

Hepatic injury signals drive proteomic reprogramming of the mesenchymal stem cells secretome to counteract liver damage

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Abstract

Paracetamol (APAP) overdose remains a leading cause of acute liver failure (ALF), a life-threatening condition with limited therapeutic options. The secretome of mesenchymal stem cells (MSCs) contain immunomodulatory and pro-regenerative factors that promote endogenous liver regeneration in ALF. *In vivo*, MSCs respond dynamically to injury microenvironments, adapting their paracrine activity to counteract damage. Accordingly, *in vitro* priming has been explored to reproduce injury-associated cues to enhance MSC therapeutic efficacy. This study aimed at uncovering the regenerative response of MSCs to hepatic pathological conditions, in specific to hepatic injury-associated signals. Human neonatal MSCs (hnMSCs) were primed with SOS signals from APAP-injured HepG2 cells. The resulting secretome (CM-pMSC) was analyzed for proteomic composition and evaluated for biological activity in APAP-injured hepatocyte-like cells (HLCs). CM-pMSC significantly increased APAP-injured HLCs viability, reduced reactive oxygen species, recovered APAP-impaired urea production and restored HLCs' gene expression of proliferation- and APAP toxicity-related genes, namely *ccnd1*, *wnt2*, *cyp2e1*, *ask1* and *gss*. Proteomic analysis revealed that CM-pMSC was enriched in proteins associated with response to stress, immunoregulation, extracellular matrix (ECM) organization and metabolism regulation. This included proteins involved in key phases of hepatocyte proliferation, e.g., IL-6, HGF, TGF- β 1, as well as proteins relevant to the modulation of the regenerative milieu, such as MMPs, ORM1, GDF15, SERPINA1, APOA1, APOE. CM-pMSC promoted a regenerative response in APAP-injured HLCs, potentially linked to proliferation, oxidative stress control, immunomodulation, ECM remodeling, and metabolic regulation. These findings support the development of targeted MSC secretome-based therapies for acute liver injury.

1. INTRODUCTION

Acute liver failure (ALF) is a life-threatening liver dysfunction that presents high mortality rates. Paracetamol (APAP) overdose is one of the most common causes of ALF (Andrade et al. 2019). Although safe at therapeutic doses, APAP overdose causes severe and potentially life-threatening hepatotoxicity. Toxicity occurs when high APAP doses saturate its primary phase II detoxification pathways, shifting metabolism towards phase I enzymes, mainly CYP2E1. This results in the accumulation of the reactive metabolite N-acetyl-*p*-benzoquinonimine (NAPQI), which rapidly depletes glutathione stores required for its detoxification. Accumulated NAPQI covalently binds to mitochondrial proteins, increasing the production of reactive oxygen species (ROS) and stimulating sustained JNK phosphorylation, which then leads to hepatocyte necrosis. Lastly, Damage-Associated Molecular Patterns (DAMPs) released by necrotic hepatocytes trigger a sterile inflammation (Fernandez-Checa et al. 2021; Woolbright and Jaeschke 2017). Initial treatment for suspected APAP overdose consists in discontinuing the drug, followed by administration of N-acetylcysteine (NAC) (Andrade et al. 2019). Nevertheless, NAC is only effective when administrated early after APAP overdose (Licata et al. 2022). Thus, when hepatotoxicity reaches a higher severity, liver transplantation remains the most effective treatment and gold standard strategy. However, liver transplantation presents a 1-year survival rate of ~80% coupled to important limitations, namely the scarcity of donors, immune rejection and lifelong

immunosuppressive treatment (Andrade et al. 2019). These challenges highlight the need to explore alternative therapeutic strategies for liver disease.

The liver exhibits an exceptional regenerative capacity, being hepatocytes quiescent under homeostatic conditions, but able to trigger a remarkable molecular and cellular program that restores cellular loss upon injury. The primary mechanism of liver regeneration to restore the original liver mass is hepatocyte proliferation, which is traditionally divided into three phases, (i) priming or initiation, (ii) proliferation or progression and (iii) termination. In the priming phase, Pathogen-Associated Molecular Patterns (PAMPs) and DAMPs released from necrotic cells activate Kupffer cells (KCs). Activated KCs secrete TNF- α and IL-6, which in turn trigger STAT3 activation in hepatocytes. This induces the expression of immediate-early genes, such as c-Jun and c-Myc, and promotes hepatocyte transition from G0 to G1 (Michalopoulos and Bhushan 2021; Ozaki 2020). During proliferation, HGF, primarily released in its inactive form, pro-HGF, by hepatic stellate cells (HSCs) and liver sinusoidal endothelial cells, is activated by extracellular matrix (ECM) remodeling agents (e.g., MMPs). Activated HGF, together with EGF, acting through c-Met and EGFR receptors on hepatocytes, activate downstream targets such as ERK, PI3K/AKT and mTOR pathways. These signaling cascades induce cyclins and cyclin-dependent kinases that drive hepatocyte transition from G1 to S (DNA synthesis) and G2 to M (mitosis). Once liver mass is restored, TGF- β 1, released by HSCs, mediates the termination phase by binding to T β RI/II, leading hepatocytes to quiescence (Michalopoulos and Bhushan 2021; Ozaki 2020). Beyond hepatocyte proliferation and hepatic progenitor cell activation, liver regeneration relies on a coordinated multi-system response that integrates stress and inflammation, angiogenesis, ECM remodeling, metabolism regulation and immunoregulation (Shi and Line 2020). Together, these processes modulate and shape the regenerative microenvironment to ensure liver repair. Stress and inflammation provide the first cues for the re-entry of hepatocytes into the cell cycle (Ozaki 2020), while angiogenesis rebuilds the sinusoidal and vascular network. This process is accompanied by induction of pro-angiogenic and ECM remodeling factors (e.g., MMPs, TIMPs), which help re-establish the structural framework of hepatic cells. The metabolic response reallocates substrates to meet bioenergetic demands for liver regeneration whilst preserving systemic metabolic homeostasis; whereas the immune crosstalk amongst KCs, macrophages and natural killer cells, along with complement activation, provide the balance that either allow or restrain regeneration (Preziosi and Monga 2017; Shi and Line 2020).

Due to the high therapeutic potential of Mesenchymal stem cells (MSCs), these cells and their respective secreted factors may constitute an alternative or adjuvant therapy to liver transplantation, either via its capacity to differentiate into hepatocyte-like cells (HLCs), or through paracrine signaling. *In vivo* differentiation of MSCs into HLCs remains controversial (Luan et al. 2021) and instead, many reports propose MSCs' paracrine signaling as the main mechanism for counteracting ALF. In fact, the secretome of MSCs present important anti-inflammatory and immunomodulatory factors, such as for example IL-6, IL-10, TGF- β 1, PGE2, IDO, as well as anti-fibrotic, e.g., MMP9, TSG-6, antioxidant, e.g., GPX1, PRDX1, and pro-regenerative factors, e.g., HGF, G-CSF, IGF-1, VEGF, that can promote regeneration of ALF damaged tissues through the activation of surrounding cells and stimulation of endogenous liver regeneration (Kim et al. 2021; Kim et al. 2020; Lee et al. 2016; Lee et al. 2015; Trigo et al. 2025; Yuan et al. 2022;

Zhang et al. 2016). Importantly, *in vivo*, upon an injury event, MSCs present a targeted response by secreting specific factors that act to counteract damage (Caplan 2016). *In vitro*, mimicking different microenvironments has shown to improve the MSCs' therapeutic response. Several *in vitro* priming strategies have been widely applied to MSCs, such as 3D culture conditions, hypoxia or stimulation with different factors to mimic the *in vivo* hepatic physiological or pathological signals (Lee et al. 2016; Lee et al. 2015; Zhang et al. 2016). 3D spheroid cultures have shown to induce the secretion of anti-fibrotic factors by human adipose-derived (AD)-MSCs, such as IGF-1, IL-6 and HGF, further decreasing cell injury and apoptosis *in vitro* as well as ameliorating hepatic fibrosis and liver function *in vivo* (Zhang et al. 2016). Moreover, lipopolysaccharide (LPS) or hypoxic conditioning of AD-MSCs have shown to upregulate the expression of regenerative mediators such as IL-6, TNF, HGF and VEGF, in which the resultant secretome promoted liver regeneration both *in vitro* and *in vivo* via STAT3 activation (Lee et al. 2016; Lee et al. 2015). The stimulation of AD-MSCs with signals from hepatitis B-transfected or thioacetamide (TAA)-pretreated hepatocytes generated an improved secretome with enhanced anti-inflammatory and anti-apoptotic properties as well as restored hepatic tissue organization and normalized liver enzymes in mouse models of hepatitis B and TAA-induced ALF (Kim et al. 2021; Kim et al. 2020). Conversely, when the secretome of TAA-induced MSCs was applied to partially hepatectomized mice, its therapeutic effect exhibited an inferior efficacy compared to the naive secretome, which was attributed to an enrichment in antioxidant proteins such as peroxiredoxin-1 that are indeed relevant for TAA-induced ALF but not relevant within the regenerative context of partial hepatectomy (Kim et al. 2020). Based on this evidence, we hypothesize that precise MSC stimulation under conditions that mimic specific injury contexts can generate a more targeted and therapeutically relevant secretome. Here, to mimic APAP-induced injury, human neonatal MSCs (hnMSCs) were stimulated with APAP-derived hepatic SOS signals and the resulting secretome was profiled in terms of proteomic composition and biological activity. This approach aimed to elucidate its contribution to liver regeneration and support the development of more targeted MSC-based therapies.

2. METHODS

2.1. Ethics statement

This study was approved by the Ethics Committee of Hospital Dr. José de Almeida (Cascais, Portugal), under a research protocol between ECBio (Research & Development in Biotechnology, S.A.) and HPP Saúde (Parcerias Cascais, S.A.). Umbilical cord donations, with written informed consents, and procurement, were made according to Directive 2004/23/EC of the European Parliament and of the Council of 31 March 2004 on setting standards of quality and safety for the donation, procurements, testing, processing, preservation, storage and distribution of human tissues and cells. Human neonatal mesenchymal stem cells were isolated from umbilical cords of healthy newborn, upon informed consent of healthy parturients, as previously described (Martins et al. 2014).

2.2. Reagents

All culture media, supplements, analytical grade solvents and reagents were acquired from Merck (Madrid, Spain), unless stated otherwise.

2.3. Cell culture

hnMSCs were isolated from umbilical cord tissue as previously described (Martins et al. 2014) and cultured, between passage 5 and 16, in α -MEM supplemented with 10% FBS according to (Miranda et al. 2019).

For hnMSCs differentiation into HLCs, a three-step differentiation protocol was applied as detailed previously (Cipriano et al. 2017). At day 27 of the differentiation (D27), HLCs seeded in collagen-coated culture plates were exposed to 15 or 30 mM of APAP for 24h, after which cells were treated with conditioned media from non-primed or primed hnMSCs. Non-injured HLCs were collected in parallel, as control (Fig. 1). Functional experiments were performed in HLCs' basal medium without dexamethasone (IMDM, 8 ng/mL oncostatin M, 1% DMSO and 1% insulin-transferrin-selenium).

HepG2 cells were cultured in α -MEM supplemented with 10% FBS, 1 mM of sodium pyruvate and 1% of non-essential amino acids.

2.4. Collagen coating

Rat-tail collagen extraction was based on Rajan *et al.* (Rajan et al. 2007), lyophilized and stored at -80 °C. When needed, collagen was dissolved in 0.1% acetic acid to a stock concentration of 1 mg/mL. To coat culture surfaces, collagen was diluted to 0.2 mg/mL in PBS and incubated 1h at 37 °C. Afterwards, surfaces were washed with PBS before inoculation. The differentiation process occurred using this collagen in t-flasks coating until D17 and onwards for cultures in well plates.

2.5. Conditioned media (CM) production

Production of the post-injury SOS media (SOS media) was performed according to Serras *et al.* (Serras et al. 2026). Briefly, HepG2 in monolayer cultures were incubated with 30 mM of paracetamol (APAP; A11240, Alfa Aesar, Karlsruhe, Germany) for 8h, followed by a 24h in serum-free media without APAP. SOS media was concentrated with Amicon® Ultra-15 3 kDa cut-off (Merck Millipore, Darmstadt, Germany), as per manufacture's recommendations. Samples were stored at -80 °C until further use.

To produce the secretome (CM) from primed hnMSCs, hnMSCs were inoculated at a density of 1.0×10^4 cells/cm² in 175 cm² flasks with α -MEM and 5% FBS. When reaching 80% confluence media was replaced by 90% of α -MEM without FBS and 10% of SOS media to a final concentration of 5x. After a 24h SOS priming, hnMSCs were washed with PBS and medium was replaced by fresh α -MEM without FBS, at a final volume of 25 mL per 175 cm² t-flask. After conditioning for 48h, CM from primed MSCs was collected under sterile conditions (CM-pMSC). CM from non-primed MSCs (CM-cMSC) was produced parallelly under the same conditions, with α -MEM without FBS nor SOS (Fig. 1). CM-cMSC and CM-pMSC were collected and centrifuged at 300xg for 10 minutes at 25 °C, following 2700xg for 30 minutes at 4

°C. For functional assays on HLCs, CM was used at a final concentration of 1 µg/mL or 10 µg/mL, achieved by concentration with Amicon® Ultra-15 3 kDa cut-off (Merck Millipore), as *per* manufacture's recommendations. Samples were stored at -80 °C until further use.

2.6. Total protein quantification

Total protein quantification of CM-cMSC, CM-pMSC and HLCs was quantified using the Bradford method, with protein dye reagent concentrate (500-0006; Bio-Rad Laboratories, Lisbon, Portugal), according to manufacturer's instructions. Absorbance at 595 nm was measured using microplate reader on SPECTROstar® Omega (BMG Labtech; Stuttgart, Germany).

2.7. LC-MS/MS analysis, proteomics data processing and bioinformatics

LC-MS/MS analysis was performed in 6 independent replicates for each experimental group, following the previously established protocol (Serras et al. 2026). Peptide mixtures were separated by NanoLC using a C18 column and analyzed on a high-resolution Orbitrap mass spectrometer (Thermo Fisher Scientific) operating in data-dependent acquisition mode (top-20). Raw spectra were processed with MaxQuant (v2.2) against the Homo sapiens UniProt database.

Data filtering was performed based on the identification of at least 2 unique peptides in each protein and the respective protein should be found in at least 5 replicates. All contaminants were removed. Data processing was performed through Log₂ transformation of all intensities. CM-pMSC/CM-cMSC ratio and list of significantly regulated proteins were calculated with PolyStest (Schwämmle et al. 2020) and statistical significance was considered with a threshold of false discovery rate (FDR)-adjusted *p*-value <0.05. Due to sample heterogeneity, one replicate per group was excluded from the analysis. Proteins were considered unique when identified in 6 replicates in the testing condition and up to 1 replicate in the control condition. STRING-DB (Szklarczyk et al. 2019) was used for identification of protein-protein interactions and their respective biological roles. MetaboAnalyst 5.0 (Pang et al. 2022), R (v4.4.3; R Studio), including *circlize* (Gu et al. 2014) and *ComplexHeatmap* (Gu et al. 2016) packages, and GraphPad Prism were used to represent enrichment data.

2.8. Immunoblotting analysis

30 µg of CM-cMSC and CM-pMSC were resolved by SDS-PAGE in 12% polyacrylamide gels and blotted onto PVDF membranes. Membranes were incubated with primary antibody overnight at 4 °C, namely anti-TXNRD1 and anti-LAMP1 diluted 1:200 (sc-20147 and sc-20011, respectively; Santa Cruz Biotechnology, Dallas, TX, USA), anti-GSS diluted 1:1000 (ZRB2624; Sigma-Aldrich, Madrid, Spain), and anti-SERPINA3 and anti-AZGP1 diluted 1:500 (MAB1295 and MAB4764, respectively; R&D Systems, Minneapolis, MN, USA) in 5% BSA, followed by horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibody diluted 1:20000 (715-035-150 or 711-035-152, respectively; Jackson ImmunoResearch, West Grove, PA, USA) for 1h at RT. Protein loading was verified by Ponceau S staining and bands were

detected by enhanced chemiluminescence (Immobilon; Millipore) and imaged using an iBright™ CL750 Imaging System (Invitrogen, Thermo Fisher Scientific, Singapore).

2.9. *In vitro* cell viability analysis

For assessing the effect of CM-cMSC and CM-pMSC in APAP-injured HLCs viability, HLCs were exposed to 15 and 30 mM of APAP at D27. After 24h exposure, cells were washed with PBS and medium was replaced by 90% of HLCs' basal medium and 10% of CM-cMSC or CM-pMSC in a final concentration of 1 µg/mL or 10 µg/mL for another 24h. HLCs' basal medium with 10% DMSO was used as negative control, HLCs' in basal medium was used as positive control and HLCs' in basal medium with 10% α-MEM was used as solvent control. Experiments were performed without dexamethasone in HLCs' basal medium. Cell viability was evaluated using the MTS reduction assay (Promega®), according to manufacturer's instructions. Three experiments in quadruplicates were performed. The absorbance was measured at 490 and 690 nm using microplate reader SPECTROstar Omega (BMG Labtech®). Results were presented as relative percentages to solvent control, which was considered as 100% of cell viability.

2.10. Intracellular ROS levels

For assessing ROS levels, HLCs were seeded at 4×10^4 cells/cm² at D17 in black 96-well plates (Sarstedt). At D27, HLCs were exposed to 15 or 30 mM of APAP for 24h, washed, and then incubated with 10% of CM-cMSC or CM-pMSC in a final concentration of 1 µg/mL for another 24h. For positive control, cells were treated with tert-butyl hydroperoxide 1 mM and incubated at 37 °C, 1h before dichloro-dihydro-fluorescein diacetate (DCF-DA) incubation. Cells were then incubated with 10 µM of DCF-DA for 1h at 37 °C and fluorescence intensity (Ex/Em 460/528) was measured on a Synergy HTX Multi-mode Microplate Reader (BioTek Instruments). Three independent experiments in quadruplicates were performed. ROS levels were normalized to total protein and expressed as percentage relative to the corresponding control, specifically HLCs injured with either 15 mM or 30 mM of APAP non-treated with CM (set as 100%).

2.11. Gene expression

To evaluate gene expression, HLCs were exposed to 15 mM of APAP for 24h, followed by exposure to CM-cMSC or CM-pMSC 1 µg/mL for another 24h, without APAP. Total RNA of 6.0×10^5 cells was isolated using TRIzol (Life Technologies, CA, EUA) and extracted according to manufacturer's instructions. RNA concentration was determined by measuring the absorbance at 260 nm using LVis plate mode (SPECTROStar Omega, BMG Labtech, Stuttgart, Germany). cDNA was synthesized from 1 µg of RNA using NZY First-Strand cDNA Synthesis Kit (NZYTech, Lisbon, Portugal), according to manufacturer's instructions. Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using PowerUp SYBR Green Master Mix (Life Technologies) for a final reaction volume of 5 µL, using 1 µL of template cDNA and 0.333 µM of forward and reverse primers. Primer sequences are provided in Table 1. Reaction was performed on QuantStudio™ 7 Flex Real-Time PCR System (Applied Biosystems, Foster City, CA,

USA), as previously described (Cipriano et al. 2017).The comparative Ct method ($2^{-\Delta\Delta Ct}$) was used to quantify gene expression, which was normalized to a reference gene (β -actin).

Table 1: Primers used for qRT-PCR characterization of HLCs gene expression.

Gene	Forward sequence (5'-3')	Reverse sequence (5'-3')
<i>β-actin</i>	CATGTACGTTGCTATCCAGGC	CTCCTTAATGTCACGCACGAT
<i>ccnd1</i>	TGGAGGTCTGCGAGGAACAGAA	TGCAGGCGGCTCTTTTTC
<i>c-met</i>	GCATTGACTGCAGGACTGGAA	TCAGGACCATCAACCCCTCAT
<i>wnt2</i>	GGGGCACGAGTGATCTGTG	GCATGATGTCTGGGTAACGCT
<i>fgf2</i>	AGAAGAGCGACCCTCACATCA	CGGTTAGCACACACTCCTTTG
<i>ask1</i>	CTGCATTTTGGGAAACTCGACT	AAGGTGGTAAAACAAGGACGG
<i>cyp2e1</i>	ATGTCTGCCCTCGGAGTCA	CGATGATGGGAAGCGGGAAA
<i>gss</i>	GGGAGCCTCTTGCAGGATAAA	GAATGGGGCATAGCTCACCAC

2.12. Urea production

Urea production was quantified in culture supernatants of HLCs injured with 15 mM of APAP for 24h and subsequently exposed to 1 µg/mL CM-cMSC or CM-pMSC for another 24h, using the QuantiChrom™ Urea Assay Kit (BioAssay Systems, Hayward, CA, USA). Absorbance was measured at 520 nm in a microplate reader (SPECTROStar Omega), according to manufacturer’s instructions. Urea production was normalized to µg/10⁶cells·h and expressed as percentage relative to non-injured HLCs.

2.13. Statistical analysis

The results are presented as Average ± SD, unless stated otherwise, and analyzed using one-way ANOVA or two-way ANOVA with GraphPad Prism. A threshold of $p < 0.05$ was considered statistically significant. Proteomics data statistical significance was calculated with PolyStest (Schwämmle et al. 2020) with a threshold of FDR-adjusted p -value < 0.05 .

3. RESULTS

3.1. The secretome from hnMSCs stimulated with SOS signals is enriched in proteins related to the response to stress, immune system process, extracellular matrix organization and metabolism regulation

Following distinct types of acute liver injury, MSCs can engage different signaling mechanisms specifically directed to each injury-associated niche. Therefore, in this study, to generate a customized

paracrine response, hnMSCs were stimulated with SOS signal media from APAP-exposed HepG2 (CM-pMSC; Fig. 1). hnMSCs non-stimulated were used as control (CM-cMSC; Fig. 1).

Given that proteins mediate most intracellular and intercellular processes, characterizing the proteomic profile of the SOS-stimulated hnMSC secretome, in comparison with CM-cMSC, provides valuable insight into the potential paracrine mechanisms activated by these cells within a liver injury-like microenvironment. LC-MS/MS proteomics analysis of CM-pMSC and CM-cMSC depicted a total of 975 different proteins, in which 881 were shared between the control (CM-cMSC) and the pre-stimulated condition (CM-pMSC), 74 were unique to CM-cMSC and 20 unique to CM-pMSC (Fig. 2a). Furthermore, stimulation of hnMSCs with the SOS signals generated a distinct proteomic profile, as shown by the volcano plot (Figure 2b) and hierarchical heatmap (Figure 2c), as well as by the PLS-DA analysis, which discriminated both group samples with moderate predictive power ($Q^2 > 0.6$; Figure 2d). Moreover, analysis of the enriched biological process Gene Ontology (GO) terms and main KEGG and Reactome pathways showed that both groups exhibited a variety of proteins related to the metabolic process (GO:0008152, hsa01100, HSA-392499), extracellular matrix organization (GO:0030198, hsa04512), response to stimulus (GO:0050896), regulation of cell death (GO:0010941), angiogenesis (GO:0001525), immune system process (GO:0002682, HSA-168256), oxidative stress (GO:0006979) and cell proliferation (GO:0042127; Fig. 3a). Importantly, when exclusively examining the unique and significantly upregulated proteins in CM-pMSC (Fig. 3b), it becomes evident that priming with SOS signals led to an enrichment of proteins specifically related to the response to stress and metabolic process regulation, whilst diminishing its protein metabolic process (Fig. 3c), but maintaining high levels of ECM organization- and immune system-related proteins (Fig. 3d).

Moreover, the biological relevance of proteins considered unique or significantly upregulated in the primed condition can give an insight into the differential role of SOS-stimulated hnMSCs on liver regeneration. STRING analysis of the unique and significantly upregulated proteins in CM-pMSC, compared to CM-cMSC, revealed a network of 25 nodes (proteins) connected by 37 edges, representing potential associations between proteins (Fig. 4a). As shown in Fig. 4b, these proteins were associated with key steps of liver injury and regeneration, including oxidative stress (e.g., APOE, ORM1), proliferation (e.g., SERPINA1, HGF), immunoregulation (e.g., APOA1, A2M), angiogenesis (e.g., APOH, ANG), extracellular matrix remodeling (e.g., FBNL1, AGT) and metabolism regulation (e.g., AGT, HMGA1), with apolipoprotein E (APOE), orosomucoid 1 (ORM1) and angiogenin (ANG) being the ones associated with the highest number of biological functions. Nonetheless, these proteins may not be the only drivers of the therapeutic effect of CM-pMSC and a deeper analysis focused on these key biological processes of, not only unique and upregulated, but also shared proteins in both conditions was performed (Fig. 4c-e). Notably, several proteins presented overlapping roles with metabolism-related proteins being the most abundantly secreted by hnMSCs, followed by proteins involved in immunoregulation, proliferation, ECM remodeling, angiogenesis and oxidative stress (Fig. 4c). Amongst individual proteins, MMP2, MMP14 and IL6 were consistently shared across all key functional roles, whereas proteins such as THBS1 and TGFBI, and PRDX4 were mainly associated with immunoregulation, proliferation, ECM remodeling as well as angiogenesis or oxidative stress, respectively (Fig. 4d, 5). Proteomic analysis was further

validated through western blotting, confirming the presence of selected proteins, namely AZGP1, SERPINA3, GSS, TXNRD1 and LAMP1, in each group (Fig. 4e). A comprehensive overview of the CM-pMSC proteome linked to each biological role is presented in Fig. 5.

3.2. CM-pMSC supports recovery of injured hepatocytes *in vitro* by improving cell viability and function while reducing ROS levels

Assessment of the biological effects of the CM-pMSC was performed on APAP-injured hepatocyte-like cells (HLCs), *i.e.*, HLCs exposed to different concentrations of APAP for 24h. CM-pMSC significantly induced cell viability of APAP-injured HLCs, suggesting its potential to counteract APAP cytotoxicity (Fig. 6a). Importantly, the biggest increase in HLCs viability was observed following treatment with CM-pMSC. In HLCs injured with 15 mM APAP, CM-pMSC at 1 and 10 µg/mL increased viability by approximately 15% compared with the respective untreated APAP-exposed HLCs (grey bars; $p < 0.001$). Similarly, in HLCs injured with 30 mM APAP, treatment with CM-pMSC at 10 µg/mL increased viability by 18.71% compared with untreated APAP-exposed HLCs ($p < 0.0001$). Furthermore, treatment with 1 µg/mL of either CM-MSC led to a reduction in ROS levels in APAP-injured HLCs of at least 20% ($p < 0.0001$). Thus, 1 µg/mL of CM was selected for subsequent assays in HLCs injured with 15 mM of APAP. In this setting, as expected, it could be observed that 24h of APAP exposure (t24) led to an overexpression of all proliferation- and APAP toxicity-related genes (Fig. 6b-c). In contrast, 24h after APAP withdrawal (t48), all treatment conditions (CM-cMSC, CM-pMSC and non-treated) led to a downregulation of all genes. However, only CM treatment could revert *wnt2*, *ask1* and *gss* gene expression to levels similar to non-injured HLCs, with CM-pMSC treatment further promoting a faster reversion of *ccnd1* and *cyp2e1* levels. Likewise, as shown in Fig. 6d, HLCs exposure to APAP led to an expected decrease in the capacity of HLCs for urea production to $36.1 \pm 16.7\%$, compared to non-injured HLCs, that was only reverted after treatment with either CM-pMSC ($127.3 \pm 31.1\%$; $p < 0.05$) or CM-cMSC ($113.1 \pm 36.4\%$; $p < 0.05$).

4. DISCUSSION

MSCs respond dynamically to cues on their surrounding microenvironment, including injury-associated signals (Caplan 2016). This knowledge has been strategically used by several authors as a mean to increase MSCs therapeutic value. Several *in vitro* MSC priming approaches have been attempted namely via cytokine stimulation mimicking *in vivo* physiological and pathological signals (Lee et al. 2016; Lee et al. 2015; Zhang et al. 2016). However, *in vitro* stimulation with only a few cytokines cannot accurately reproduce the complexity that these cells encounter *in vivo*. Thus, studying the MSCs response to complex hepatic SOS signals would both enable the understanding of their physiological role and to set the grounds for the development of novel targeted therapies for liver injury conditions.

In this study, to better mimic APAP-liver injury microenvironment, hnMSCs were cultured with a cocktail of factors and cytokines resulting from APAP injury (SOS media), which resulted from the exposure of HepG2 cells to paracetamol (Fig. 7). These stimulated/primed hnMSCs to produce a secretome targeted for APAP-injury resolution (CM-pMSC) (Fig. 7). Indeed, it could be observed that CM-pMSC significantly

induced APAP-injured HLCs viability, reduced ROS levels, accelerated the return of proliferation-related and APAP toxicity-related genes to the same basal levels of the non-injured cells and reverted the decrease in urea production capacity caused by APAP injury, suggesting its potential to counteract APAP hepatotoxicity. Upon deep analysis of the secretome in terms of protein content, this therapeutic effect could be attributed to the enriched proteins within the SOS-stimulated hnMSCs' secretome (CM-pMSC), which not only depicted several proteins characteristic of the classical phases of hepatocyte proliferation during liver regeneration (e.g., IL-6, HGF, TGF- β 1), but also a set of proteins that could be supportive for the regenerative microenvironment (Fig. 7). Stress and inflammation, ECM remodeling and metabolism regulation represent three important hallmarks of liver regeneration (Shi and Line 2020). Consistently, hnMSCs stimulated with the hepatic SOS signals secreted more stress-related proteins (e.g., SERPINA1, A2M), as well as proteins involved in metabolic process regulation (e.g., APOA1, AGT), whilst maintaining a high expression of extracellular matrix organization- (e.g., COL1A1, TIMP1) and immune system-related proteins (e.g., SERPINA3, CXCL1, C3). Moreover, it is known that global translation is reduced when cells are exposed to stress, in order to save energy and switch specific translation to proteins that are indeed required for cell survival (Holcik and Sonenberg 2005), and, in fact, protein synthesis-related proteins were diminished (e.g., EIF4H, RPS5) in CM-pMSC (Fig. 7).

Classical liver regeneration involves an initial priming of the hepatocytes by inflammatory cytokines such as IL-6, followed by hepatocyte proliferation by growth factor stimulation such as HGF, and termination of cell proliferation when liver mass is restored by anti-mitogenic signals, namely TGF- β (Michalopoulos and Bhushan 2021; Ozaki 2020). IL-6, which was found in both CM, is crucial for hepatocyte re-entry into the cell cycle via STAT3 activation (Michalopoulos and Bhushan 2021; Ozaki 2020) whilst its absence compromises regeneration after APAP hepatotoxicity (Ma et al. 2025). In addition, MSC-derived IL-6 have shown to promote M2 macrophage polarization (Liu et al. 2022; Philipp et al. 2018) and reduce T cells proliferation (Djouad et al. 2007), whilst promoting urea synthesis in hepatocytes (Gu et al. 2022). Thus, the restoration of urea-producing capacity of APAP-injured HLCs only after CM treatment further suggests a role for hnMSCs, partly through IL-6 secretion, in the modulation of APAP-induced metabolic disruption. Following priming, HGF, which was predominantly enriched in the SOS-stimulated hnMSCs' secretome, activate ERK, PI3K/AKT and mTOR signaling to induce cyclins and cyclin-dependent kinases for cell cycle entry and division (Michalopoulos and Bhushan 2021; Ozaki 2020). In fact, CM-pMSC not only promoted APAP-injured HLCs' viability but also accelerated the leveling of *cyclin D1* (*ccnd1*) and *wnt2* genes, as evidenced by its transversal upregulation during HLCs injury, subsequent downregulation during recovering from APAP injury, and further reduction following CM-pMSC treatment. Importantly, HGF secreted by MSCs has previously shown comparable therapeutic efficacy to NAC in APAP-induced liver injury (Wang et al. 2022a), inhibit HSCs activation, improve liver function (Choi et al. 2019), and prevent liver failure as well as liver fibrosis (Kim et al. 2014; Yu et al. 2007). As for TGF- β 1, similar levels were observed in both CM conditions, and it has been previously shown to contribute to ECM remodeling and angiogenesis during the proliferation stage, whereas, when liver mass is restored, activated TGF- β terminates hepatocyte proliferation and suppress HGF production (Michalopoulos and Bhushan 2021; Ozaki 2020). MSC-derived TGF- β 1 has also shown to promote M2 macrophage polarization, stimulate

regulatory T cells (Treg) (Melief et al. 2013) and reestablish Treg/TH17 cells balance under inflammatory conditions (Xue et al. 2022). All and all, these findings suggest that IL-6, HGF and TGF- β secreted by SOS-stimulated hnMSCs may contribute to the priming, proliferation and termination phases of hepatocyte proliferation while also acting at metabolic and immunoregulatory levels (Fig. 7).

In addition to mitogenic proteins, CM-pMSC and CM-cMSC were enriched in several ECM remodeling factors, including metalloproteinases (e.g., MMP1, MMP2, MMP14), tissue inhibitors of MMPs (e.g., TIMP1, TIMP2) and collagens (e.g., COL1A1, COL3A1, COL4A2, COL6A1). These factors support proteolytic activation of HGF and promote tissue reconstruction through coordinated stages of MMP-mediated degradation, TIMP-mediated stabilization, and collagen-mediated deposition (Arteel and Naba 2020; Michalopoulos and Bhushan 2021). Notably, the SOS-primed hnMSCs' secretome presented additional proteins linked to oxidative stress, inflammation and metabolism modulation, such as ORM1, growth differentiation factor 15 (GDF15), alpha-2-macroglobulin (A2M), alpha-1-antitrypsin (SERPINA1), alpha-1-antichymotrypsin (SERPINA3), apolipoprotein 1 (APOA1) and APOE. ORM1 has shown to support hepatocyte proliferation (Qin et al. 2017), diminish TNF- α -induced inflammation (Lee et al. 2010), and reduce oxidative stress and apoptosis via Nrf2 antioxidant response (Cheng et al. 2020). Similarly, GDF15, or non-steroidal anti-inflammatory drug (NSAID)-activated gene-1 (NAG-1), has shown to reduce inflammasome activation, ROS production and mitochondrial dysfunction whilst promoting hepatocyte proliferation and lipid metabolism (Li et al. 2023; Wang et al. 2022b). Protease inhibitors such as A2M, SERPINA1 and SERPINA3 protect against excessive proteolysis during inflammation (de Mezer et al. 2023; Remih et al. 2025; Vandooren and Itoh 2021). Notably, SERPINA1 also shown to promote hepatocyte proliferation (Chen et al. 2025), correlate with better prognosis in acute liver failure patients (Remih et al. 2025), and act as a systemic metabolic regulator by promoting mitochondrial activation, energy consumption and improved glucose tolerance (Okagawa et al. 2024). APOA1 and APOE further contribute to this metabolic adaptation, constituting important regulators of cholesterol transport, inflammation, oxidative stress and energy metabolism. APOA1 has shown to promote hepatocyte proliferation and mitochondrial biogenesis (Li et al. 2018), whereas its deficiency impairs liver regeneration via autophagy inhibition (Wang et al. 2024) and correlate with poorer survival in liver cirrhosis patients (Trieb et al. 2020). APOE has shown to suppress macrophage activation and T helper (TH)-1 immune response (Zhang et al. 2011), and reduce oxidative stress-induced apoptosis (Gao et al. 2021), whilst its deficiency exacerbates hepatic inflammation, oxidative stress and apoptosis whilst decreasing mitochondrial function and autophagy (Lu et al. 2020). All these proteins likely mediate CM-pMSC' observed effects, driving accelerated hepatocyte regeneration through stimulation of hepatocyte viability, proliferation and metabolic function, whilst diminishing oxidative stress and immune response in the context of APAP-induced liver injury (Fig. 7).

Beyond supporting hepatocyte regeneration, the hnMSCs' secretome may also act at the APAP toxicity mechanism level. APAP overdose induces CYP2E1-dependent oxidative stress that leads to the depletion of glutathione (GSH) pools and subsequently activates the ASK1-JNK pathway, leading to hepatocyte necrosis and liver failure (Fernandez-Checa et al. 2021; Woolbright and Jaeschke 2017). Our results demonstrated that both CM treatment significantly downregulated *gss* and *ask1*, as well as

reduced oxidative stress levels, whilst only CM-pMSC was able to revert *cyp2e1* induction in APAP-injured HLCs. In fact, previous studies showed that AD-MSCs transplantation could restore hepatic GSH levels via activation of the Nrf2 antioxidant response and reduction of CYP2E1 expression and MAPK signaling in APAP-induced ALF (Huang et al. 2016). Together with our results, these findings suggest that CM-pMSC may reduce APAP-induced toxicity by limiting reactive metabolite formation, restoring antioxidant capacity, limiting necrosis and reducing subsequent downstream injury mechanisms.

5. CONCLUSION

Our study provides a set of results demonstrating that CM-pMSC accelerated HLCs regeneration after APAP injury, potentially via key signaling for hepatocyte proliferation, oxidative stress control and metabolism regulation. Specific stimulation of hnMSCs with the SOS hepatic signals triggered a targeted paracrine response associated with an increased secretion of proteins related to the response to stress and metabolic process regulation, whilst maintaining high levels of ECM organization- and immune system-related proteins, which represent important hallmarks of liver regeneration. In fact, CM-pMSC presented several proteins not only typically associated to the main phases of hepatocyte proliferation, such as IL-6, HGF or TGF- β 1, but also an enrichment in proteins detrimental for liver regeneration and modulation of the regenerative milieu, such as MMPs, TIMPs, ORM1, GDF15, SERPINA1, APOA1, APOE, amongst others. These proteins can act in a coordinated manner to promote liver regeneration through stimulation of hepatocyte proliferation, redox homeostasis, metabolism regulation and excessive inflammation control, establishing the ideal conditions for hepatocyte survival and effective liver regeneration. Our results not only uncover the potential physiological function of MSCs but also provide a rationale for the development of targeted therapeutic strategies.

Abbreviations

APAP: paracetamol

ALF: acute liver failure

MSCs: mesenchymal stem cells

hnMSCs: human neonatal mesenchymal stem cells

HLCs: hepatocyte-like cells

NAPQI: N-acetyl-*p*-benzoquinonimine

ROS: reactive oxygen species

DAMPs: damage-associated molecular patterns

NAC: N-acetylcysteine

PAMPs: pathogen-associated molecular patterns

KCs: Kupffer cells

HSCs: hepatic stellate cells

ECM: extracellular matrix

LPS: lipopolysaccharide

TAA: thioacetamide

FBS: Fetal Bovine Serum

SOS media: post-injury SOS media

CM: secretome

CM-pMSC: conditioned media derived from SOS-primed hnMSCs

CM-cMSC: conditioned media derived from non-primed hnMSCs

FDR: false discovery rate

DCF-DA: dichloro-dihydro-fluorescein diacetate

GO: gene ontology

BP: biological process

APOE: apolipoprotein E

ORM1: orosomucoid 1

ANG: angiogenin

CCND1: cyclin D1

Treg: regulatory T cells

GDF15: growth differentiation factor 15

A2M: alpha-2-macroglobulin

SERPINA1: alpha-1-antitrypsin

SERPINA3: alpha-1-antichymotrypsin

APOA1: apolipoprotein 1

TH: T helper cells

GSH: glutathione

Declarations

Competing Interests

The authors declare no conflict of interest.

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Author Contribution

Joana P. Miranda and Ana S. Serras: Conceptualization and Funding acquisition; Joana P. Miranda, Ana S. Serras, Rui Vitorino: Methodology; Ana S. Serras: Formal analysis, Writing - Original Draft, and Visualization; Ana S. Serras, Sérgio P. Camões, Joel M. Vej-Nielsen, Rui Vitorino: Investigation and Data Curation; Joana P. Miranda, Sérgio P. Camões, Rui Vitorino: Writing - Review & Editing; Joana P. Miranda: Supervision and Project administration.

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Data Availability

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE(Perez-Riverol et al. 2025) partner repository with the dataset identifier PXD078434 (Token: BbYP0JmmBfry).

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Figures

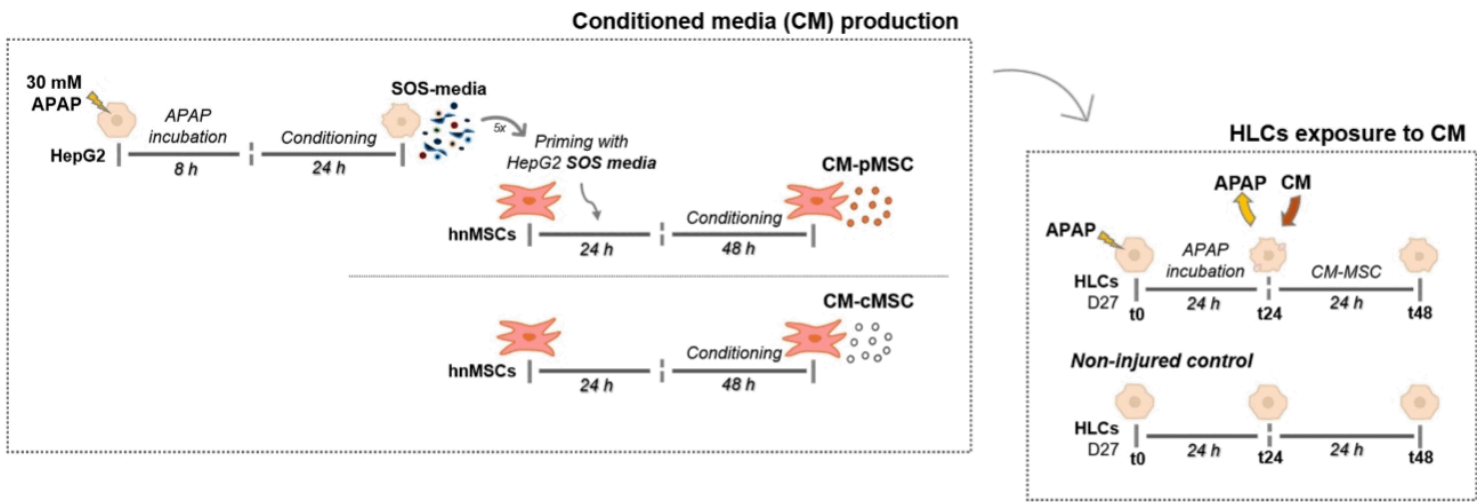


Figure 1

Schematic representation of the methodological approach for production of conditioned media derived from SOS-primed hnMSCs (CM-pMSC) and non-primed hnMSCs (CM-cMSC) and subsequent treatment of APAP-injured hepatocyte-like cells (HLCs).

Legend: APAP, paracetamol; CM, hnMSCs conditioned media/secretome; hnMSCs, human neonatal mesenchymal stem cells.

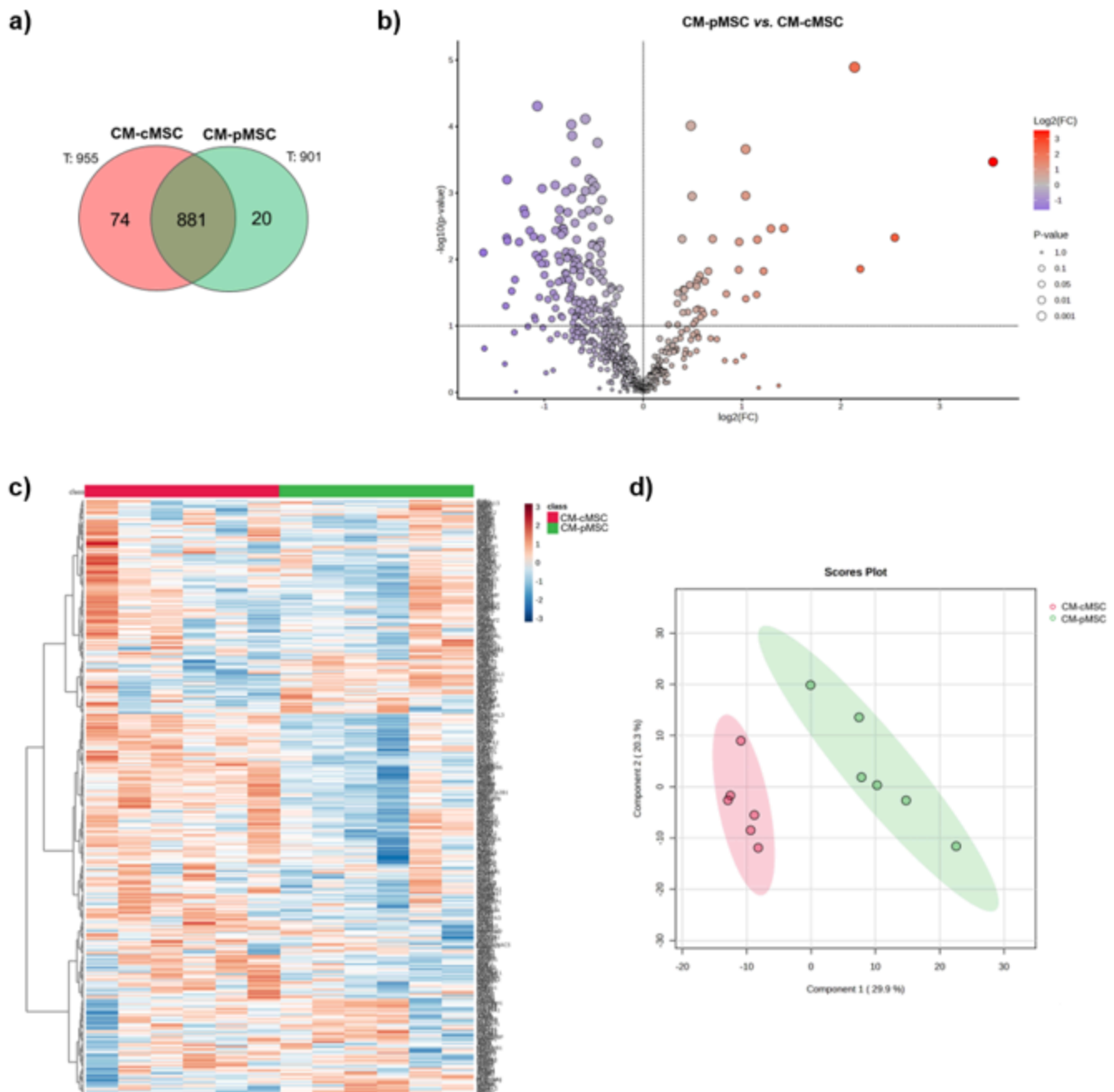


Figure 2

Stimulation of hnMSCs with the SOS signals generated a distinct proteomic profile, characterized by the presence of priming-specific proteins and an overall reduction in protein secretion. **(a)** Venn diagram, representing the overlapping of proteins within samples, **(b)** volcano plot, **(c)** hierarchical clustering heatmap and **(d)** PLS-DA plot of the hnMSCs' proteomics dataset.

Legend: CM, conditioned media/secretome; cMSC, hnMSCs non-primed; hnMSCs, human neonatal mesenchymal stem cells; pMSC, hnMSCs primed with SOS signals.

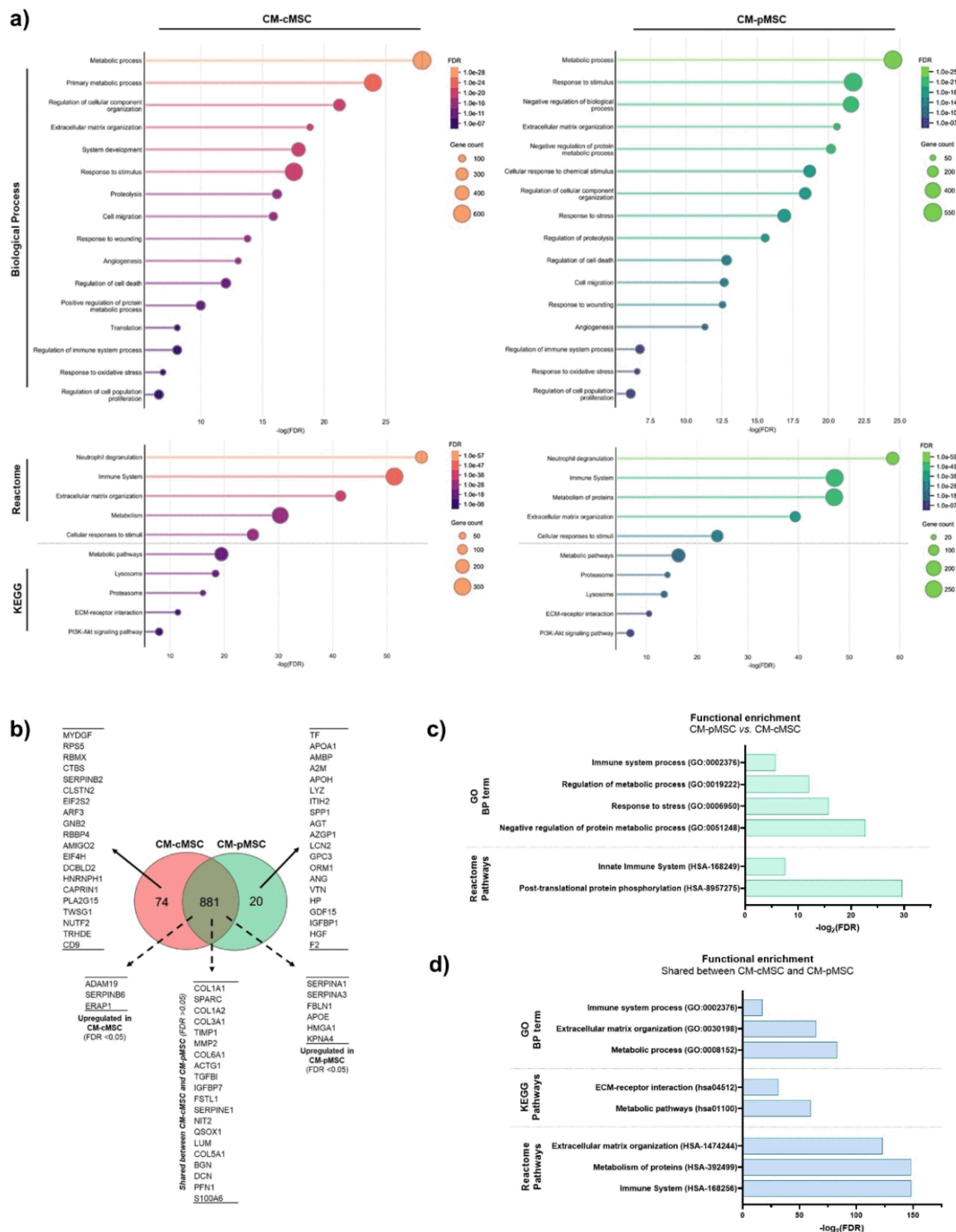


Figure 3

SOS-primed hnMSCs' secretome showed an enrichment in proteins related to the response to stress, immune system process, metabolism and extracellular matrix organization, whilst diminishing their protein metabolism. **(a)** STRING representation of Biological Process (BP) GO terms, Reactome and KEGG pathways enriched in whole proteome of CM-cMSC and CM-pMSC, based on FDR and gene count; **(b)** Venn diagram depicting top unique, significantly upregulated and shared proteins within sample

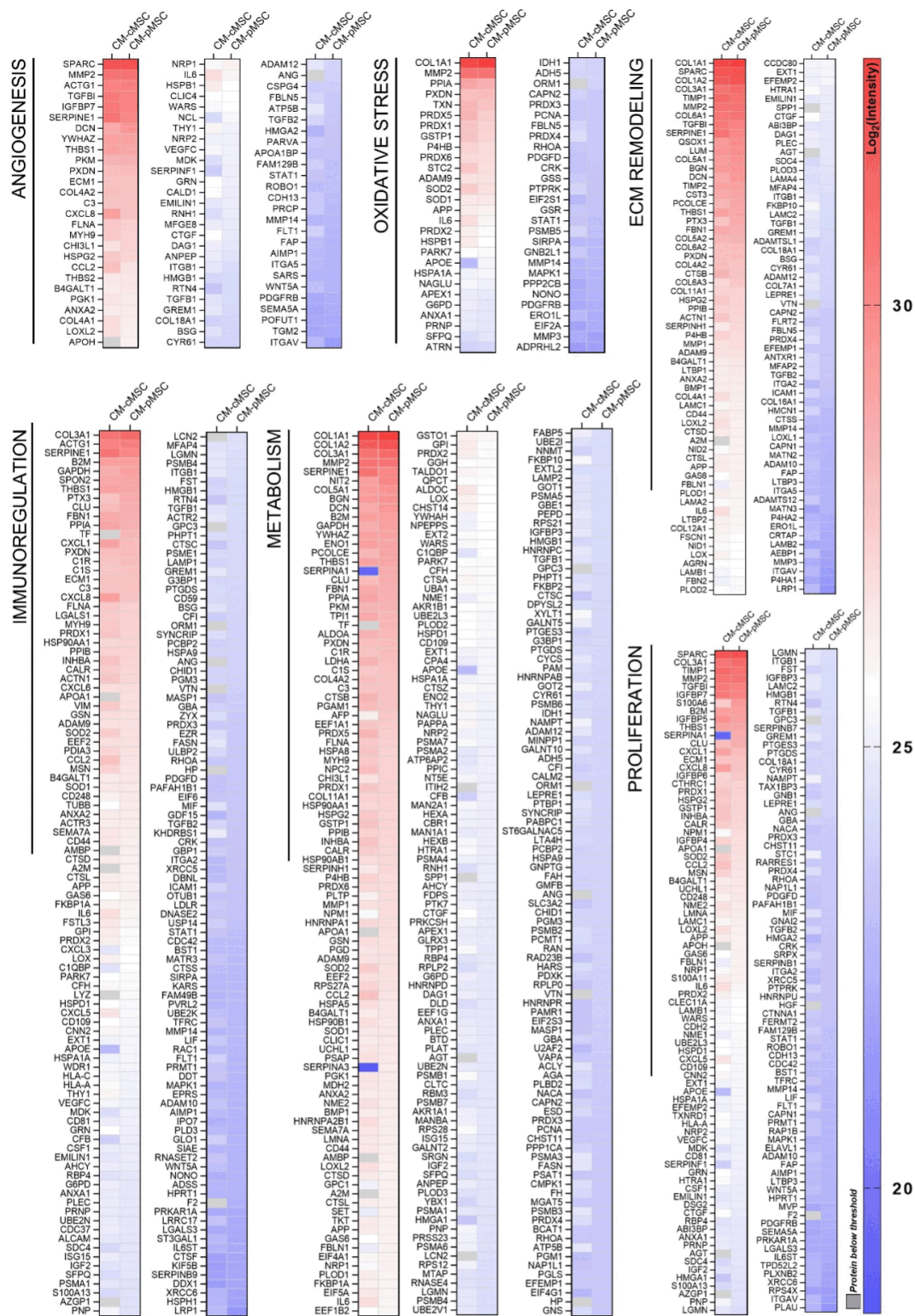


Figure 5

Comprehensive overview of the secretome from primed hnMSCs' proteome linked to each biological process role. Heatmaps of CM-pMSC proteins associated to each biological role, namely angiogenesis, oxidative stress, extracellular matrix remodeling, immunoregulation, metabolism and proliferation.

Legend: CM, conditioned media/secretome; cMSC, hnMSCs non-primed; ECM, extracellular matrix; FDR, false discovery rate; hnMSCs, human neonatal mesenchymal stem cells; pMSC, hnMSCs primed.

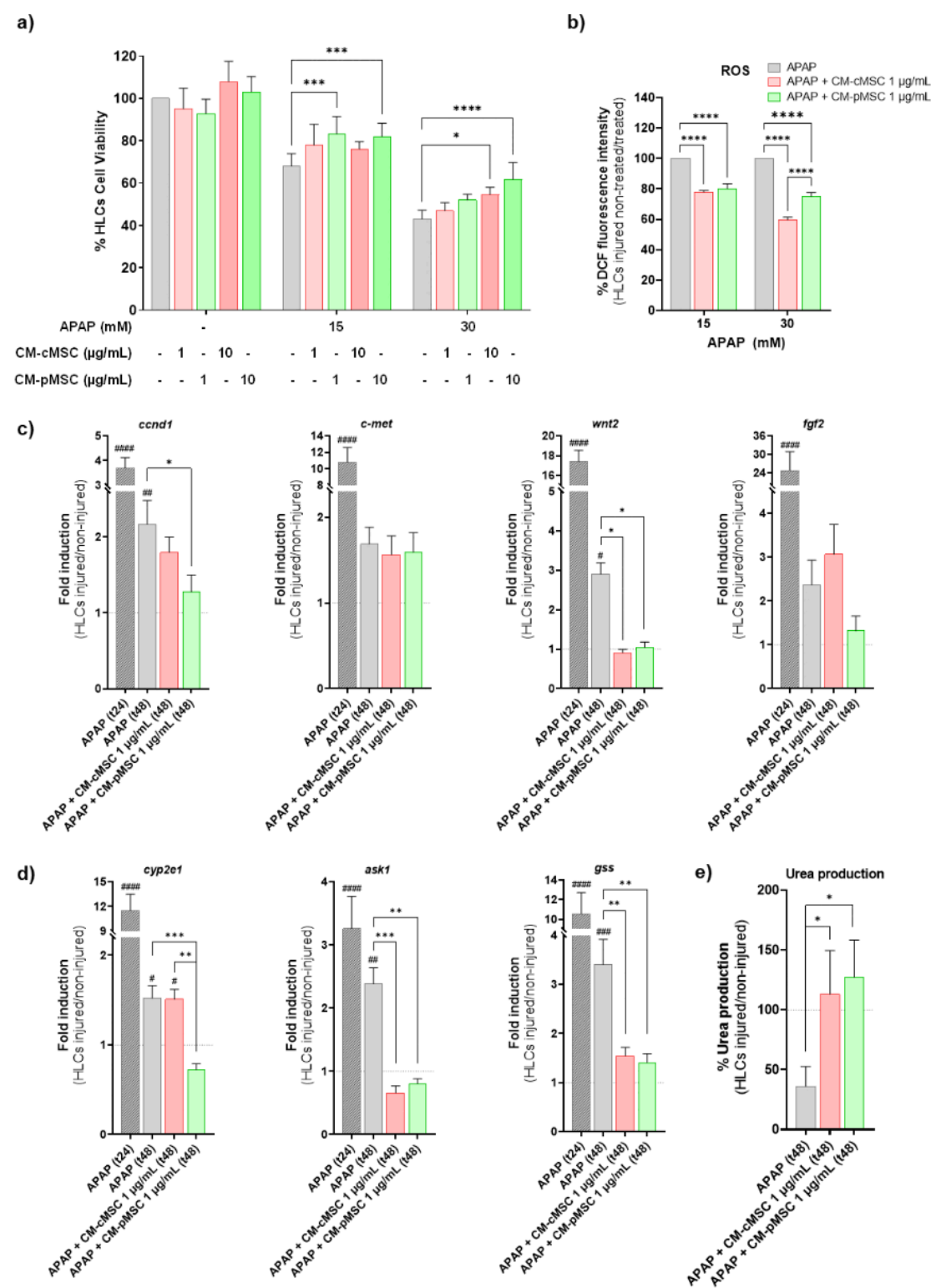


Figure 6

Secretome derived from hnMSCs increased APAP-injured hepatocytes viability and urea production capacity, decreased ROS levels and stimulated a return of gene expression to basal levels. **(a)** Cell

viability of HLCs exposed to 0-30 mM of APAP and subsequently treated with CM-MSC 1-10 µg/mL, compared to non-injured HLCs (100%; n=3); (b) ROS levels of HLCs exposed to 15-30 mM of APAP and subsequently treated with CM-MSC 1 µg/mL using the DCF-DA fluorescent probe (values are expressed relative to APAP-injured HLCs untreated; n=3); (c) Proliferation- and (d) APAP toxicity-related gene expression levels of HLCs exposed to 15 mM of APAP for 24h (t24; dotted grey), followed by 24h with no treatment (grey) or treatment with 1 µg/mL of the secretome derived from non-primed (CM-cMSC, red) or primed hnMSCs with SOS signals (CM-pMSC, green), compared to non-injured HLCs (n=3-4). Grid line represents fold induction equals to 1 (non-injured HLCs). Data is normalized to the reference gene *b-actin*; (d) Urea production of APAP-injured HLCs injured with 15 mM of APAP, treated and non-treated with 1 µg/mL of CM-cMSC or CM-pMSC, compared to non-injured HLCs (100%; n=2-3). Data is represented as Average ± SEM. Statistical significance as * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ and **** $p < 0.0001$; and # $p < 0.05$; ## $p < 0.01$; ### $p < 0.001$ and #### $p < 0.0001$, compared to non-injured control.

Legend: APAP, paracetamol; cMSC, hnMSCs non-primed; CM, conditioned media/secretome; HLCs, hepatocyte-like cells; pMSC, hnMSCs primed with SOS signals; t24, 24h of APAP exposure; t48, 24h after APAP withdrawal.

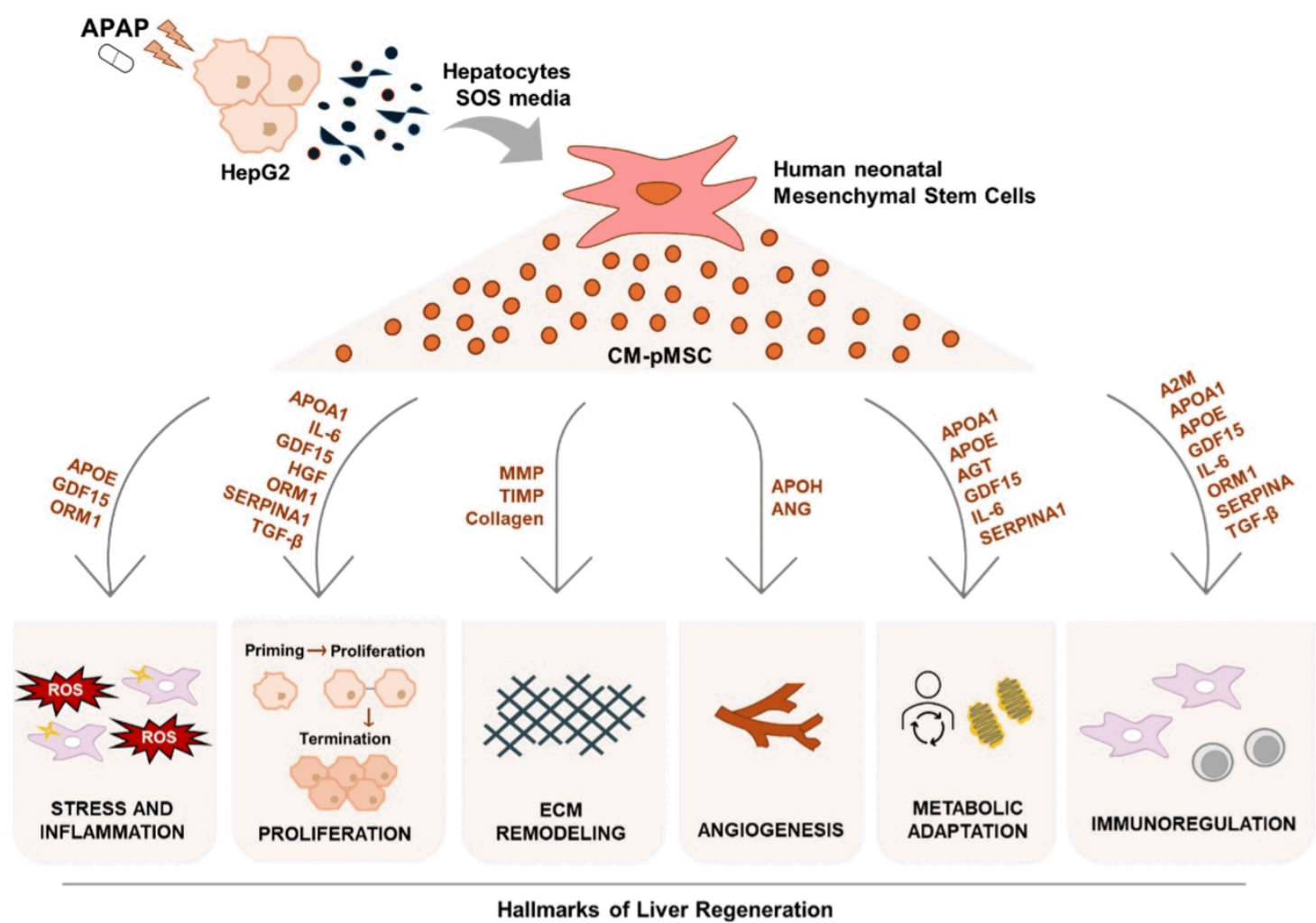


Figure 7

Schematic representation of the potential role of the secretome derived from human neonatal mesenchymal stem cells stimulated with specific hepatic SOS signals (CM-pMSC) on the hallmarks of liver regeneration. CM-pMSC exhibit a multitude of proteins that may support the liver regeneration process through a modulation of not only hepatocyte proliferation, via secretion of APOA1, IL-6, GDF15, HGF, ORM1, SERPINA1 or TGF- β ; but also through modulation of stress and inflammation, via secretion of APOE, GDF15 and ORM1; extracellular matrix remodeling, via MMPs, TIMPs or collagen secretion; angiogenesis, via APOH and ANG; metabolic adaptation, through APOA1, APOE, AGT, GDF15 and SERPINA1; and immunoregulation, via A2M, APOA1, APOE, GDF15, IL-6, ORM1, SERPINA and TGF- β secretion.

Legend: APAP, paracetamol; CM, conditioned media/secretome; ECM, extracellular matrix; pMSC, hnMSCs primed with SOS signals.