Plasma-derived microparticle biomarkers of paracetamol-induced hepatotoxicity

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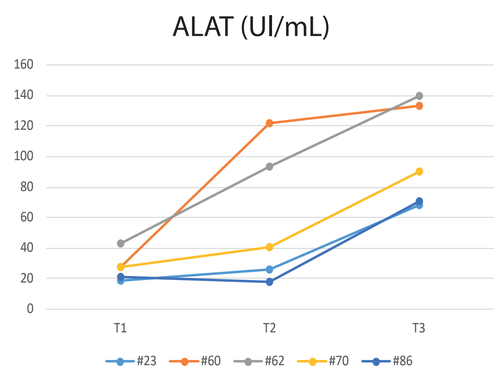
Drug-induced liver injury (DILI) is a main cause of acute liver failure (ALF) and a major problem for both clinicians and the drug industry. Acetaminophen (APAP), the most commonly used over-the-counter antipyretic and painkiller worldwide, is a well-described hepatotoxic agent and the leading cause of ALF in developed countries after an overdose (estimated overall mortality 28%). At therapeutic doses (4g/day), up to one third of healthy volunteers develops liver test elevation and cases of ALF have been described in the presence of certain risk factors.
APAP-induced hepatotoxicity is currently diagnosed on the bases of aspartate transaminase (AST) and alanine transaminase (ALT) circulating levels, considering these measures as "gold standards". They can however not be used to predict developing injury as they only detect existing injury, and suffer from their lack of specificity as they cannot determine the etiology of liver injury. This therefore underlies the need of new and early predictive biomarkers of APAP-induced hepatotoxicity in order to improve patient management.
In the field of biomarker research, microvesicles (MVs) emerged progressively as potential fruitful biomarker holders. MVs are circulating vesicles released from almost all cell types, and are composed of a huge variety of biomolecules such as mRNAs, miRNAs, proteins and lipids. Interestingly, their composition is related to their original cell, tissue or organ, but moreover, is dependent of any stimulation or the micro-environmental change of the donor cell. This gives them "signatures" of a physiological state. They are of highest value in biomarker research as they are rapid responders, and are rapidly released in the blood after a stimuli or a change of condition. MVs are therefore potential early indicators of a physiological state, containing precious information for the monitoring of pathologies.
We here hypothesize that hepatocytes release, directly in the blood via MVs, specific molecules that could be efficient candidates for liver injury early detection. These markers would therefore be of great interest to improve patient treatment management.

PATIENT SELECTION AND MVs PREPARATION

A/



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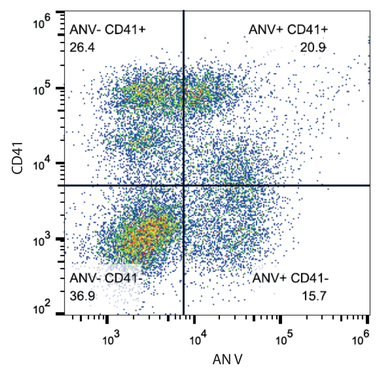
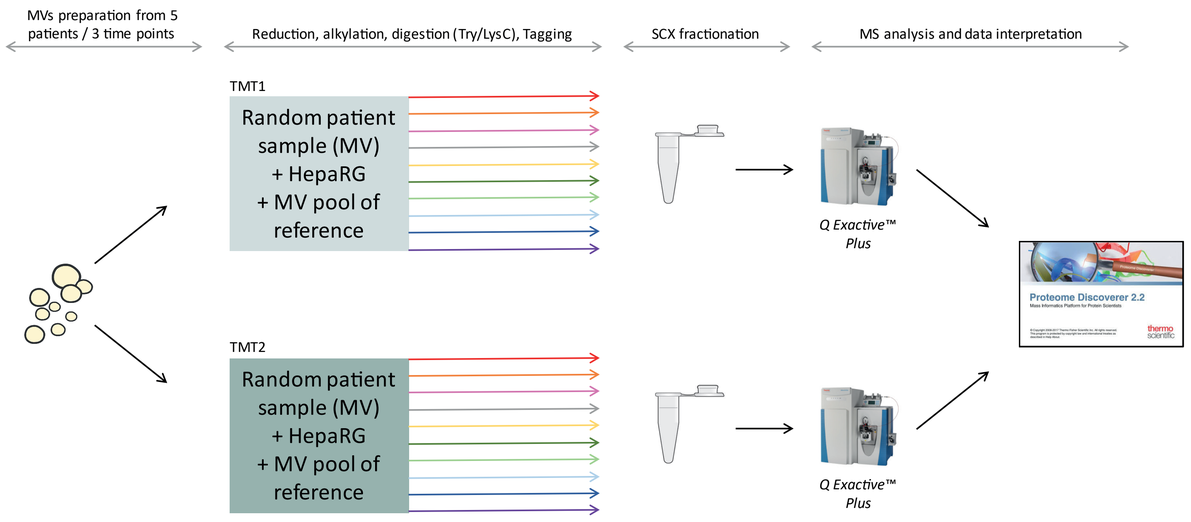


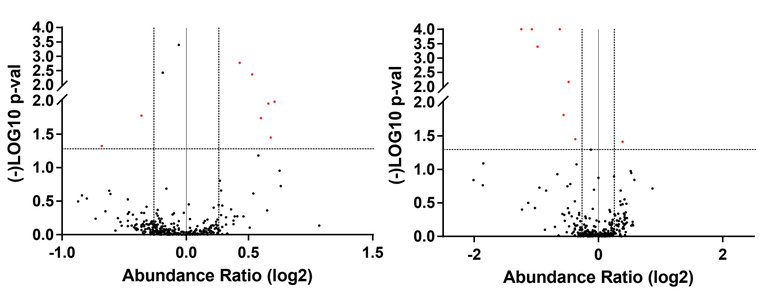
▲ Blood sample (EDTA) were collected from patients taking 4g/day of APAP post-surgery. ALAT levels were daily evaluated. To point out early biomarkers, a kinetic quantitative analysis was performed on MVs proteins. A/ For the discovery phase, MVs were isolated from 5 patient plasmas at 3 time points: an early time in the first days after surgery (T1), then at an intermediary time before hepatic test elevation (T2), and the third time point correspond to the day of hepatic test elevation (T3). MPs were isolated by differential centrifugations, and pelleted from platelet-free plasma after centrifugation at 18'000xg for 45 min. B/ Validation of MVs enrichment was performed with FACS. Closed microvesicles (CFSE-positive events) were gated and analyzed according to CD41 expression and Annexin V fluorescence (representative figure from patient #70-T2).

QUANTITATIVE PROTEOMIC APPROACH



▲ Quantitative proteomics strategies (10plex isobaric Tandem Mass Tag - TMT) were applied to compare the MV protein content from patients under APAP treatment that encountered liver test elevation (n=5), at 3 different time points. Additional tags were used as control (MV pools) and as reference (HepaRG cells). Quantitative analysis was performed with Proteome Discoverer (v2.2).

(T2) / (T1) **(T3) / (T1)**



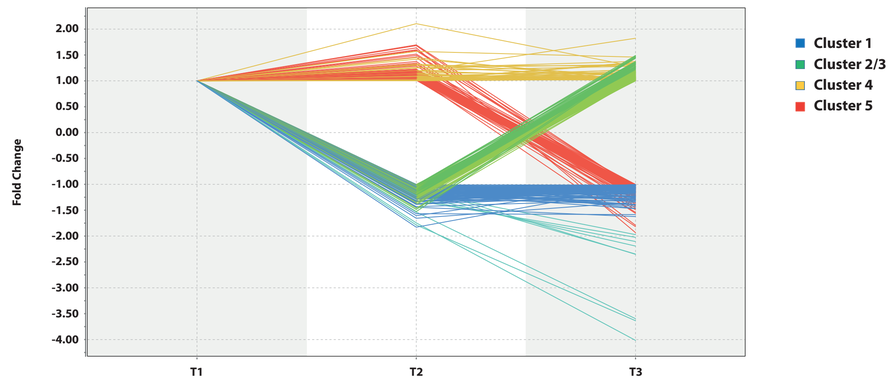
◄ More than 430 proteins were identified (1%FDR, 2 unique peptides).
Fifty-five percent of these proteins are known to be circulating MV Core Proteins (Ostergaard et al. 2012), and 90% were present in our MV protein list of reference, which confirmed the MV enrichment.
Twenty of these proteins are shown to be regulated in at least one time point of APAP patient (two-way ANOVA, adj. p-val (Tukey)<0.05)

Early regulated proteins (T2/T1)		
Ras GTPase-activating-like protein	Q13576	**0.62 (*)**
Serum amyloid A-2	P0DJI9	**1.64 (*)**
Cathelicidin antimicrobial peptide	P49913	**0.78 (*)**
Spectrin beta chain, erythrocytic	P11277	**1.45 (**)**
Fibrinogen alpha chain	P02671	**1.52 (*)**
Fibrinogen gamma chain	P02679	**1.6 (*)**
Spectrin alpha chain, erythrocytic	P02549	**1.58 (*)**
Hyaluronan-binding protein 2	Q14520	**1.34 (**)**
Lysozyme C	P61626	**0.88 (**)**

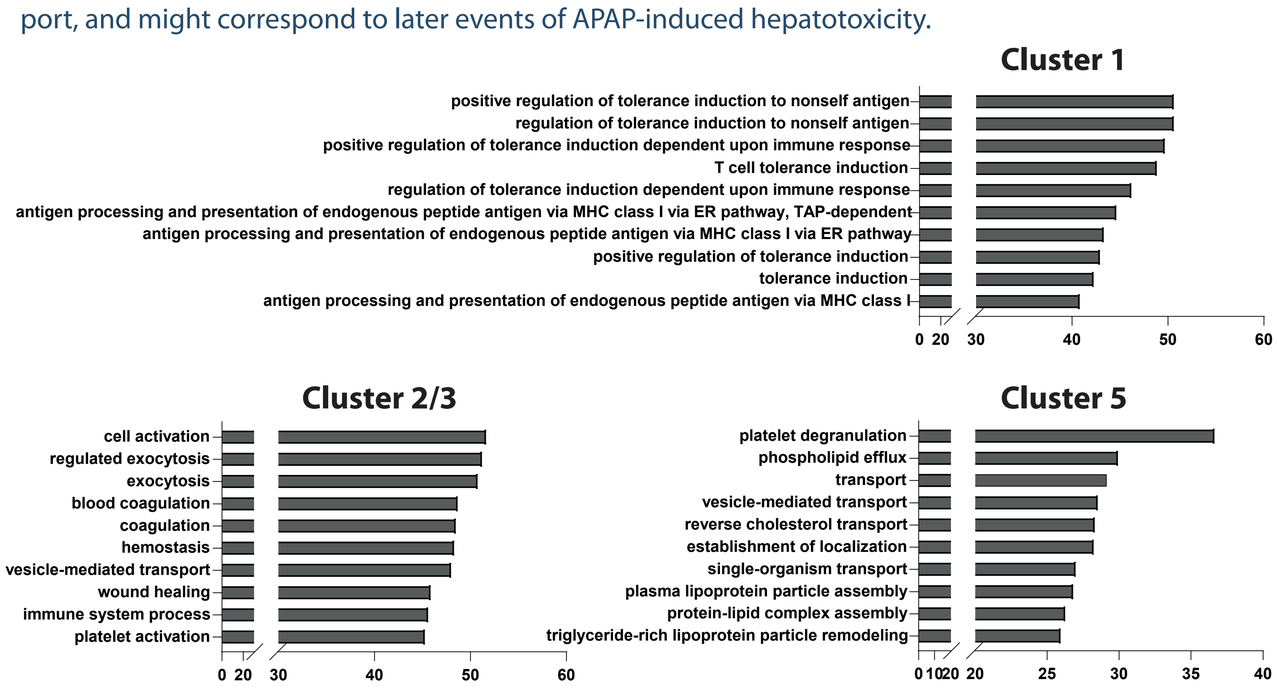
▲ Among the 20 proteins significantly modulated in MVs during the time course, the 9 proteins in this table are early modulated (T2/T1), and are therefore potential candidate as early biomarkers of APAP-induced hepatotoxicity. Adjusted p-values (Tukey's test) <0.05=* ; <0.01=**.

MECHANISTIC APPROACH

To better understand the mechanism of APAP-induced hepatotoxicity, proteins clustering (X-means) and pathway analysis was performed. Proteins identified in all patients (372 proteins) were selected.
Five disctinct clusters of proteins were drawn along APAP exposure.



▼ The enrichment of Gene Ontology allowed pointing out biological processes which are enriched in each cluster. The cluster of proteins (Cluster 1) wich are quickly decreasing during the time course are related to immune response for instance. The second interesting cluster is composed of proteins which are shortly downregulated (Cluster 2/3). These proteins are mainly linked to exocytosis mechanisms and are therefore of high interest to understand MV release in hepatotoxicity condition. Cluster 4 is grouping proteins with no variation of expression. The last interesting group is the cluster 5, in which proteins are lately downregulated. This cluster is associated with platelet mechanisms and vesicles transport, and might correspond to later events of APAP-induced hepatotoxicity.



CONCLUSIONS

- Circulating MVs from patients with APAP-induced hepatotoxicity were enriched and analyzed using TMT-based approach.
- The time course of blood samples allowed the confident identification of 20 regulated proteins
- Regulated proteins are giving previous informations on the APAP-induced hepatotoxicity mechanisms, revealing the modulation of biological patways related to immunity and vesicule trafficking for instance.

OUTLOOK

- The different candidates highlighted in the individual experiments described here will be validated on a larger cohort of patients, with orthogonal methods.
- Plasma-derived MVs will also be studied from patients with APAP intoxication (samples of acute intoxication cases of APAP (>10g)). This candidates could also be of particular interest for the early diagnosis of APAP-induced hepatotoxicity.

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