types of patients needing platelet support [25–28]. Whereas Intercept-treated platelets are in routine use in several European regions without any increase in product use [25–28], some recent clinical studies have shed some doubts on the haemostatic efficacy of Intercept-treated platelets compared to non-treated ones. The HOVON study showed that transfusion of Intercept-treated platelets resulted in lower corrected count increments (CCI) at 1 h and 24 h compared to non-treated platelets, and increased bleeding scores in patients receiving Intercept-treated platelets [29]. Whereas this study has been criticized mainly because it was non-randomized, non-blinded, and because bleeding scores were not assessed by trained clinical research personnel, it stimulated a strong debate about the use of Intercept-treated platelets [30,31]. Vamvakas further conducted meta-analyses of clinical studies involving Intercept-treated platelets by including data of randomized, blinded studies (i.e. not including the HOVON study) and concluded that Intercept-treated platelets indeed result in increased bleeding scores of moderate gravity, but that there is no modification of high severity bleeding scores compared to non-treated platelets [32,33]. This analysis has also been the subject of hot debate [34]. The situation of the debate, to the

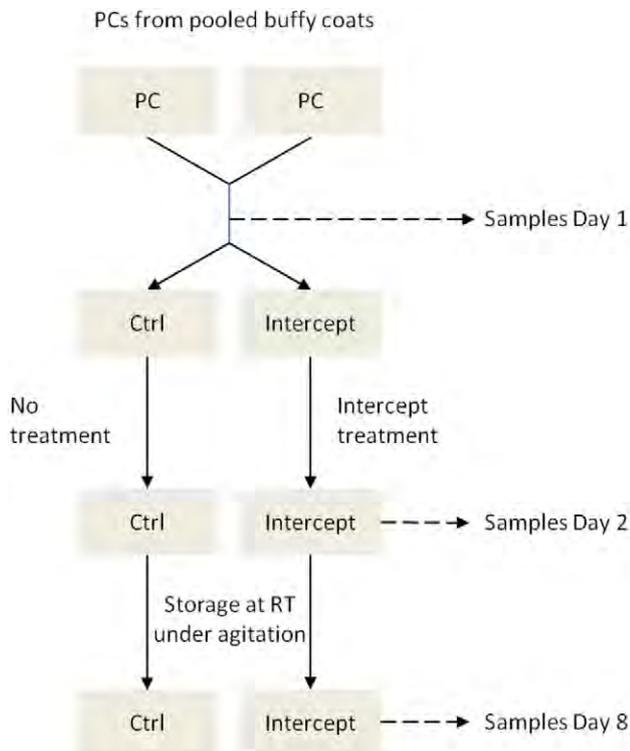
best of our understanding, is that there is a consensus from clinical studies that Intercept-treated platelets have a somewhat diminished haemostatic activity as demonstrated by CCIs and bleeding scores; on the other hand, retrospective haemovigilance data from places where Intercept is in routine use do not show any increase in product use nor in bleeding events [26,27].

The gist of the present study was to compare Intercept-treated platelets with non-treated ones by proteomic analysis in order to evaluate if the Intercept treatment has any detrimental effect at the protein level in PCs.

2. Material and methods

2.1. Samples and reagents

All samples used in the experiments described below came from whole blood units of regular donors who gave their consent for research use of their blood components. For each experiment, two ABO-matched PCs were prepared by the blood component preparation laboratory of our blood bank by the Buffy coat technique: briefly, whole blood units (450 ± 50 mL supplemented with 63 mL CPD) were kept at 22 °C overnight, and centrifuged at 3500 *g* for 14 min to separate RBCs and plasma, which were expressed on an Optipress II (Fenwal, Lake Zurich, IL, USA). Five buffy coats (eligible for platelet production, i.e. from donors who did not take any non steroid anti-inflammatory drug) that would otherwise have been trashed were pooled with 280 mL of Intersol additive solution (Fenwal, Lake Zurich, IL, USA), and centrifuged at 500 *g* for 10 min. Platelets were expressed on a manual plasma extractor (4R4414, Fenwal, Lake Zurich, IL, USA), and further filtrated to remove leukocytes (filtration kit R7013, Fenwal, Lake Zurich, IL, USA). Resulting PCs had a volume of 330 mL with 39% plasma and 61% Intersol additive solution and a platelet content of $3.7 \pm 0.1 \cdot 10^{11}$ platelets per bag. Two ABO-matched PCs were pooled and split to yield two strictly identical products, each of those coming from 10 donors (see Scheme 1). One of these pooled PCs was kept in standard blood banking conditions up to 8 days after collection (container of kit R7013, Fenwal, Lake Zurich, IL, USA, 1300 mL, polyolefin plastic PL2410, under agitation at 22 °C). The other pooled PC was processed with the Intercept kit large volume (INT2203B, Cerus, Concord, USA): 17.5 mL of Amotosalen hydrochloride 3 mM solution was added sterilely to the PC, and the mixture was further illuminated with the Intercept illuminator (INT100, Cerus, Concord, USA) to yield 3.9 J/cm². After illumination, the platelet mixture was transferred to the Compound Adsorbing Device container and kept under agitation for 14 h to remove residual Amotosalen. The treated PC was then transferred to the final storage bag (1300 mL, polyolefin PL2410 plastic, under agitation at 22 °C). The same experiment was performed on 5 different preparations (i.e. 10 PCs were pooled by pairs, each one coming from five different donors, amounting for 50 individual blood donations, which enables to diminish biological variability). The characteristics of PCs, i.e. platelets, leukocytes and erythrocytes concentrations, are reported in Table 1.



Scheme 1 – Workflow for proteomic analysis of Intercept-treated platelet. Two PCs from pooled buffy coats (5 donors per PC) are mixed and split. One of these PCs is stored in standard blood banking conditions as control and the other one is treated with the Intercept system. PC: Platelet concentrate, Ctrl: control PC untreated, Intercept: Intercept-treated PC.

2.2. Two-dimensional gel electrophoresis and image analysis

Samples were taken for proteomic analysis at three time points (see Scheme 1): before Intercept treatment (Day 1), after Intercept treatment (both in the treated PC and in the control one, Day 2), and after storage; because of logistical constraints, sampling at the end of storage was performed at Day 7 for sample 1 and Day 8 for all other samples.

Three aliquots of 1 mL platelet solution were centrifuged for 10 min at 2370 g at RT and each washed twice with 1 mL NaCl 0.9%. The three samples were pooled in 3 mL NaCl 0.9%, and platelets were counted by an automated haematology analyser (Sysmex KX-21N, Sysmex Digitana, Horgen, Switzerland), and total proteins were measured by the BCA technique. Each platelet sample contained $919 \pm 116 \cdot 10^9$ platelets/L and 0.44 ± 0.08 mg/mL of proteins. Samples were aliquoted at 50 or 200 μ g

total protein (see below), centrifuged at 14,000 g for 10 min at 4 °C, and the pellet was suspended in 350 μ L of a solution composed of 7 M urea, 2 M thiourea, 2% CHAPS, 10 mM DTE and 0.5% v/v Pharmalytes pH 4.0–7.0 (30 min at room temperature under agitation) for two dimensional gel electrophoresis (2D-GE) analysis.

Isoelectric focusing (IEF) was performed under paraffin oil, using linear IPGs (Immobiline Dry-Strip, pH range 4–7, 18 cm, GE Healthcare). The strips were rehydrated overnight in 340 μ L of a solution containing 8 M urea, 2% CHAPS, 10 mM DTE, 2% Pharmalytes pH 3–10 (GE Healthcare), and traces of bromophenol blue. The sample (either 50 μ g for image analysis or 200 μ g for mass spectrometric identification) was then loaded on the gels. The voltage was progressively increased from 300 to 3000 V during the first 3 h, followed by 1 h at 3500 V, and finally stabilized at 5000 V for a total of 100 kWh. The strip was then equilibrated during 12 min in the equilibration solution supplemented with 2% DTE, followed by a 5 min incubation under agitation in the equilibration solution supplemented with 2.5% iodoacetamide. The strip was then transferred on top of a 9–16% PAGE. Second dimension was run in a Biorad large gel electrophoresis tank with a current of 40 mA/gel (2000 V, 240 mA, 300 W) during 4 to 6 h.

Ammoniacal silver staining was done according to standard protocols [35–37]. Briefly, at the end of the run, the gels were washed in deionized water, then soaked in ethanol: acetic acid:water (40:10:50, v/v/v) for 1 h and ethanol:acetic acid:water (10:5:85, v/v/v) overnight. After a water wash, the gels were soaked for 30 min in glutaraldehyde (1%) buffered with sodium acetate (0.5 M) and the glutaraldehyde was removed by deionized water washes. The gels were then soaked in a fresh solution of 2,7-naphthalenedisulfonic acid fresh solution (0.05% w/v) for 30 min and rinsed again with deionized water. The gels were stained in a freshly made ammoniacal silver nitrate solution for 30 min and then rinsed with deionized water. The images were finally developed in a solution containing citric acid (0.01% w/v) and formaldehyde (0.1% w/v). Development was stopped with an acetic acid: water (5:95, v/v) solution. Alternatively, for mass spectrometric identification, gels were stained with colloidal coomassie blue [38]. All incubations were performed on an orbital shaker.

The silver-stained or coomassie-stained gel images were captured with high resolution densitometry reading with the Personal Laser Densitometer (GE Healthcare). Using Melanie software Version 6.0 (GeneBio, Geneva, Switzerland), gels were manually compared by pairs (for example Day 2 Intercept versus Day 2 control, or Day 8 versus Day 2 sample) in large zooming mode over the entire gels. Whenever differences were visually identified, spots were matched and spot volumes were computed in all gels to extract histograms.

2.3. Statistics

SigmaStat 3.10 software 355 (SYSTAT Software Inc., Point Richmond, CA, USA) was used to evaluate the significant evolution of protein abundance (spot intensities in %volume). Spot numbers 1524, 1518, 1776, 1784, and 1644 were analyzed using the One Way Analysis of Variance whereas t-test between Days 1/2 and Day 8 of storage for spot numbers

Table 1 – Characteristics of platelet concentrates.

	Platelet/bag	Leukocyte/bag	RBC/bag
Specifications	$>2.4 \cdot 10^{11}$ ($>2.5 \cdot 10^{11}$ before treatment)	$<1.0 \cdot 10^6$	$<1.3 \cdot 10^9$
Measured (n=50)	$3.3 \pm 0.1 \cdot 10^{11}$ ($3.8 \pm 0.1 \cdot 10^{11}$ before treatment)	$0.07 \cdot 10^6$	$0.2 \cdot 10^9$

1361 and 1386 was considered (see reports in Supplementary Information, SI).

2.4. Western-blot analysis

For 2D-Western blot analysis, isoelectric focusing on 11 cm strips and equilibration steps were performed as described above, and second dimension was performed on precast 4–12% gels (Invitrogen, Carlsbad, CA, USA).

2.5. Mass spectrometry

Protein spots from 2D-GE were digested with trypsin according to a described protocol [39,40]. Tryptic peptides were recovered in the supernatant of the digestion, dried by evaporation, reconstituted in 20 μ L of H₂O:ACN 97:3 v/v with 0.1% formic acid and analyzed by LC-MS/MS with an Agilent 1100 nano HPLC system (Agilent Technologies, Waldbronn, Germany). The chromatographic system was interfaced to an LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) through a TriVersa Nanomate (Advion Biosciences, Ithaca, NY, USA). Samples were loaded onto a trapping microcolumn ZORBAX 300SB C18 (5 mm \times 300 μ m id, 5 μ m, Agilent Technologies) in H₂O:ACN 97:3 v/v+0.1% formic acid at a flow rate of 10 μ L/min. After 5 min they were back-flush eluted and separated on a reversed-phase nanocolumn ZORBAX 300SB C18 column (75 μ m id \times 15 cm, 3.5 μ m, Agilent Technologies). Solvents used for the mobile phase were 95:5 H₂O:ACN v/v with 0.1% formic acid (A) and 5:95 H₂O:ACN v/v with 0.1% formic acid (B). The flow rate was 300 nL/min and the gradient used lasted 20 min. In data-dependent acquisition controlled by Xcalibur 2.0 software (Thermo Fisher Scientific), the six most intense precursor ions detected in the full MS survey performed in the LTQ Orbitrap (range 350–1500 m/z, resolution 60,000 at m/z 400) were selected and fragmented. MS/MS was triggered by a minimum signal threshold of 10,000 counts, carried out at relative collision energy of 35% and with isolation width of 4.0 u. Only precursors with a charge higher than one were selected for CID fragmentation and fragment ions were analyzed in the linear trap. The m/z of fragmented precursors was then dynamically excluded, with a tolerance of 0.01 u, from any selection during 120 s.

Tandem mass spectra were exported as mgf files (Mascot Generic File, text format) using Mascot Distiller 2.3.2 (Matrix Science, Boston, MA, USA). Charge state deconvolution and deisotoping were not performed. All MS/MS samples were analyzed using Mascot (Matrix Science, London, UK; version Mascot). Mascot was set up to search the sptrembl_15-12_ database (selected for Homo sapiens, 97055 entries) assuming the digestion enzyme trypsin. Mascot was searched with a fragment ion mass tolerance of 0.50 Da and a parent ion tolerance of 10.0 ppm. Iodoacetamide derivative of cysteine was specified in Mascot as a fixed modification. Deamidation of asparagine and glutamine, oxidation of methionine and acetylation of the N-terminus were specified in Mascot as variable modifications.

Scaffold (version Scaffold_3_00_08, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability as

specified by the Peptide Prophet algorithm. Protein identifications were accepted if they could be established at greater than 99.0% probability and contained at least 2 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony.

3. Results

3.1. New platelet proteins identified

Fig. 1 shows a typical 2D-GE map of platelet proteins. There was no difference in the number of spots seen on 2D maps between the analyzed conditions (see Table 2); the number of spots detected is well in line with the literature for 2D-GE of platelets stained with silver in various conditions: for example, the reference map of platelets in the Swiss-2D-PAGE database contains 2193 spots on a pH 3.5–10 range.

In the course of our study, we identified proteins (not necessarily from the spots that show variations of intensity in the studied conditions) that have not been reported to be present in platelets, to the best of our knowledge [41,42]: glutaredoxin 5 (gene GLRX5, <http://www.uniprot.org/uniprot/Q86SX6>), Replication protein A 14 kDa subunit (gene RPA3, <http://www.uniprot.org/uniprot/P35244>), GRIP1-associated protein 1 (gene GRIPAP1, <http://www.uniprot.org/uniprot/Q4V328>), HD domain-containing protein 2 (gene HDDC2, <http://www.uniprot.org/uniprot/Q7Z4H3>), Histone H2B type 1-B (gene HIST1H2BB, <http://www.uniprot.org/uniprot/P33778>), Protein BRICK1 (gene BRK1, Uniprot AC Q8WUW1). The identifications are shown in SI Figure S1.

It has to be noticed that the PCs were centrifuged and filtered for leukoreduction during the production process. In these conditions, the contamination by leukocytes and red blood cells is 7 orders of magnitude and 3 orders of magnitude lower than the platelet concentration, respectively. The contamination is thus very weak and the proteins identified certainly belong to platelets.

3.2. Effect of Intercept treatment

Direct comparison of 2D-GE maps of Intercept-treated platelets versus controls at Day 2 (after completion of the Intercept treatment) identified three spots that were significantly modified upon Intercept treatment: as shown in Fig. 2, spot 1524 increases upon Intercept treatment, a difference that persists throughout storage (3-fold increase at Day 2 compared to control, $p=0.003$, 4-fold increase at Day 8 compared to control, $p<0.001$, whereas controls remain unchanged). Mass spectrometry identified spot 1524 as protein DJ-1, as shown in Fig. 3 and in Table S1. Whereas some other proteins were identified in spot 1524 (see Table S1), mass spectrometric semi-quantitative analysis shows that DJ-1 is by far the most abundant protein of the spot (quantitative value of 51 over 8 for second most abundant protein). Because it is well known that protein DJ-1 can undergo cysteine oxidation and shift in pI on 2D-GE [43,44] [45,46], we performed Western blot analysis of DJ-1 protein on 2D-GE; as shown in Fig. 4, oxidized DJ-1 (spot 1524) accounts for

10% of total DJ-1 spots at Day 2 in controls, whereas it accounts for 22% of total DJ-1 in Intercept-treated platelets at Day 2. This difference is further increased at Day 8, where oxidized DJ-1 accounts for 28% (respectively 42%) in control platelets (respectively in Intercept-treated platelets). Unfortunately, due to our sample preparation workflow where proteins are reduced and alkylated after the first dimension of separation (isoelectric focusing), our mass spectrometric data cannot confirm that spot 1524 corresponds to oxidized DJ-1; only similarity of 2D-GE Western blots with those published in the literature allows us to identify spot 1518 as native, non-oxidized DJ-1 protein, and spot 1524 as reversibly oxidized DJ-1 protein [45].

Spot 1776 is increased at Day 2 upon Intercept treatment compared to control ($p=0.002$), a difference that persists throughout platelet storage (even though the significance is at the limit, $p=0.054$, the difference remains clear because of the absence of spot 1776 in untreated samples—except for one gel), as shown in Fig. 5, in direct opposition with spot 1784, that complementarily decreased upon Intercept treatment (the spot intensity is below the limit of detection in Intercept treated samples). Mass spectrometry identified primarily Guanine nucleotide-binding protein G(i) subunit alpha-2 (G(i)alpha2), glutaredoxin 5, and Galectin in spot 1776, whereas glutaredoxin 5 was also identified in spot 1784. Glutaredoxin 5 was identified from the same peptides without any documented modification in both spots. Only one additional amino acid was covered by the mass spectrometric identification of glutaredoxin 5 in spot 1784 due

to the miscleavage in peptide (K)LGIHSAALLDEKK(D). Unfortunately, our MS data does not allow to explain the molecular differences in the two spots; however, Johansson et al. showed that apo-glutaredoxin 5 purification from recombinant constructs expressed in *E. coli* yields three different species of masses 13,469 Da, 13,158 Da, and 12,851 Da, attributed by authors to singly glutathionylated, doubly glutathionylated and non-glutathionylated glutaredoxin with an intramolecular disulfide bond [47]. Again, the sample preparation workflow used here only allows to speculate about potential glutathionylation of glutaredoxin 5 in the more acidic spot 1776. Interestingly, the most abundant protein of spot 1776 is G(i)alpha-2 (see Table S1), followed by Galectin-1. We were able to confirm by Western blotting that Galectin-1 does not vary in intensity between control and Intercept-treated platelets (data not shown), which leaves as main candidates G(i)alpha2 and glutaredoxin 5 to account for the change in spot intensity. Whereas the complementarity of intensity variation between spots 1776 and 1784 pleads for a modification of glutaredoxin 5, it is interesting that G(i)alpha2 be present at this position on the gel: the whole protein G(i)alpha 2 has a mass of 40,320 Da whereas it appears in a region of mass 12–16 kDa on the gel. Mass spectrometric identification covers the C-terminal region 250–346 of the protein, which means that only a C-terminal fragment of the protein is detected. Given the mass range in which the spot is found, this fragment might not be much longer than the 250–355 (Fig. 6).

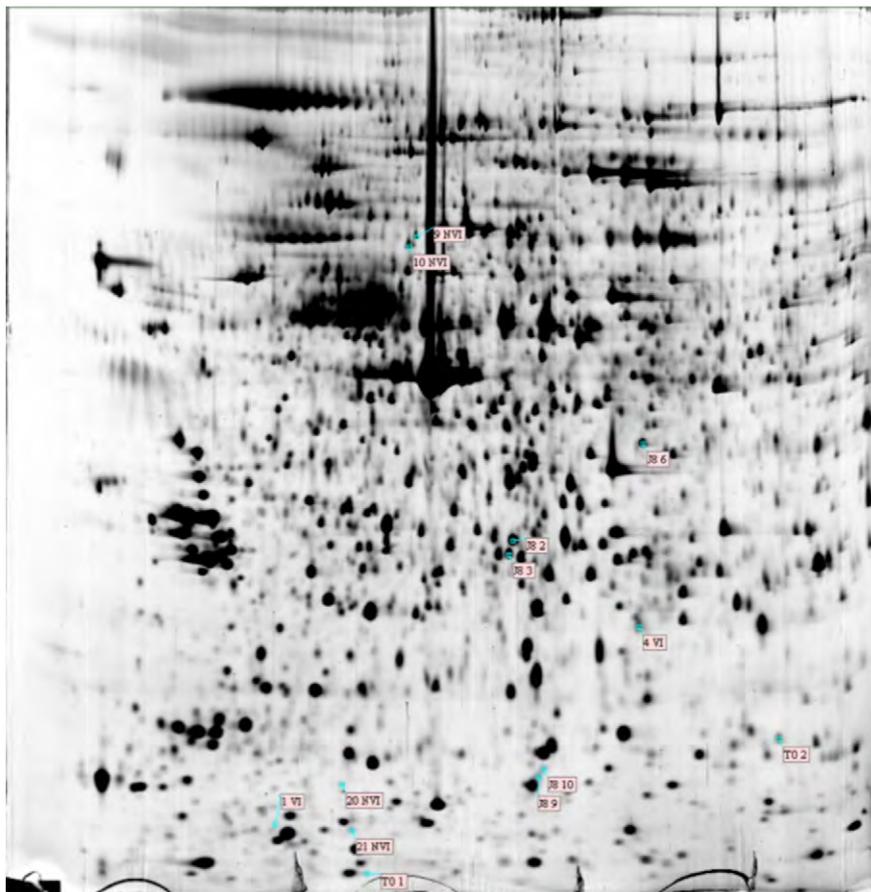


Fig. 1 – Representative 2D-GE map of platelet proteins.

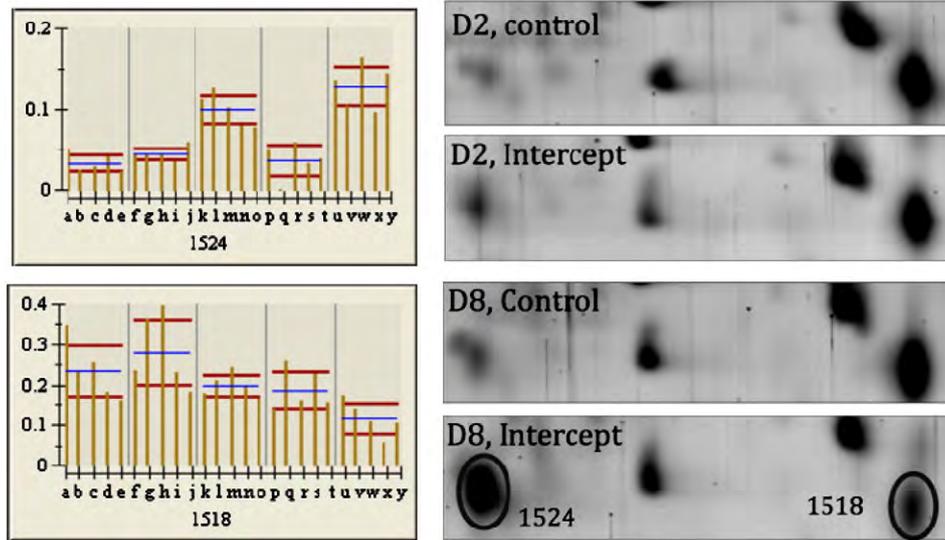


Fig. 2 – Evolution of DJ-1 spots in Intercept-treated platelets and in controls at Day 2 and Day 8. In the histograms, spot intensities for individual samples are depicted: a–e: Day 1; f–j: Day 2 controls; k–o: Day 2, Intercept-treated; p–t: Day 8, controls; u–y: Day 8, Intercept-treated.

3.3. Effect of platelet storage

When comparing 2D-GE maps at Day 8 versus Day 1, there are three additional spots that vary, independently of the Intercept treatment: even though the variation is not systematically statistically different (as shown in Fig. 7) the intensity of spot 1644 tends to diminish through time in controls and in Intercept-treated platelets ($p=0.064$ and $p=0.013$, respectively). Mass spectrometry identified Cofilin-1 as the major protein in this spot (see Table S1).

Two other spots increase in intensity through storage (see Fig. 8): spot 1386 (t-test with a p -value <0.001 between Days 1/2 and Day 8, treated and untreated) proved to be constituted of chloride intracellular channel protein 4 (CLIC4) and actin, and spot 1361 of actin (t-test with a p -value <0.001 between Days 1/2 and Day 8 treated and untreated). In both spots, despite good sequence coverage (35%), MS identification does not allow to discriminate cytoplasmic Actin 1 from cytoplasmic Actin 2, and peptides are identified in the C-terminal half of the protein. Additionally, the apparent mass on the gel is lower than that of intact Actin (28 kDa on gel versus 41 kDa for whole actin), indicating that the detected Actin might be N-terminally processed. Conversely, CLIC4 is identified with 67% sequence coverage, from peptides spanning the entire protein.

In summary, two families of spots showed variations in intensity whether directly upon Intercept treatment, or throughout storage irrespective of the Intercept treatment. In the first category, DJ-1 protein undergoes a modification induced by the Intercept treatment that persists throughout

platelet storage. Similarly, glutaredoxin 5 is also modified upon treatment, and this modification also persists throughout storage. Additionally, a C-terminal fragment of G(i)alpha2 appears upon Intercept treatment. In the second category, a spot with Cofilin-1 diminishes up to Day 8 of platelet storage, and spots containing the CLIC4 and actin increase up to Day 8 of storage. Based on the statistical analysis, these storage lesions are not affected by the Intercept treatment, which differs from the Mirasol treatment where VASP Ser 157 phosphorylation increase is accelerated by the treatment [48].

4. Discussion

The first striking result of our analysis is the relatively low number of proteins that appear to be modified upon Intercept treatment of platelets. For comparison, Mohr et al. compared the proteome of platelets prepared by the Buffy-coat method, suspended in SSP+additive solution to a plasma ratio of 35%, and subsequently treated with UV-B, UV-C and gamma irradiation by DIGE analysis (pH 4–7 in the first dimension, SDS-PAGE with 12.5% gel in the second dimension): they found 67 proteins modified by UV-B treatment, 48 by UV-C and 87 by gamma irradiation, compared to controls, with variations in spot intensities from 1.6 to 23 between controls and treated samples [21]. More recently, Schubert et al. compared the proteome of platelets treated with Mirasol with the control ones, and identified 26 proteins over 14 1D-GE bands that were differentially expressed at Day 6 of storage compared

Table 2 – Number of spots detected on 2D gels in each condition tested.

	Day 1	Day 2 control	Day 2 Intercept	Day 8 control	Day 8 Intercept
Number of spots	1439	1375	1393	1310	1325
Standard deviation	190	190	160	232	230

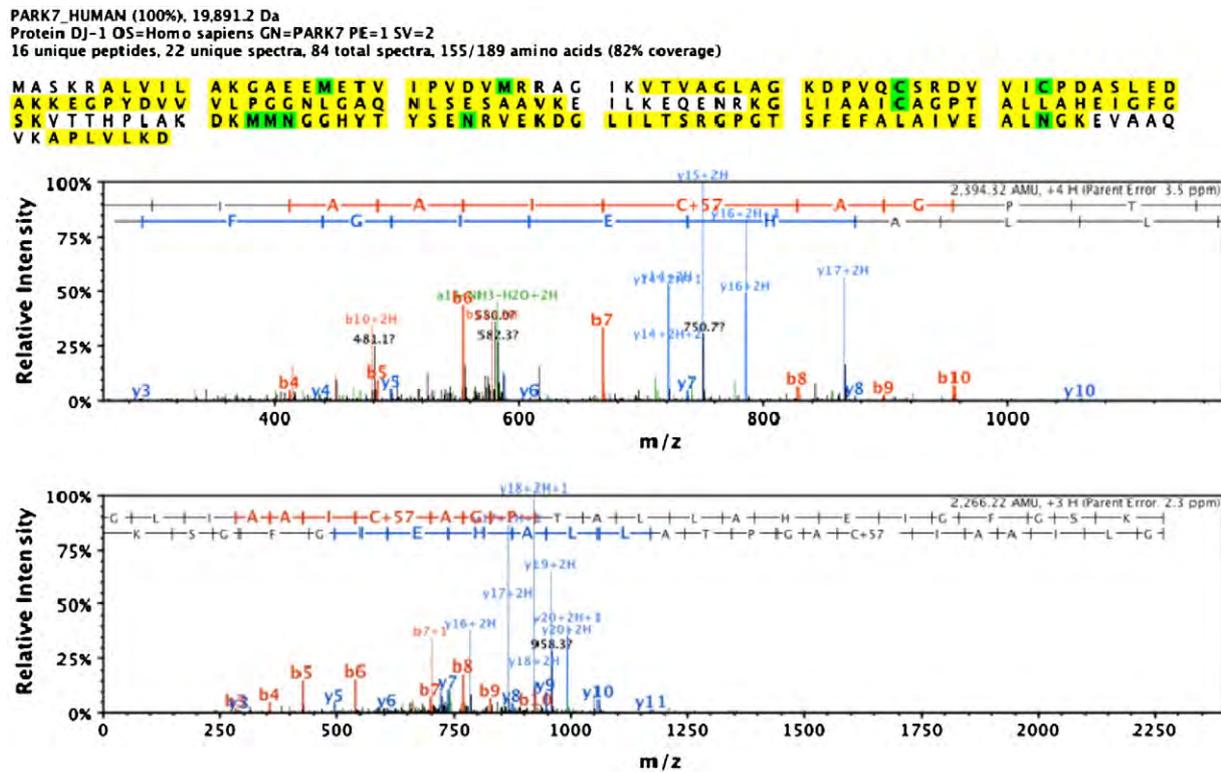


Fig. 3 – Identification of DJ-1 in spot 1524. Top panel shows the sequence coverage (green boxes show carbamidomethylated cysteines, oxidized methionines, and deamidation of asparagine). Two other panels show the tandem mass spectra where C106 is carbamidomethylated.

to the day of production and treatment [48]. Interestingly, authors could not identify any difference just after the Mirasol treatment, but only at the end of platelet storage. Changes of spot intensity were in this study in the range 1.3-3.0. This year, another proteomic analysis on Intercept-treated platelet, revealed the alteration of 23 proteins at Days 1 and 58 proteins at Day 5 [49]. Inside this set of proteins, only three showed consistent changes after treatment and storage: platelet endothelial aggregation receptor 1 precursor (PEAR-1), protein-tyrosine sulfotransferase 2 and CLIC4. Those proteins are different from the ones found here, except CLIC4. Their approach was slightly different from the one presented here. Indeed, gel-free LC-MS/MS analysis was used whereas our strategy was based on 2D-GE. In such conditions, the final outcome can be different due to the advantages and drawbacks

of gel-based and gel free separation techniques [50–54]. Complementary techniques would have to be considered to take into account technical variations [55,56].

The reasons why we detect less altered proteins might be two-fold: first, our methodology rely on silver staining of 2D-GE maps, that has a steeper and narrower linearity range when compared with the fluorescent staining used in DIGE, and may thus be less sensitive to variations of very low or very high abundance proteins. Secondly, our manual detection of variations in spot intensities might miss some subtle differences, the advantage being the confidence in the data; for example, if fold changes in the study by Mohr et al. are restricted to two-fold changes in either direction, only 10 proteins appear as differentially expressed in the UV-C treated sample compared to the control one [21].

Secondly, pathogen reduction technologies proceed through different mechanisms: the Intercept technique makes use of a psoralen-based photosensitizer, Amotosalen S-59, to cross-link DNA and RNA strands and prevent their replication or transcription. The Mirasol process uses riboflavin as a photosensitizer that induces direct lesions to DNA and RNA and prevents their replication, and the Theraflex-UV process uses germicidal properties of direct UV-C irradiation. Both Intercept and Mirasol are thought to work through photo-excitation of the sensitizer, that further reacts directly with its target through electron transfer (type I reaction), or through the generation of reactive oxygen species (type II reaction), or by direct photoaddition to its target without electron transfer. In this respect, Intercept and Mirasol may behave differently, because

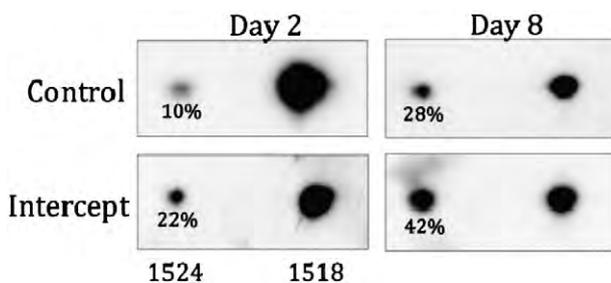


Fig. 4 – Western blot analysis of platelet samples with Anti-DJ-1 antibody.

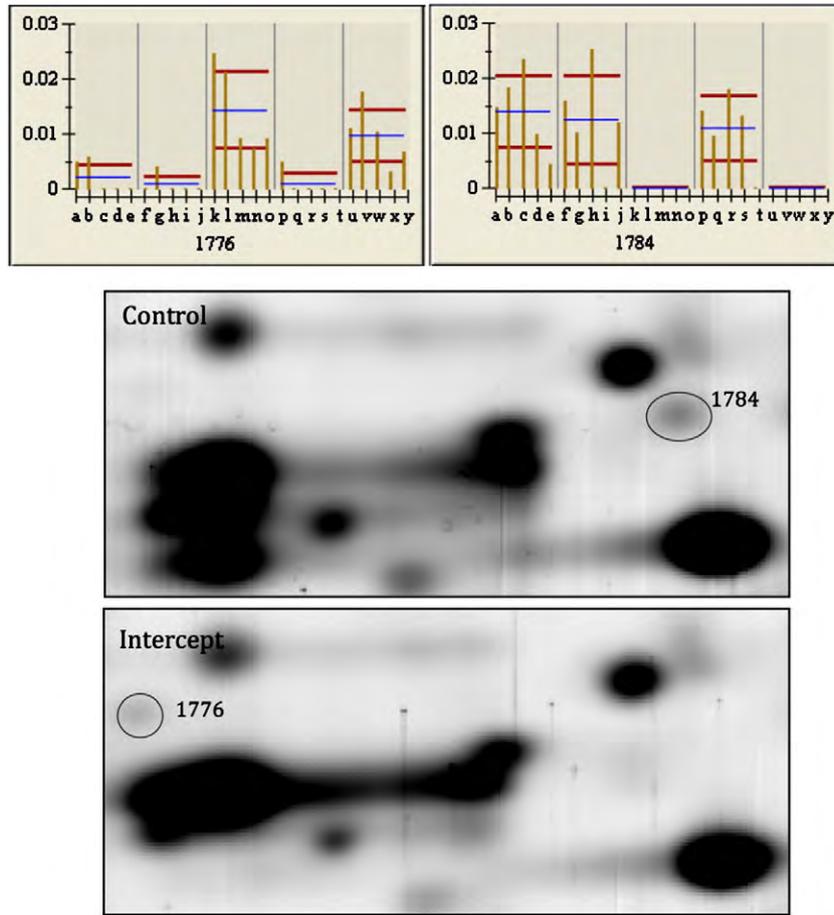


Fig. 5 – Image analysis of spots 1776 and 1784, showing complementary evolution of the two spots. In the histograms, spot intensities for individual samples are depicted: a–e: Day 1; f–j: Day 2 controls; k–o: Day 2, Intercept-treated; p–t: Day 8, controls; u–y: Day 8, Intercept-treated.

Intercept is thought to proceed through type I or III mechanisms (without the generation of oxygen species) whereas Mirasol is thought to proceed mainly through type II mechanism, implying the generation of reactive oxygen species ($O_2^{\bullet-}$, H_2O_2 , $\bullet OH$) with broad range of reactivities. Conversely, UV-C treatment is thought to work through direct absorption of UV light by nucleic acids, thus forming cyclobutane pyrimidine and pyrimidine–pyrimidone dimers that block any replication. At the same time, UV-C irradiation of an aqueous solution causes

the formation of reactive oxygen species (either single oxygen and $O_2^{\bullet-}$, H_2O_2 , $\bullet OH$) that may in turn have a non-specific, deleterious effect on proteins. It could thus not be totally surprising that the Intercept treatment might be less harmful to proteins compared to Mirasol and UV-C treatment. However, the Intercept study carried out by Thiele and coworkers revealed a more pronounced changes at Day 5 of storage with 58 proteins identified due to the Intercept treatment [49]. Even though their number of proteins displaying consistent

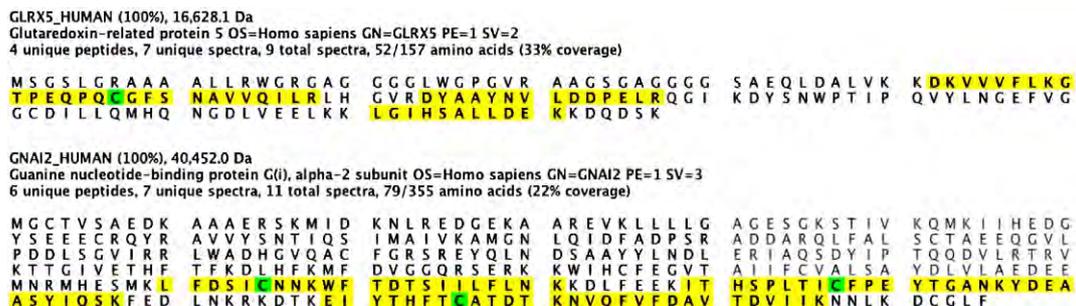


Fig. 6 – Top: Sequence coverage of glutaredoxin 5 in spot 1776. Amino acids 1–31 are thought to be a potential transit peptide. Bottom: Sequence coverage of G(i)alpha2 in spot 1776. Green box shows carbamidomethylated cysteines.

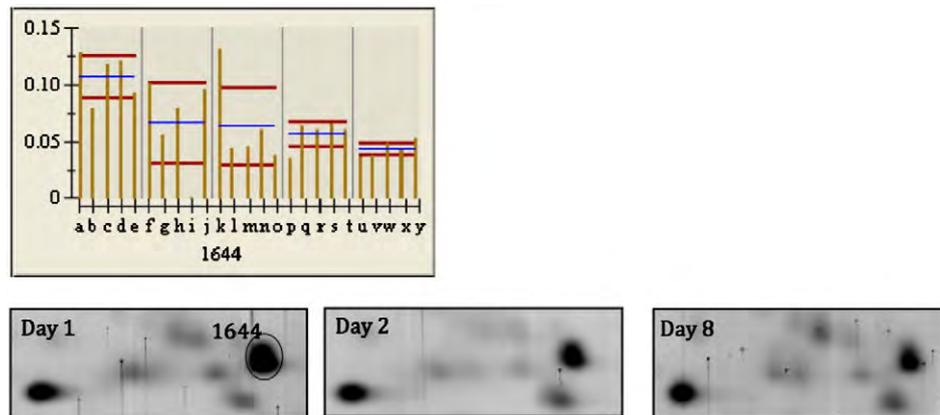


Fig. 7 – Evolution of spot 1644 upon storage. In the histograms, spot intensities for individual samples are depicted: a–e: Day 1; f–j: Day 2 controls; k–o: Day 2, Intercept-treated; p–t: Day 8, controls; u–y: Day 8, Intercept-treated.

changes is equivalent to the number reported here and below the number of altered proteins in the Mirasol study [48], it is difficult to conclude straightforwardly.

Our study evidences that a few proteins are directly altered by the Intercept treatment (Day 2 compared to control). First the modified form of DJ-1 protein increases two-fold upon Intercept treatment, whereas the unmodified form seems to slightly diminish ($p=0.05$ for comparison between Days 1 and Day 8 Intercept-treated). Duan et al. extensively studied the influence of oxidative stress on DJ-1 protein in pneumocytes, and the similarity of their Western blot images with ours let us conclude that the acidic spot of DJ-1 protein is probably the Cys106 sulfenic acid form of DJ-1; in pneumocytes, the authors found that the oxidized, inactive form of DJ-1 slightly diminishes whereas newly synthesized DJ-1 rescues the basal protein pattern after removal of the oxidative stress [45]. In platelets, we found that the DJ-1 pattern is not rescued at Day 2 (i.e. 14 h after the UV illumination) nor after 8 days of storage. Protein DJ-1 (gene PARK7) has been intensively studied in the context of Parkinson disease [57–60]; whereas its function in

neurons is still not fully understood, it seems that DJ-1 takes part in a specialized network of proteins encompassing Hsc70/Hsp90 chaperones, Pink1 and Lrrk2 as targets, and connections to the proteasome through the interaction of Hsc70 with two ubiquitin 3 ligases, parkin, and the carboxyl terminus of Hsc70-interacting protein [61–64]. This implication of DJ-1 in chaperone and proteasome-targeting activities in neurons is completed by its interactions with antioxidant enzymes such as peroxiredoxins and superoxide dismutase [65], which makes it a partner in oxidative stress-sensitive chaperone machinery. However, the potential function of DJ-1 in platelets is totally unknown to the best of our knowledge, and most published data relates to neurons, which makes any extrapolation to blood cells difficult.

The variations of glutaredoxin 5 (diminution of the native form with concomitant appearance of an acidic form that might be the glutathionylated form of the enzyme) clearly show that defenses against oxidative stress are challenged in Intercept-treated platelets. Glutaredoxin 5 is a poorly studied monothiol glutaredoxin [47], which has been shown to be expressed

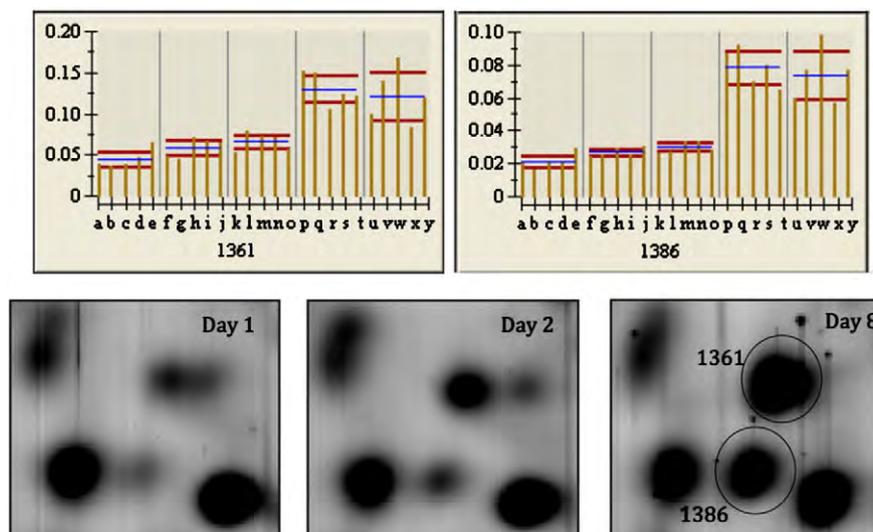


Fig. 8 – Evolution of spots 1386 and 1361 upon storage. In the histograms, spot intensities for individual samples are depicted: a–e: Day 1; f–j: Day 2 controls; k–o: Day 2, Intercept-treated; p–t: Day 8, controls; u–y: Day 8, Intercept-treated.

ubiquitously during development of blood cells [66] and whose mutation leads to sideroblastic microcytic anemia and iron overload [67]. In yeast, glutaredoxin 5 has been shown to regulate protein S-glutathionylation, a mechanism that protects proteins against irreversible cysteine oxidation [68]. Unlike dithiol glutaredoxins that depend on glutathione reductase for recycling, monothiol glutaredoxins are passively recycled in presence of glutathione [69]. The appearance of oxidized glutaredoxin upon Intercept treatment thus points out to a decreased recycling mechanism, and most probably to a decreased glutathione system activity. Burch et al. reported a rapid decrease of glutathione with a half-life of 2 days post-collection for aphaeresis platelets collected in 100% plasma [70], which may be even quicker in platelets stored in 35% additive solution.

The identification of G(i)alpha2 as a truncated form in an overexpressed spot in Intercept-treated samples is intriguing: G(i)alpha2 is a part of the G-protein coupled to receptors P2Y₁₂ (one of the two ADP receptors together with P2Y₁) [71]: upon ADP binding to the receptor, G(i)alpha2 is released and inhibits adenylyl cyclase, preventing the synthesis of cAMP, a major prerequisite of platelet activation [72–74]. At the same time, subunits β/γ migrate to activate phosphatidylinositol 3-kinases (PI₃Ks)[75]; this enzyme produces phosphatidylinositol 3,4,5-triphosphate (PIP₃), which in turns activate the Akt serine/threonine kinase, protein kinase B, and Rap1b, a central control point in platelet activation [76,77] that is an activator of integrin $\alpha_{IIb}\beta_{III}$ through kindling and talin. A modification of the Gi protein alpha 2 subunit thus implies an alteration of the signaling in platelet activation. The reason for the presence of this fragment of G(i)alpha2 is unknown; as the fragment appears at Day 2 after the Intercept treatment, it is highly improbable that the apoptotic mechanisms identified to be activated in stored platelet (such as caspase activation, see below), are already active.

Interestingly, none of the proteins we identified to be modified at Day 2 in Intercept-treated platelet appears in the list of proteins identified by Mohr et al. after treatment by UV-B, UV-C or gamma irradiation [21]. In their studies of Mirasol-treated platelets and Intercept-treated platelets, Schubert et al. and Thiele et al. did not identify the proteins identified in our Intercept study by comparing effect of treatment and storage [48,48]. Beyond the technical differences, these facts tend to show that the various pathogen inactivation methods (Intercept, Mirasol and UV-C) may have different impacts on platelets. Indeed, proteins altered due to Mirasol treatment were mainly cytoskeletal protein in addition of a treatment-dependent increase in phosphorylation of VASP, which links the signal transduction caused by the treatment to platelet activation, whereas proteins reported in Intercept studies were mainly linked to catalytic activity and platelet function (G(i)alpha2 here and PEAR-1 and protein-tyrosine sulfotransferase 2 in the Thiele's study). Additionally, several groups have studied the in vitro differences between Intercept-treated platelets and control ones from a metabolic, or functional point of view: there is a relatively clear consensus that Intercept-treated platelets suffer from an increased metabolism, as shown by glucose consumption and lactate production [78–81], an increased fragility as demonstrated by lower hypotonic shock response [78,82], and increased activation, as identified through CD62P

expression, Annexin V release and mitochondrial potential [82]. Here we have shown that DJ-1 and glutaredoxin 5 are directly affected by the Intercept treatment, which directly points to an implication of oxidative stress in the Intercept-induced lesion to platelets, a phenomenon that can be put in perspective with the loss of mitochondrial potential after Intercept treatment. In vitro studies have reported the cellular injury after pathogen reduction treatment, in particular showing a reduction in ATP generation [82,83]. In spite of the relation between G(i)alpha2 and cAMP, ATP and cAMP, and activation of platelets, it is difficult to directly draw a link on the activation/aggregation mechanism between the alteration of this protein and in vitro studies. Indeed, the role played by this fragment within the activation pathway in response to ADP, increase or inhibition, is not clear. The targets are now identified and complementary studies will be required.

In parallel, we have identified a number of proteins that are modified during platelet storage independently of the Intercept treatment: both Cofilin-1 and actin modifications are well-known effects of platelet storage, which have been previously associated with altered cytoskeleton modifications [21,55]. We also identified the CLIC4 in a spot with increased intensity at Day 8 versus Days 1 and 2 irrespective of the Intercept treatment. CLIC4 is a low specificity chloride transporter which inserts into nuclear membranes in a redox sensitive manner in various cell types [84,85], and whose selectivity is pH-dependent. Its function in platelets is totally unknown, but its increased presence in the soluble fraction of the platelet proteome, as seen by 2D-GE, is indicative of an altered function of the transporter, which plays a role in platelet homeostasis and can be related to altered platelet response to hypotonic shock after storage [82]. The fact that several authors identified CLIC/actin interactions as a key regulator of the channel activity [86] also relates to altered cytoskeleton regulation in platelets stored for 8 days. Interestingly, CLIC4 was also involved in Myc-induced apoptosis in fibroblasts: Myc binds to a mtCLIC4 promoter which induces its transcription; at the same time, anti-apoptotic Bcl-2 and Bcl-xL were decreased and Bax was increased, showing that CLIC4 expression from mtRNA is tightly associated with apoptotic mechanisms [87].

Interestingly, Reid et al. have recently shown that the Mirasol treatment triggers pro-apoptotic mechanisms, as testified by Annexin V externalization, cytochrome C release, increased expression of Bak and Bax proteins, and detection of caspase-3 activity [88]. Comparable results have also been demonstrated with the Intercept process [89]. These features are known players in the platelet storage lesion irrespective of pathogen reduction techniques, as already demonstrated by Dasgupta et al. [90].

In summary, the most striking feature of our study is the limited number of proteins that are altered by the Intercept treatment in platelets. Among the proteins identified to be directly altered by the Intercept treatment, both DJ-1 and glutaredoxin 5 point to an oxidative stress-associated lesion that may overcome platelet antioxidant defenses. However, as no other major oxidative damage is identified, these results suggest that oxidative lesions induced by the Intercept treatment are highly specific to particular pathways. The fact that these lesions are persistent or even amplified throughout storage after Intercept treatment suggest that lesions cannot

be rescued by new protein synthesis [91] as in other cell types. The extent of de novo protein synthesis in platelets is largely unknown, but in principle the Intercept treatment should inhibit any protein synthesis. Additionally, the detection of an abundant truncated form of G(i)alpha2 in Intercept-treated platelets directly links the Intercept treatment with a potential functional lesion, as G(i) protein is coupled with P1Y₁₂ ADP receptor and inhibits cAMP synthesis, a major event in platelet activation and aggregation, and triggers the PI₃K pathway, a central point in platelet functional response. The other proteins identified to be altered due to storage only are well in line with published results and points to cytoskeleton alterations [49,56,92]. A particular case is the CLIC4 that links cytoskeleton reorganization, apoptotic mechanisms and oxidative stress.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.jprot.2012.07.008>.

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