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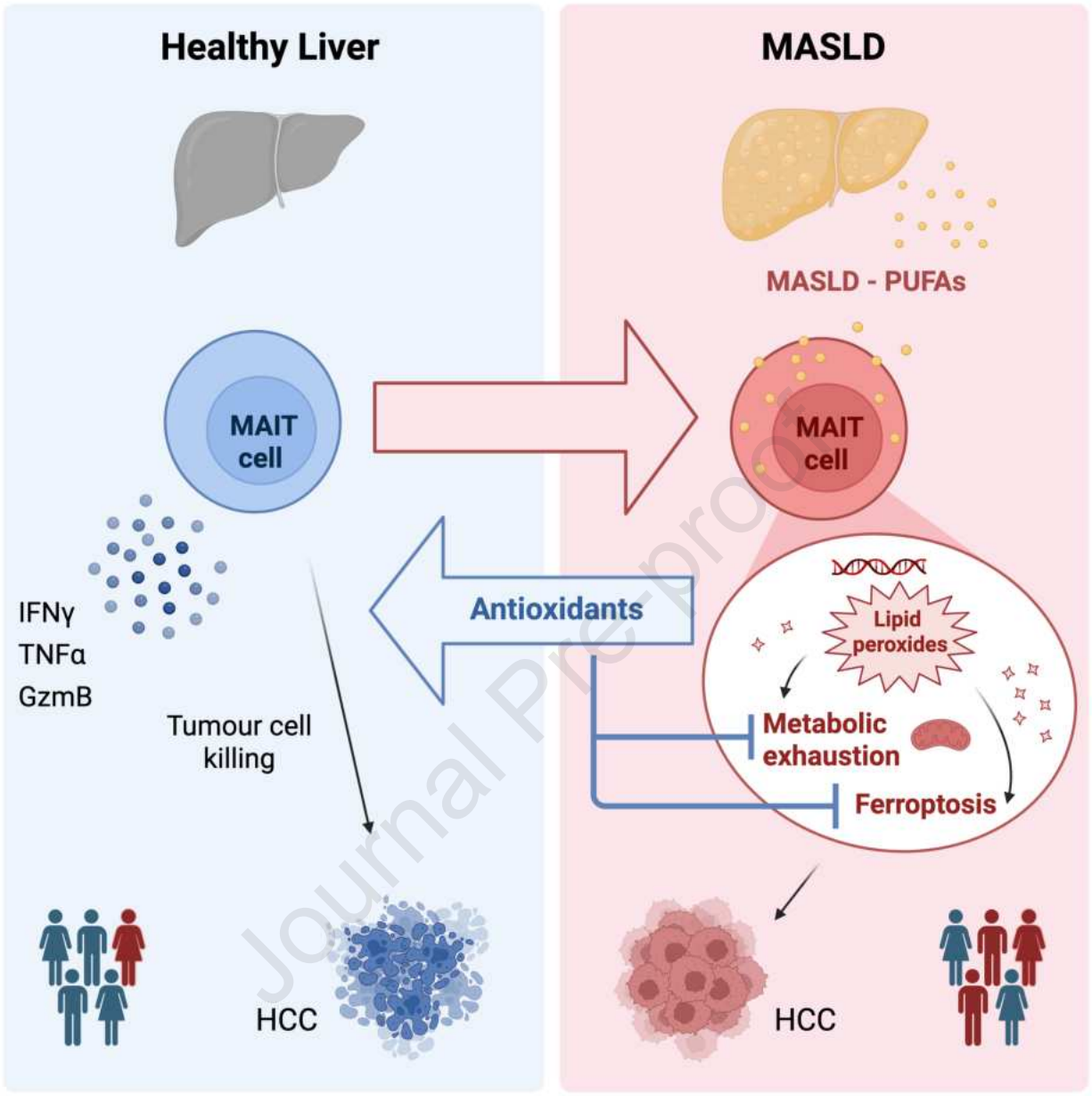

Polyunsaturated fatty acid-induced metabolic exhaustion and ferroptosis impair the anti-tumour function of MAIT cells in MASLD

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Competing interests

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Keywords

MAIT cells; MASLD; HCC; lipid peroxidation; immunometabolism; ferroptosis

Abstract

Background & Aims: Mucosal-associated invariant T (MAIT) cells constitute a highly abundant innate-like T cell population in the human liver that is critical for immune surveillance of hepatic cancers but often dysfunctional in human hepatocellular carcinoma (HCC) for unclear reasons. Here, we sought to determine mechanisms that drive MAIT cells dysfunction in metabolic dysfunction-associated steatotic liver disease (MASLD), a chronic liver disease predisposing patients for HCC development.

Methods: We studied MAIT cell functionality, metabolism and anti-cancer activity directly *ex vivo* in patients with MASLD, as well as in co-culture models mimicking MASLD. (Single-cell) RNA sequencing was used for translation into clinical cohorts of patients with MASLD and MASLD-associated HCC.

Results: We show that MAIT cells have lost their effector functions in patients with MASLD. We uncover that MAIT cell dysfunction is caused by MASLD-associated polyunsaturated fatty acids (PUFAs), which selectively accumulate in MAIT cells but not conventional CD8+ T cells or NK cells. Mechanistically, PUFAs drive MAIT cell dysfunction through intracellular formation of lipid peroxides that promote a state of 'metabolic exhaustion' characterised by compromised mitochondrial respiration and glycolysis in MAIT cells. Excessive signalling through this MASLD-PUFA-lipid peroxide axis results in MAIT cell death by ferroptosis. Interference with PUFA-induced lipid peroxide formation in MAIT cells reversed their metabolic exhaustion and prevented ferroptotic MAIT cell death, thereby restoring MAIT cell effector function and anti-cancer activity. In patients with HCC, high enrichment of the MAIT cell-PUFA gene signature linked to MAIT cell dysfunction was associated with poor survival.

Conclusions: Our findings uncover a novel immunometabolic axis that serves as a functional barrier for MAIT cell-mediated anti-cancer immunity and could be exploited for enhancement of immunotherapy.

Impact and Implications

This study identifies a novel immunometabolic axis by which polyunsaturated fatty acids (PUFAs) accumulating in MASLD liver tissue drive MAIT cell dysfunction through lipid peroxide-induced

metabolic exhaustion and ferroptosis, thereby impairing their anti-tumour activity. These findings reveal how MASLD creates an immune-permissive environment that may facilitate HCC development and -progression. Targeting the PUFA-lipid peroxide axis could restore MAIT cell function and enhance current immunotherapeutic anti-cancer strategies.

Introduction

Metabolic dysfunction-associated steatotic liver disease (MASLD) is the most common chronic liver disease affecting more than 1.6 billion adults worldwide[1-3]. MASLD is characterised by hepatic

accumulation of fatty acids and local liver inflammation, resulting in impaired hepatic immune surveillance. This is reflected by an increased risk of MASLD patients for the development of hepatocellular carcinoma (HCC) [4]. Only a minority of MASLD-HCC patients respond to immunotherapy targeting the PD-1 axis[5,6]. Therefore, there is an urgent need to understand the cellular and molecular mechanisms that determine reduced intrahepatic immune surveillance in MASLD and to advance HCC immunotherapy.

Mucosal-associated invariant T (MAIT) cells are innate-like T cells present in high abundance in the human liver[7,8] that have emerged as key cellular players in the development of liver fibrosis [7,9,10] and immunosurveillance of HCC[11,12]. The anti-cancer role of MAIT cells is attributed to their ability to rapidly secrete effector cytokines and cytolytic molecules with anti-cancer activity upon recognition of vitamin B derived antigens[13,14] and antigen-independent triggering by innate immune stimuli[7,15,16]. However, in patients with chronic liver disease and HCC, MAIT cells often display a dysfunctional phenotype[7,17,18] that is thought to contribute to a failure of cancer immune control[12,19]. The mechanisms controlling MAIT cell function in tissues remain poorly characterized, and it is unclear whether their dysfunction in the liver is promoted by factors derived from the liver microenvironment. In MASLD, accumulation of fatty acids causes intrahepatic oxidative stress including lipid peroxidation, that is thought to promote liver injury[20-22]. Whether accumulating fatty acids modulate the redox homeostasis and effector function of intrahepatic T cell populations in MASLD patients, and to what extent this impacts intrahepatic immunity remains poorly understood. Here, we show that MAIT cells are highly dysfunctional in patients with MASLD and reveal that their dysfunction is caused by polyunsaturated fatty acids (PUFAs) accumulating in MASLD. Mechanistically, PUFAs abrogate the ability of human MAIT cells to express effector molecules and to kill HCC tumour cells by inducing intracellular formation of lipid peroxides, thereby promoting a state of 'metabolic exhaustion' that is responsible for MAIT cell dysfunction. We further provide evidence that excess quantities of PUFAs commit dysfunctional MAIT cells to ferroptotic cell death. Finally, we demonstrate that interference with PUFA-induced lipid peroxidation protects MAIT cells from metabolic exhaustion, restores their

anti-cancer effector function and prevents MAIT cell ferroptosis. In conclusion, as the MAIT cell-PUFA axis is relevant for clinical outcomes of patients with HCC, we identify an immunometabolic axis that controls MAIT cell function in MASLD patients and could be targeted for MAIT cell-based anti-cancer immunotherapy, thereby enhancing current treatment strategies against HCC.

Results

MAIT cells are dysfunctional in MASLD patients

To determine the impact of MASLD on human MAIT cell biology and function, we analysed MAIT cells in a cohort of 30 MASLD patients and healthy controls (HC). Using MR1-5-OP-RU tetramer staining to reliably identify MAIT cells among T lymphocytes[13,23] (Fig. S1A), we found that circulating MAIT cells in MASLD patients displayed markedly increased expression of CD38, HLA-DR (Fig. S1B), PD-1 and CD39 (Fig. 1A), suggesting a dysfunctional state of MAIT cells in MASLD patients[24-27]. We therefore probed MAIT cells from MASLD patients for their capacity to express key effector molecules. Circulating MAIT cells from MASLD patients were substantially compromised in their ability to produce key effector molecules such as IFNγ (Fig. 1B), IL-17A (Fig. S1C), granzyme B (Gzmb) and perforin (Fig. 1C) upon TCR-independent stimulation by IL-12 and IL-18[15] or similarly following TCR-dependent stimulation (Fig. S1D). MAIT cells from MASLD patients further showed markedly reduced proliferation in response to *ex vivo* stimulation with *E. coli*-derived antigen (Fig. 1D), indicative of impaired MAIT cell proliferative expansion[28]. Consistently, we found a reduced frequency (Fig. 1E, Fig. S1E) and absolute number (Fig. 1F) of circulating MAIT cells in our MASLD patient cohort as reported previously[7]. Taken together, these data demonstrate that (1) circulating MAIT cells are dysfunctional in human MASLD patients and that (2) MAIT cell paucity is a characteristic feature of MASLD patients. Next, we extended our analyses to MAIT cells in liver tissue. First, we analysed RNA-sequencing (RNA-seq) datasets for hepatic immune cells from two independent cohorts of human MASLD patients[29,30]. Using CIBERSORTx[31], we determined the frequency of intrahepatic MAIT cells and found that they

were significantly reduced in both patient cohorts (Fig. 1G). To investigate the functionality of intrahepatic MAIT cells, we analysed a publicly available singe-cell RNA sequencing (scRNA-seq) dataset of intrahepatic CD45+ immune cells from human MASLD and control liver tissue[5]. MAIT cells were identified by *SLC4A10*[32,33]expression, which marked a cluster of MAIT cells in MASLD patients and healthy controls (Fig. 1H) that was confirmed by applying other established MAIT cell scores (Fig. S1F). Consistent with our analyses of circulating MAIT cells, intrahepatic MAIT cells from MASLD patients showed significant upregulation of marker genes for activation-induced T cell dysfunction (*ENTPD1, PDCD1, CD38, HLA-DRA*) (Fig. 1I and Fig. S1G). Consistently, gene set enrichment analysis (GSEA) revealed lower expression of gene signatures for T cell cytotoxicity and T cell-mediated anti-cancer immunity in intrahepatic MAIT cells from MASLD patients compared to controls (Fig. 1J). We extended our analyses to a murine model of western diet-induced MASLD[34] (Fig. S1H-I). Here, compared to normal chow controls and similar to human patients, we observed a clear decline of intrahepatic MAIT cell frequency at early-stage MASLD, which was preserved late-stage disease (Fig.S1J), although the latter did not reach statistical significance in this experiment. Importantly, we also found that intrahepatic MAIT cells display an activated phenotype in early-stage MASLD, which becomes even more pronounced in late-stage MASLD (Fig. S1K), where MASLD-associated HCC has already developed (Fig. S1I). *Ex vivo* analyses further demonstrated a MASLD-associated functional impairment of intrahepatic MAIT cells, again apparent in both early-stage MASLD and late-stage MASLD/MASLD-HCC (Fig. S1L), indicating that MAIT cells become dysfunctional upon MASLD induction. Taken together, these data indicate that MASLD causes the dysfunction and paucity of MAIT cells, raising the question as to which mechanisms determine their functional impairment and reduced abundance in this disease.

MAIT cell dysfunction is mediated by PUFAs accumulating in MASLD

Intrahepatic accumulation of free fatty acid (FFA) species is a characteristic feature of MASLD[21,22]. Mass spectrometry profiling of FFAs previously described to accumulate in MASLD patients[35,36]

revealed significant enrichment of multiple saturated-, mono-/di-unsaturated- and polyunsaturated long-chain FFAs in the peripheral blood in our MASLD patient cohort (Fig. 2A, Fig. S2A). To test the effect of these distinct long-chain FFA classes on MAIT cell effector function, we exposed circulating and hepatic MAIT cells from healthy individuals to the saturated FFAs palmitic acid (C16:0) and stearic acid (C18:0), the mono- and di-unsaturated FFAs oleic acid (C18:1) and linoleic acid (C18:2), or the polyunsaturated FFAs arachidonic acid (C20:4) and docosahexaenoic acid (C22:6), which were abundant in steatotic liver tissue (Fig. 2B). We found that such treatment resulted in intracellular lipid accumulation for (poly-) unsaturated but not saturated fatty acids (Fig. 2C). Next, we asked to what extent FFAs impact MAIT cell effector functions. Strikingly, these analyses revealed an inhibitory effect of PUFAs on the production of IFNγ, TNFα and GzmB (Fig. 2D-F, Fig. S2B-D) by both circulating and hepatic MAIT cells. Of note, this inhibitory effect was selective for polyunsaturated FFAs and not observed upon MAIT cell treatment with unsaturated, mono-unsaturated or di-unsaturated FFA species (Fig. 2 D-F, Fig. S2B-D). We also confirmed that PUFAs inhibited effector cytokine expression by MAIT cells in a dose-dependent manner (Fig. S2D) and that PUFA serum concentrations correlated with expression of MAIT cell activation and exhaustion marker expression (Fig. 2G). Of note, in contrast to MAIT cells, conventional CD8+ T cells including CD8+ effector memory T cells (TEM), and natural killer (NK) cells largely failed to accumulate FFAs (Fig. S2E). In line with their reduced accumulation of fatty acids, their functionality was largely unaltered by PUFAs (Fig. S2F). Collectively, these findings point towards a so-far unknown PUFA-mediated mechanism for MAIT cell dysfunction in MASLD patients.

MASLD-associated PUFAs inhibit MAIT cell killing of HCC tumour cells

Studies in preclinical mouse models suggest that MAIT cells contribute to immunosurveillance of cancer including HCC[11,37]. However, whether MAIT cells can directly eliminate tumour cells, and if this applies to human MAIT cells remains unclear. To test this possibility, we established an *in vitro* co-culture system for analysis of human MAIT cell-mediated killing of HCC cells. We found that *ex vivo*-

activated MAIT cells exhibited potent tumour cell killing capacity against multiple HCC cell lines including HepG2, PLC/PRF/5, SNU398 (Fig. 3A) and HuH-7 (Fig. S3A) but not primary hepatocytes (Fig.3B), clearly demonstrating that human MAIT cells can efficiently and specifically eliminate human HCC cells. Mechanistically, killing of HCC cells by MAIT cells required TCR-dependent activation of MAIT cells (Fig. 3C) and direct cell-cell contact (Fig. 3D), but was independent of MR1 (Fig. S3B). Cell contact-dependent immune cell-mediated killing of cancer cells often involves Fas signalling[38]. MAIT cells robustly expressed Fas ligand (FasL), which was further upregulated upon MAIT cell stimulation (Fig. 3E). Similarly, co-culture with activated MAIT cells (Fig. 3F) or exposure to the MAIT cell effector cytokines IFNγ and TNFα (Fig. 3G) resulted in increased Fas expression by HCC cells. To explore the relevance of MAIT cell effector cytokines and the Fas-FasL axis for MAIT cell cytotoxicity against HCC cells, we added neutralising antibodies against FasL, IFNγ and TNFα to MAIT cell – HCC co-cultures. We found that neutralisation of both MAIT cell derived IFNγ and TNFα in combination with anti-FasL was necessary to prevent killing of HCC cells by MAIT cells (Fig. 3H, Fig. S3C).

We next asked to what extent MASLD-associated PUFAs can interfere with this newly discovered MAIT cell anti-cancer function. Strikingly, MAIT cells exposed to MASLD-PUFAs almost completely lost their tumour cell killing capacity (Fig. 3I-J, Fig. S3D), which was not the case for MAIT cells treated with saturated long-chain FFA (Fig. 3I-J). Taken together, these data suggest that MAIT cell dysfunction caused by MASLD-PUFAs limits MAIT cell anti-cancer activity against HCC tumour cells.

MASLD-associated PUFAs cause lipid peroxidation in MAIT cells

To explore the mechanism by which MASLD-associated PUFAs promote MAIT cell dysfunction, we isolated MAIT cells from healthy human individuals, treated the cells with saturated-, di-unsaturated- or polyunsaturated FFAs, and determined their gene expression profiles by RNA-seq. Principle component analysis (PCA) revealed that MAIT cells treated with PUFAs clustered separately from MAIT cells treated with di-unsaturated- or saturated fatty acids, which clustered together with non-FFA treated MAIT cells (Fig. 4A). Analysis of differentially expressed genes (DEGs) revealed that both

PUFAs C20:4 and C22:6 induced upregulation of a core set of 311 genes and downregulation of 167 genes (Fig. S4A, supplementary table S2), pointing towards a distinct transcriptional programme associated with PUFA-induced MAIT cell dysfunction. Pathway analyses uncovered that pathways related to T cell activation and -signalling were among the top underrepresented pathways in PUFA-exposed MAIT cells compared to non-PUFA-treated controls (Fig. 4B, Fig. S4B, supplementary table S3). In contrast, pathways related to oxidative stress and ferroptosis, a cell death pathway caused by excessive intracellular accumulation of lipid peroxides[39], were enriched in PUFA-exposed MAIT cells (Fig. 4B, Fig. S4B, supplementary table S3). We corroborated these findings by GSEA, which demonstrated PUFA-dependent enrichment of gene sets related to the regulation of oxidative stress and ferroptosis, and downregulation of gene sets related to TCR- and cytokine-mediated signalling (Fig. 4C). Consistently, genes associated with MAIT cell activation were downregulated in PUFA-exposed MAIT cells, while reactive oxygen species (ROS) and ferroptosis-related genes were upregulated (Fig. 4D). Of note, these alterations were selective for MAIT cells exposed to PUFAs but not di-unsaturated and saturated FFAs (Fig. 4C, D, Fig. S4B). Non-stimulated MAIT cells phenocopied stimulated MAIT cells regarding PUFA-induced transcriptional changes (Fig. S4C). Collectively, these findings suggest that PUFA-induced MAIT cell dysfunction is linked to oxidative stress and the emergence of lipid peroxides.

By flow cytometry, we confirmed that PUFAs selectively induce the formation of mitochondrial ROS (mito-ROS) (Fig. 4E) and lipid peroxides (Fig. 4F) in MAIT cells. Of note, prevention of mito-ROS formation by the mitochondria-targeted antioxidant mitoTEMPO (Fig. S4D) diminished lipid peroxide formation in MAIT cells (Fig. S4E), suggesting that lipid peroxidation is a consequence of mito-ROS formation. To determine the relevance of lipid peroxides for MAIT cell function, we induced lipid peroxidation in MAIT cells by cumene hydroperoxide (Fig. S4F), which, in a dose-dependent manner, reduced the ability of MAIT cells to express GzmB and IFNγ (Fig. 4G). Taken together, these data demonstrate that MASLD-associated PUFAs induce lipid peroxidation in MAIT cells and suggest that this process underlies MAIT cell dysfunction in MASLD patients.

Consistently, we found increased expression of genes associated with oxidative stress and lipid peroxidation (*CHAC1, NFE2L2, KEAP1, ALOX5, ALOX12, ALOX15)* in steatotic human liver tissue compared to non-steatotic human liver tissue (Fig. 4H). Moreover, analysis of scRNA-seq data of intrahepatic MAIT cells revealed enrichment of ROS pathway and KEAP1-NFE2L2 gene signatures in intrahepatic MASLD MAIT cells (Fig. 4I). These findings indicate that MAIT cells experience oxidative stress in FFA-rich MASLD liver tissue and that this may cause their functional impairment. To test this possibility, we derived gene signatures for PUFA-induced MAIT cell dysfunction from our RNA-seq analyses and probed their expression in scRNA-seq data of intrahepatic MAIT cells from MASLD patients. Indeed, we found significantly increased enrichment of signatures derived from the top 50 upregulated genes by C20:4 and C22:6 in intrahepatic MAIT cells from MASLD patients (Fig. 4J) as well as enrichment of key genes relevant for several top-upregulated pathways by PUFAs in intrahepatic MAIT cells (Fig. S4G-H), arguing that PUFA-induced transcriptional changes are inflicted on MAIT cells within the liver microenvironment in MASLD patients. Together, these data suggest that PUFAs accumulating in MASLD patients promote MAIT cell dysfunction through induction of oxidative stress including MAIT cell-intrinsic lipid peroxidation.

PUFA-induced lipid peroxidation leads to metabolic exhaustion in MAIT cells

Oxidative stress is closely linked to oxidative phosphorylation (OXPHOS) [40,41], which occurs in mitochondria. Extracellular flux analyses of oxygen consumption rate (OCR) as a measure of OXPHOS revealed a rapid decrease of OCR in MAIT cells exposed to MASLD-associated PUFAs but not mono-/di-unsaturated or saturated FFAs (Fig. 5A). Moreover, PUFAs selectively caused a substantial reduction of MAIT cell mitochondrial potential (Fig. 5B) and -mass (Fig. 5C) in a dose-dependent manner (Fig. S5A-C). Interestingly, PUFA-mediated impairment of mitochondrial function in MAIT cells was linked to a subsequent, dose-dependent decline of extracellular acidification rate (ECAR) (Fig. 5D-E and Fig. S5D), indicating that PUFA-induced oxidative stress also negatively impacts on MAIT cell

glycolysis. We hereafter refer to this state of corrupted mitochondrial respiration and glycolysis caused by MASLD-associated PUFAs as 'metabolic exhaustion'.

ROS-mediated damage or functional impairment of mitochondria can result in compromised cellular functions[42]. We therefore tested whether PUFA-induced ROS and lipid peroxides are responsible for metabolic exhaustion of MAIT cells. Indeed, we observed rapid ROS-formation in MAIT cells after PUFA exposure (Fig. S5E). MAIT cell treatment with the ROS scavenger α-tocopherol[43] however prevented PUFA-induced formation of mito-ROS (Fig. 5F) and lipid peroxides (Fig. 5G). Strikingly, treatment of PUFA-exposed MAIT cells with α-tocopherol or N-acetylcysteine (NAC), a second clinically-approved antioxidant drug[44], resulted in a marked increase of both mitochondrial respiration (Fig. 5H), glycolysis (Fig. 5I) and mitochondrial membrane potential (Fig. 5J). Collectively, these findings suggest a model by which MASLD-associated PUFAs promote the generation of ROS and lipid peroxides that lead to metabolic exhaustion of human MAIT cells, which can be prevented by antioxidants.

Excessive PUFA-dependent lipid peroxidation culminates in MAIT cell ferroptosis

The reduced abundance of MAIT cells in MASLD patients raised the question to which extent MASLD-associated PUFAs, beyond promoting MAIT cell dysfunction, promote MAIT cell death. This idea is additionally supported by our finding on increased expression of genes related to ferroptosis, a cell death pathway caused by excessive accumulation of lipid peroxides[39]. To test whether PUFA-induced lipid peroxidation can lead to MAIT cell ferroptosis, we first analysed MAIT cell survival following treatment with different FFAs. We observed a significant reduction of MAIT cell viability after exposure to MASLD-associated PUFAs but not other FFA types (Fig. 6A). Along these lines, PUFA-exposed MAIT cells showed enhanced production of the bioactive lipid prostaglandin E2 (PGE2) (Fig. S6A), a marker indicative of ferroptosis[45]. We therefore treated PUFA-exposed MAIT cells with a selective inhibitor for lipid peroxidation and ferroptosis, Liproxstatin-1[46], which effectively prevented lipid peroxide formation in MAIT cells (Fig. 6B) and restored their survival (Fig. 6C). In contrast to Liproxstatin-1, iron depletion did not affect lipid peroxide formation in PUFA-exposed MAIT cells (Fig.

S6B) and did therefore not prevent MAIT cell metabolic exhaustion (Fig. S6C) and dysfunction (Fig. S6D).Complementary assays revealed that low lipid peroxide levels in MAIT cells promote MAIT cell dysfunction, whereas MAIT cell ferroptosis only occurred at high lipid peroxide levels (Fig. 6D-E). Moreover, PUFA-induced MAIT cell death occurred much later than reduction of their killing capacity and metabolic exhaustion (Fig. S6E, Fig. 3I, Fig. 5A, D). Thus, MAIT cells can exist in a dysfunctional, lipid-peroxide enriched state, while excessive accumulation of lipid peroxides causes their ferroptotic death.

Comparative analyses of MAIT cells, conventional T cell subsets and NK cells revealed increased vulnerability towards PUFA-induced lipid peroxidation and ferroptosis by MAIT cells compared to their conventional and innate counterparts (Fig. 6F-G, Fig. S6F-G). Consistently, analyses of scRNA-seq data on lymphocytes from MASLD patients revealed selective enrichment of a ferroptosis driver gene signature in MAIT cells but not CD8+ T cells (Fig. 6H). Notably, serum PUFA concentrations (Fig. 6I) as well as hepatic fat content (Fig. 6J) negatively correlated with MAIT cell frequency. These findings indicate a higher susceptibility of MAIT cells to ferroptotic cell death induced by MASLD-associated PUFAs and imply that this is at least partly responsible for the selective reduction of MAIT cells in MASLD patients (Fig. 6K).

Targeting lipid peroxide-induced metabolic exhaustion and ferroptosis restores MAIT cell tumour killing capacity and enhances their viability

We finally aimed to determine whether MAIT cell effector function and viability can be rescued by targeting the PUFA-lipid peroxide axis we have identified. To this end, we prevented lipid peroxide-induced metabolic exhaustion by α-tocopherol and NAC in PUFA-exposed MAIT cells and analysed MAIT cell effector functions. Antioxidant treatment resulted in a prominent rescue of effector cytokine (Fig. 7A-B) and cytolytic molecule (Fig. 7C) expression by MAIT cells. Strikingly, α-tocopherol and NAC also restored the capacity of PUFA-exposed MAIT cells to kill HCC cells (Fig. 7D-E). These data strongly suggest that targeting metabolic exhaustion of MAIT cells caused by PUFA-induced lipid

peroxidation can prevent MAIT cell dysfunction. Finally, we found that NAC and α-tocopherol substantially enhanced the survival of MAIT cells exposed to MASLD-associated PUFAs (Fig. 7F). This demonstrates that prevention of PUFA-induced metabolic exhaustion by antioxidant-mediated scavenging of lipid peroxides restores MAIT cell effector function, tumour cell killing and survival, and may thereby enhance immune responses against HCC.

To investigate the relevance of this PUFA-lipid peroxide axis for disease progression and -outcome in human patients with MASLD, we analysed an RNA-seq dataset of hepatic immune cells from MASLD patients with different disease stages[30]. We observed higher expression of the MAIT cell-PUFA score that was linked to MAIT cell dysfunction in patients with advanced MASLD stages, i.e. higher non-alcoholic fatty liver disease activity (NAS) scores[48] (Fig. 7G). Consistently, expression of the MAIT cell-PUFA signature correlated with patients' NAS and fibrosis scores (Fig. 7H), suggesting that PUFA-induced MAIT cell dysfunction is linked to MASLD progression. We next probed for expression of gene signatures associated with PUFA-induced MAIT cell dysfunction in a scRNA-seq dataset from liver tissue of HCC patients with different underlying liver diseases[49]. These analyses revealed a significantly higher expression of dysfunction-related signatures in intrahepatic MAIT cells from HCC patients with non-viral liver disease (including patients with MASLD), compared to MAIT cells from patients with HBV-/HCV-associated HCC (Fig. 7I). This suggests that PUFA-induced MAIT cell dysfunction is specific to liver diseases associated with hepatic steatosis. Finally, we assessed whether the MAIT cell PUFA-lipid peroxide axis we have identified is relevant for HCC patient outcome. Therefore, we analysed gene expression data in a cohort of HCC patients derived from The Cancer Genome Atlas (TCGA). We found that high expression of MAIT cell-PUFA signatures was correlated with poorer HCC patient survival in patients with steatotic, i.e. non-viral- (Fig. 7J), but not non-steatotic, i.e. viral-associated HCC (Fig. S7A), arguing that PUFA-induced MAIT cell dysfunction contributes to impaired HCC immune control.

Discussion

MAIT cells are emerging key players in liver immunosurveillance[7,37,50] but often dysfunctional in patients with chronic liver diseases or HCC[7,19,37]. There is an urgent need to identify the mechanisms underlying MAIT cell dysfunction in these patients to help the development of novel, MAIT cell-based strategies to advance current immunotherapy against HCC.

Our finding that human MAIT cells directly exert cytotoxic effector function against HCC cells, implies a contribution of MAIT cells to anti-HCC immunity in humans. In line with this idea, the reduction of hepatic MAIT cells negatively correlates with HCC patient survival, and MAIT cells protect against HCC development in preclinical mouse models[37,51]. Here, we uncover that MAIT cells are dysfunctional in patients with MASLD, a chronic liver disease with rapidly increasing worldwide incidence that drives HCC development[1-3,52,53], and that MAIT cell dysfunction is caused by an immunometabolic MASLD-PUFA-lipid peroxide axis. We thereby reveal a link between MASLD and impaired MAIT cell function that is mediated by a distinct class of metabolites accumulating in MASLD liver tissue, i.e. PUFAs.

Energy metabolism is crucial for immune cell effector function as it provides bioenergetic building blocks for cell proliferation and effector differentiation[54,55]. Our results now demonstrate that PUFAs accumulating in metabolic diseases curtail mitochondrial function by inducing lipid peroxidation, demonstrating a link between altered metabolic environments and immune cell metabolism and -function. Limiting mitochondrial respiration in MAIT cells will likely impair MAIT cell effector functions by limiting their energy supply. Moreover, increased lipid peroxidation is known to limit the functional properties of membranes[56]. Thus, lipid peroxidation at the mitochondrial membrane may in particular compromise electron transport essential for OXPHOS and ATP generation[57]. In addition, this may hinder binding of hexokinase, the rate-limiting enzyme of glycolysis, to the mitochondrial membrane[58] and thereby hamper an essential step of glycolysis, which may explain our finding that glycolysis is also compromised in PUFA-exposed MAIT cells.

Our data clearly demonstrate that by rescuing MAIT cell mitochondrial respiration and glycolysis, antioxidants restore their cytotoxic effector function. This suggests that the metabolic exhaustion we

identify as a hallmark of impaired MAIT cell effector function may have important implications for advancing immunotherapeutic strategies targeting MAIT cell anti-cancer function. It will be of great interest to determine in future studies whether targeting lipid peroxidation, e.g. by antioxidants, in MAIT cells can reverse their metabolic exhaustion in patients and thereby enhance their functionality against cancers. Notably, vitamin E, an antioxidant we show to reverse MAIT cell metabolic exhaustion, can improve features of MASLD[59,60] and may therefore be used for treatment of patients with MASLD who are at risk of developing HCC.

Our results further suggest that the PUFA-lipid peroxide axis only minimally affects the function of conventional CD8+ T cells and NK cells, implying the existence of mechanisms that selectively promote MAIT cell versus CD8+ T cell dysfunction. Indeed, the key features of PUFA-mediated MAIT cell dysfunction are fundamentally different to the mechanisms responsible for dysfunction of conventional T cells that are mediated by transcriptional and epigenetic programming through transcription factors such as TOX[61,62]. It will be important to determine whether existing approaches to improve CD8+ T cell-mediated anti-tumour immunity may be further enhanced by concurrently targeting the specific mechanisms driving MAIT cell dysfunction in liver cancer. Our data demonstrate a dose-dependent effect of MASLD-PUFAs on MAIT cell effector function and -survival. While low PUFA concentrations cause lipid peroxidation leading to a state of metabolic exhaustion, high PUFA concentrations trigger MAIT cell ferroptotic death. These findings help to explain why the functional impairment of MAIT cells in MASLD is accompanied by MAIT cell paucity[7,9]. Targeting this mechanism may thus be an attractive therapeutic means to overcome the scarcity of MAIT cells in diseased liver tissue and to elicit their anti-cancer activity. The sensitivity of MAIT cells to PUFA-induced ferroptotic death may be linked to HCC development in early stages of MASLD that are characterised by hepatic fat accumulation[63,64]. Notably, hepatic fat accumulation is a common feature of chronic liver disease independent of the disease aetiology[65-67], implying that PUFA-mediated MAIT cell dysfunction and -ferroptosis may be relevant for reduced immune surveillance across liver disease entities. Targeting

the PUFA - lipid peroxide axis in MAIT cells could therefore be an attractive therapeutic target for simultaneously enhancing MAIT cell functionality and abundance in human patients.

Figure Legends

Fig. 1: MAIT cells are dysfunctional in MASLD patients

(A) *Ex vivo* surface expression of PD-1 and CD39 on MAIT cells from peripheral blood analysed by flow cytometry. (B, C) Intracellular cytokine expression in MAIT cells following *ex vivo* stimulation with IL-12 and IL-18 for 24 h. (D) Proliferation of MAIT cells after stimulation with fixed *E. coli* for 7 days. (E) MAIT cell frequency and (F) absolute numbers in peripheral blood. (G) MAIT cell abundance in human liver samples from GSE126848[29] and GSE135251[30] calculated by CIBERSORT. (H) UMAP of intrahepatic immune cells and feature plot of SLC4A10 expression (GSE159977 from Pfister et al., Nature 2021[5]). (I, J) Gene expression/ gene signature scores of indicated genes or gene sets (GO:0002711, GO:0001916, GO:0002424) in single cell transcriptomes of MAIT cells from human liver tissue (GSE159977). (A-F) MAIT cells in a PBMC pool were analysed. Data represent mean +/- SEM, pooled data from at least 3 independent experiments. (G) Data represent mean +/- SD. (I-J) Data represent median with IQR. ***p<0.001, **p<0.01, *p<0.05, ns = p>0.05 compared to healthy control or as indicated assessed by Welch's t-test (A-F), One-way ANOVA with Tukey's multiple comparison test (G) or Wilcoxon-test (I-J). Each dot represents one individual (A-G).

Fig. 2: MAIT cell dysfunction is mediated by poly-unsaturated fatty acids (PUFAs) accumulating in MASLD

FFA profiles in (A) blood serum of MASLD patients (n=11) and HC (n=15) or (B) MASLD liver tissue measured by mass spectrometry (MS). (C) Intracellular lipid content in purified MAIT cells (HC) after incubation with FFAs for 16 h. Intracellular cytokine expression in purified circulating (D-E) and intrahepatic (F) MAIT cells after stimulation with aCD3/CD28 and IL-12/IL-18 for 24 h in the presence of FFAs. (G) Spearman correlation between MAIT cell expression of surface activation markers and PUFA serum concentrations. Data represent mean +/- SEM (C, E, F), pooled data from at least 3 independent experiments, or mean +/- SD (B). ***p<0.001, **p<0.01, *p<0.05, ns = p>0.05 compared

to untreated control or as indicated, assessed by Welch's t-test or Repeated measures ANOVA (C, E, F). Each dot represents one individual (B-C, E-G).

Fig. 3: MASLD-associated PUFAs inhibit MAIT cell killing of HCC tumour cells

MAIT cell killing activity against hepatic tumour cell lines (A) or primary hepatocytes (B) after stimulation with aCD3/CD28. MAIT cell killing activity against HepG2 cells after stimulation with IL-12/IL-18 and/or aCD3/CD28 (C) and restriction of cell-cell contact by transwells (D). (E) FasL surface expression by MAIT cells. Fas surface expression by HepG2 cells co-cultured with activated MAIT cells (F) or after treatment with TNFα and/or IFNγ (200ng/mL) (G). MAIT cell killing activity against HepG2 cells in the presence of blocking antibodies against FasL, TNFα and IFNγ (H) or FFAs (I-J). Data represent mean +/- SD, pooled data from at least 3 independent experiments (A-D, F, H, J). ***p<0.001, **p<0.01, *p<0.05, ns = p>0.05 compared to untreated control or as indicated assessed by One-way ANOVA (C-H) or Welch's test (A-B). Each dot represents one individual (A-F, H (right panel), J).

Fig. 4: MASLD-associated PUFAs cause lipid peroxidation in MAIT cells

(A-D) RNA-seq analysis of purified MAIT cells following treatment with different FFAs. (A) Principal component (PC) analysis based on differentially expressed genes in purified MAIT cells following treatment with FFAs +/- *ex vivo* stimulation with aCD3/CD28 and IL-12/IL-18 for 12 h. (B) Gene Set Enrichment Analysis (GSEA) results for gene sets derived from WikiPathways and (C) selected gene sets from WikiPathways (WP100, WP2884, WP5072, WP4313) and KEGG (hsa04060, hsa04062). (D) Heatmap showing z-transformed gene expression values for indicated genes. (E) 2h mito-ROS and (F) 16h lipid peroxide expression in purified MAIT cells after culture with different FFAs. (G) Intracellular cytokine expression in stimulated purified MAIT cells incubated with cumene hydroperoxide (CuOOH) for 24 h. (H) Relative gene expression in steatotic and non-steatotic liver tissue. (I) Gene signature scores in single cell transcriptomes of hepatic MAIT cells (GSE159977). Gene sets derived from MSigDB (M5938) and Reactome (R-HSA-9755511). (J) Gene expression score based on the top 50 upregulated genes in MAIT cells following PUFA treatment. Data represent mean +/- min to max (E-F) or mean +/- SD (G-H), pooled data from at least 2 independent experiments. (I-J) Data represent median with IQR. ***p<0.001, **p<0.01, *p<0.05, ns = p>0.05 compared to untreated control or as indicated, assessed by Repeated measures ANOVA (E-G), Welch's t-test (H) or Wilcoxon test (I-J). Each dot represents one individual (E-H).

Fig. 5: PUFA-induced lipid peroxidation leads to mitochondrial exhaustion in MAIT cells

(A) Real-time oxygen consumption rate (OCR) in MAIT cells following treatment with different FFAs, right panel: summary data at 300 min. (B) 16h Mitochondrial membrane potential (TMRE) and (C) 24h mitochondrial mass (MitoTracker Green, MTG) in MAIT cells after culture with FFAs. (D) Real-time extracellular acidification rate (ECAR) in MAIT cells following treatment with different FFAs, right panel: summary data at 300 min. (E) OCR 50% and ECAR 50% in MAIT cells following PUFA treatment. (F) 2h mitochondrial ROS, (G) 16h lipid peroxide formation, (H) OCR 50%, (I) mitochondrial membrane potential and (J) ECAR 50% in MAIT cells after treatment with PUFAs +/- α-tocopherol/NAC. Data represent mean +/- SEM (A-E, G-H, J), pooled data of at least 3 independent experiments or mean +/- SD (F, I), pooled data of at least 2 independent experiments. ***p<0.001, **p<0.01, *p<0.05, ns = p>0.05 vs untreated control or as indicated, assessed by Repeated measures ANOVA (A-D), Welch's t-test (E) or Paired samples t-test (F-J). Each dot represents one individual.

Fig. 6: Excessive PUFA-dependent lipid peroxidation culminates in MAIT cell ferroptosis.

(A) Viability of MAIT cells after FFA treatment for 24 h. (B) Lipid peroxide staining in MAIT cells treated with PUFAs and liproxstatin-1 for 16 h. (C) Viability of MAIT cells after treatment with PUFAs and liproxstatin-1 for 24 h. (D) 16h lipid peroxide staining and (E) 24h viability/cytokine expression of MAIT cells incubated with cumene hydroperoxide (CuOOH). Lipid peroxide formation (F) and viability (G) of different immune cell populations in a PBMC pool after 16h FFA exposure. (H) Ferroptosis driver gene signature[47], in single cell transcriptomes of hepatic MAIT cells and CD8+ T cells (GSE159977). Spearman correlation between circulating MAIT cell frequency and PUFA serum concentration (I) or liver steatosis measured by transient elastography (K). Frequency of different immune cell subsets (gating strategy see material and methods) in peripheral blood. Data represent mean +/- SEM (A-G, K), pooled data of at least 3 independent experiments, or median with IQR (H). ***p<0.001, **p<0.01, *p<0.05, ns = p>0.05, assessed by Welch's t-test (K), Paired samples t-test (B, C, E), Repeated measures ANOVA (A, D, F, G) or Wilcoxon-test (H). Each dot represents one individual (A-G, I- K).

Fig. 7: Targeting mitochondrial exhaustion and ferroptosis restores anti-tumour effector function and viability of MAIT cells

(A-C) Intracellular cytokine expression, (D-E) killing activity against HCC cells and (F) viability of PUFA treated, activated MAIT cells in presence of NAC + α-tocopherol. (G) Gene Set Variation Analysis (GSVA) of human liver samples (GSE135251[30]) showing enrichment of a MAIT cell-PUFA signature in MASLD patients and HC. (H) Spearman correlation between MAIT cell-PUFA signature and NAS or fibrosis score in liver tissue from MASLD patients and HC (GSE135251[30]). (I) Gene signature scores in

hepatic MAIT cell single cell transcriptomes (GSE149614[49]). (J) Prognostic value of MAIT cell-PUFA signatures for HCC patient overall survival, comparing top and bottom thirds in an HCC TCGA dataset. Data represent mean +/- SEM (A-C, F), pooled data of at least 3 independent experiments or mean +/- SD (D, E, G).***p<0.001, **p<0.01, *p<0.05, ns = p>0.05, assessed by Paired samples t-test (A-C, E-F), One-way ANOVA with Tukey's multiple comparison test (G) or Wilcoxon test (I). Each dot represents one individual (A-C, E-G).

Material and methods

The materials and methods used in this manuscript are described in detail in the supplementary information.

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Author names in bold designate shared co-first authorship.

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prospective sample collection.

Data and materials availability

The raw sequence data of bulk RNAseq analyses of MAIT cells exposed to different types of fatty acids have been deposited to GEO with accession number GSE250131. All other data and materials are available in the main text or the supplementary materials.

Tables

Table 1

Characteristics		MASLD	Healthy controls
Cases	number	33	46
Age (years)	median (range)	49 (24-68)	30 (19-66)
Male	number (%)	18 (54.5)	22 (47.8)
Female	number (%)	15 (45.5)	24 (52.2)
Liver stiffness (kPa)	median (range)	6.8 (3.5 - 58.8)	n/a
Controlled Attenuation Parameter (CAP; dB/m)	median (range)	329 (222-400)	n/a
Fib-4 score	median (range)	1.10 (0.47 - 14.47)	n/a
BMI (kg/m2)	median (range)	31.83 (23.67 - 48.21)	n/a
HCC/CCC		2 (HCC)	0
Liver metastasis		0	0
Other malignant disease		2 1 melanoma (1975), 1 AML (2006)	0

Table 1: Characteristics of PBMC donors

Table 2

Characteristics		MASLD	Controls
Number of cases	number	12	21
Age (years)	median (range)	61 (24-77)	61 (34-78) (n/a 2)
Male	number (%)	9 (75.0)	14 (66.7)
Female	number (%)	3 (25.0)	7 (33.3)
reason for resection	CRC metastasis	5	12
	haemangioma/adenoma/lipoma	0	6
	CCA	2	0
	unsuitable for transplantation	3	0
	NET	2	0
	HCC	0	2
	GIST metastasis	0	1

Table 2: Characteristics of liver tissue donors

Characteristics		MASLD	Healthy controls
Number of cases	number	33	46
Age (years)	median (range)	49 (24-68)	30 (19-66)
Male	number (%)	18 (54.5)	22 (47.8)
Female	number (%)	15 (45.5)	24 (52.2)
Liver stiffness (kPa)	median (range)	6.8 (3.5 - 58.8)	n/a
Controlled Attenuation Parameter (CAP; dB/m)	median (range)	329 (222-400)	n/a
Fib-4 score	median (range)	1.10 (0.47 - 14.47)	n/a
BMI (kg/m2)	median (range)	31.83 (23.67 - 48.21)	n/a
HCC/CCC		2 (HCC)	0
Liver metastasis		0	0
Other malignant disease		2 1 melanoma (1975), 1 AML (2006)	0

Characteristics		MASLD	Controls
Number of cases	number	12	21
Age (years)	median (range)	61 (24-77)	61 (34-78) (n/a 2)
Male	number (%)	9 (75.0)	14 (66.7)
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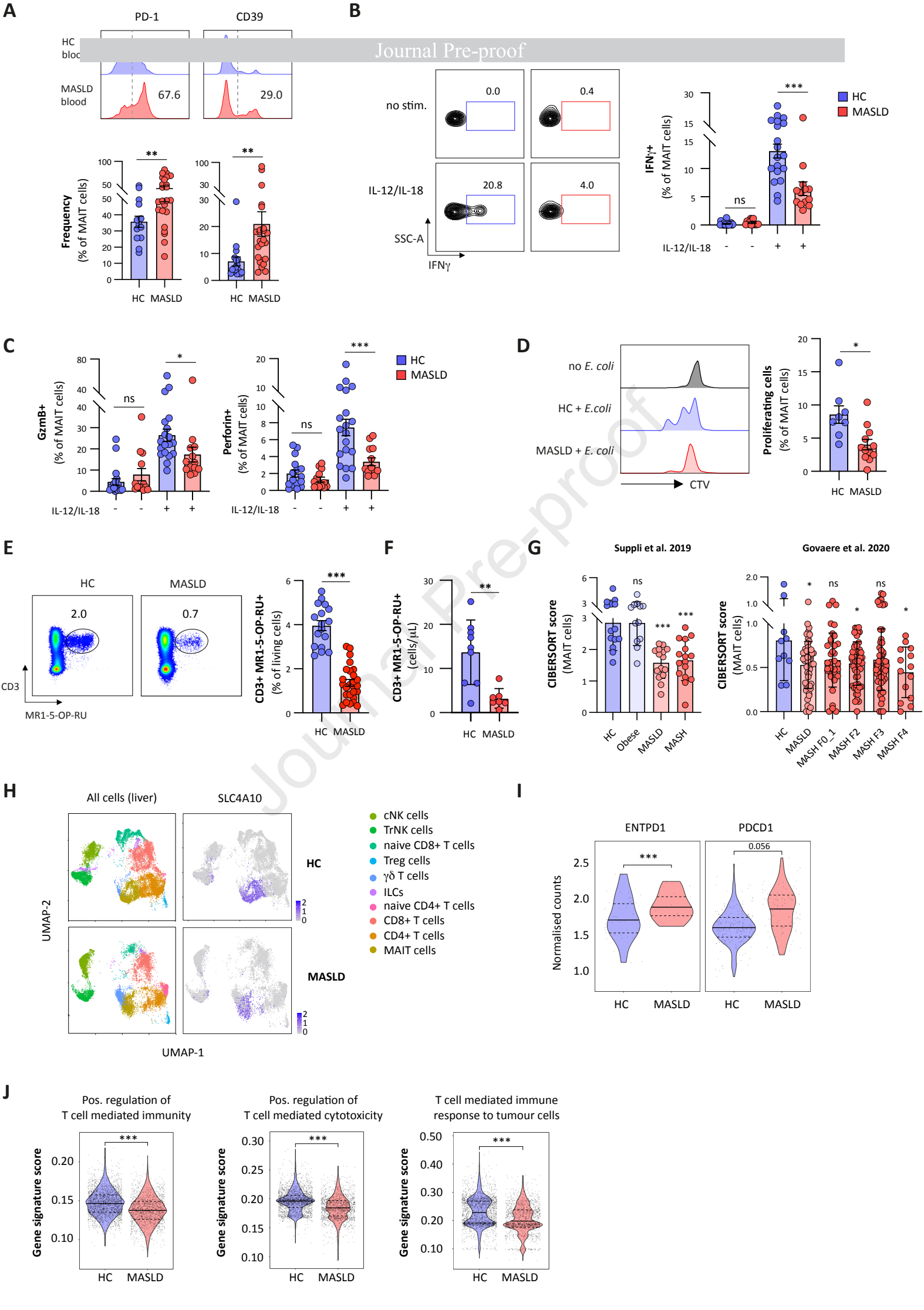
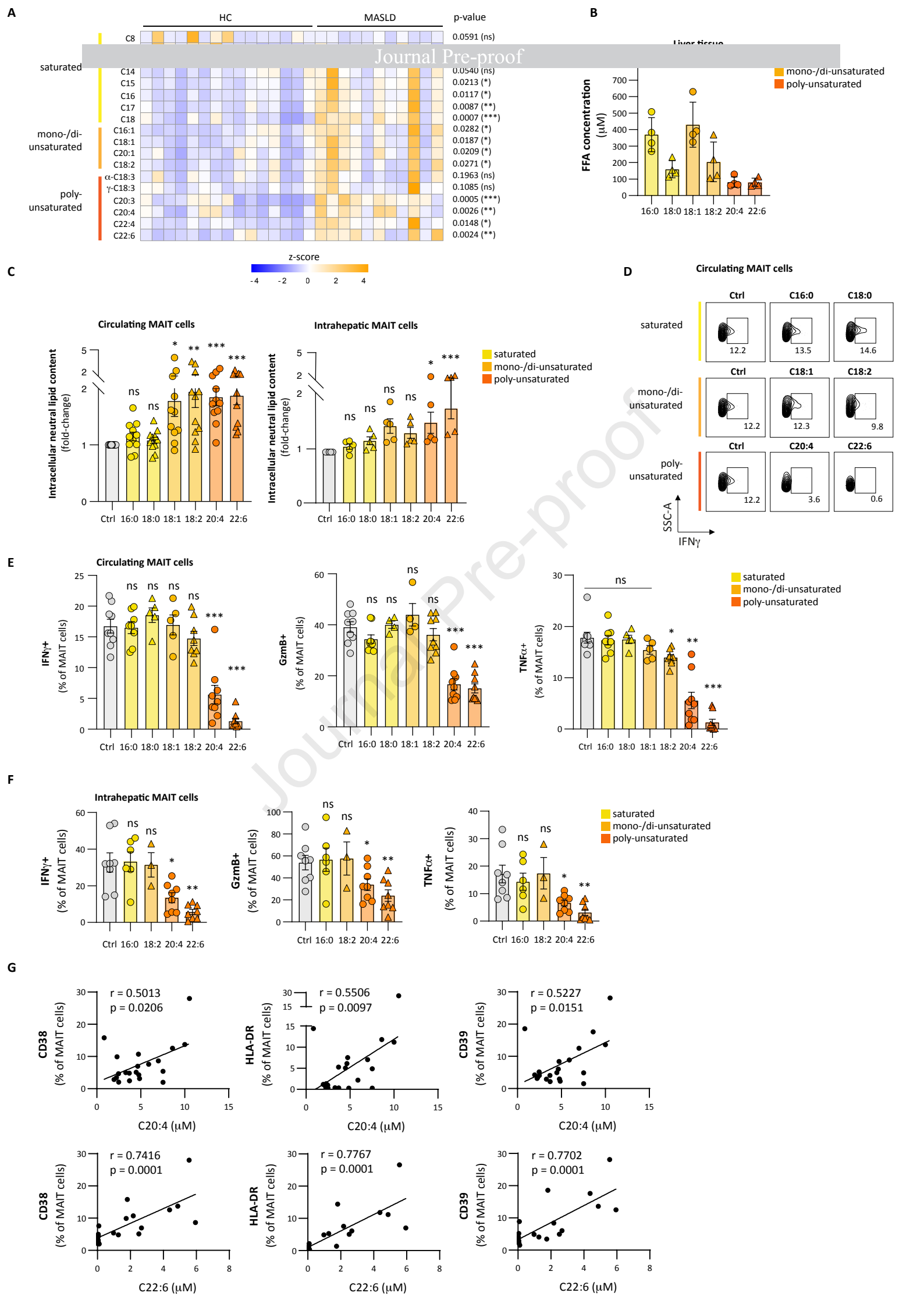
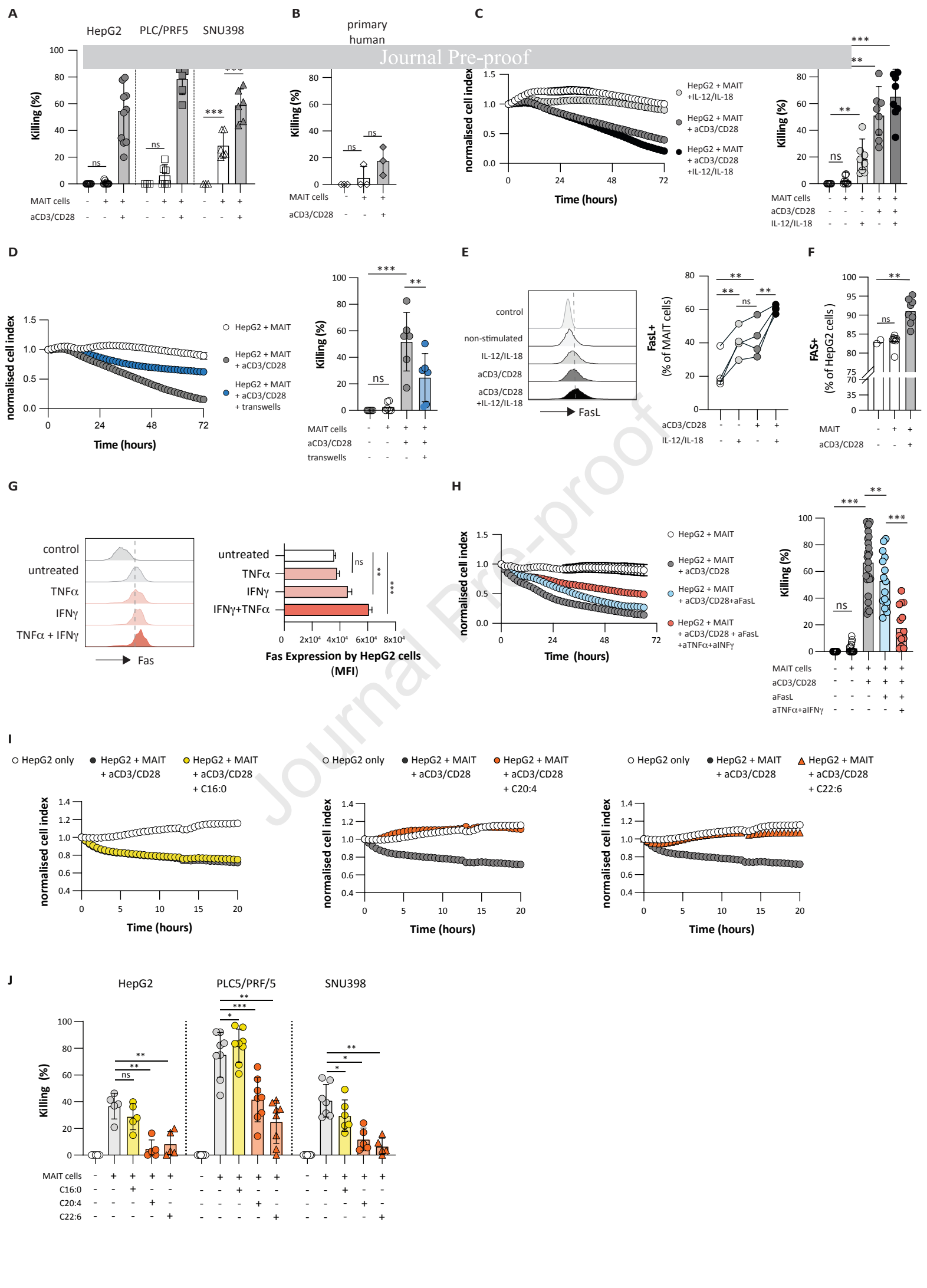
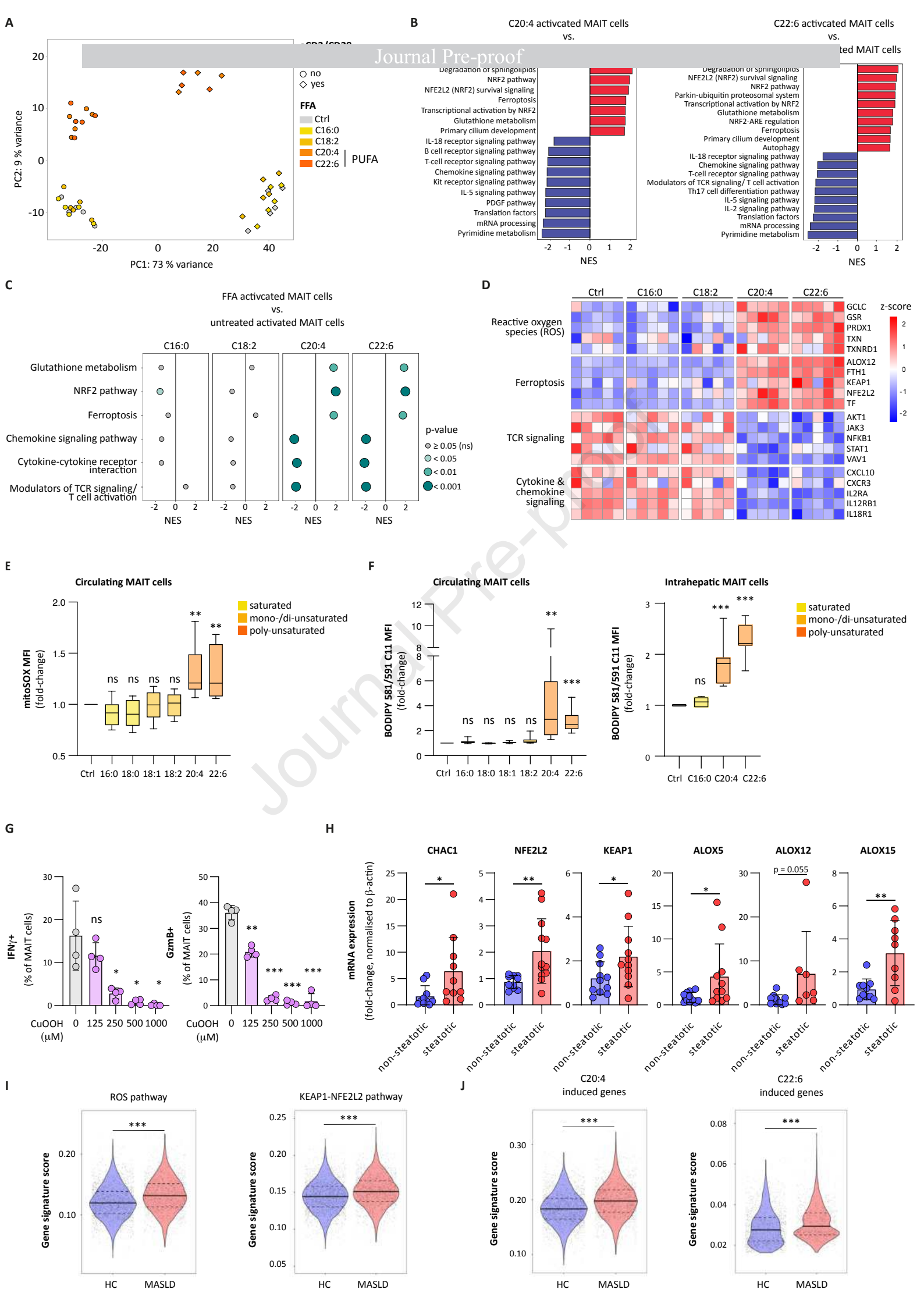
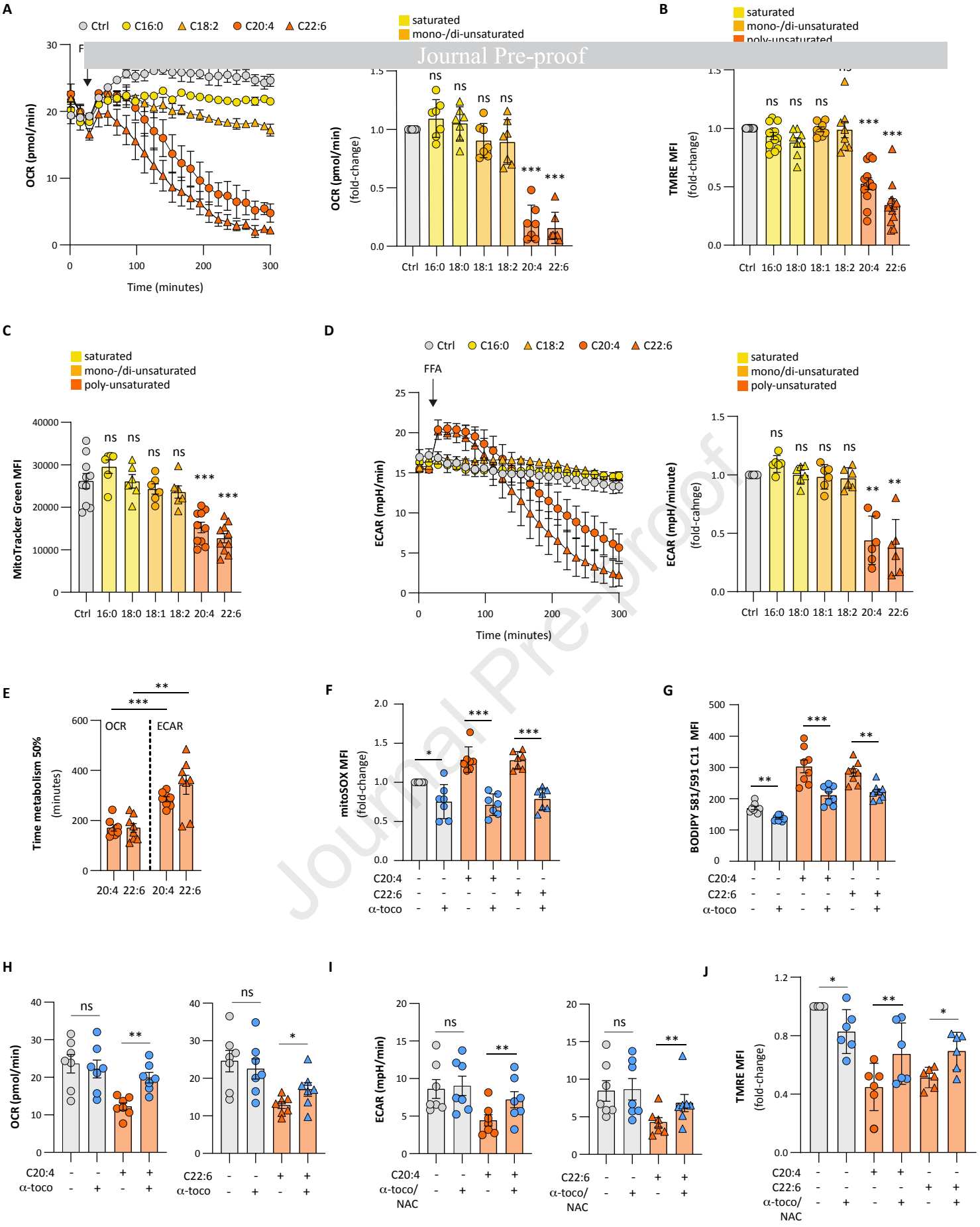
reason for resection	CRC metastasis	5	12
	haemangioma/adenoma/lipoma	0	6
	CCA	2	0
	unsuitable for transplantation	3	0
	NET	2	0
	HCC	0	2
	GIST metastasis	0	1

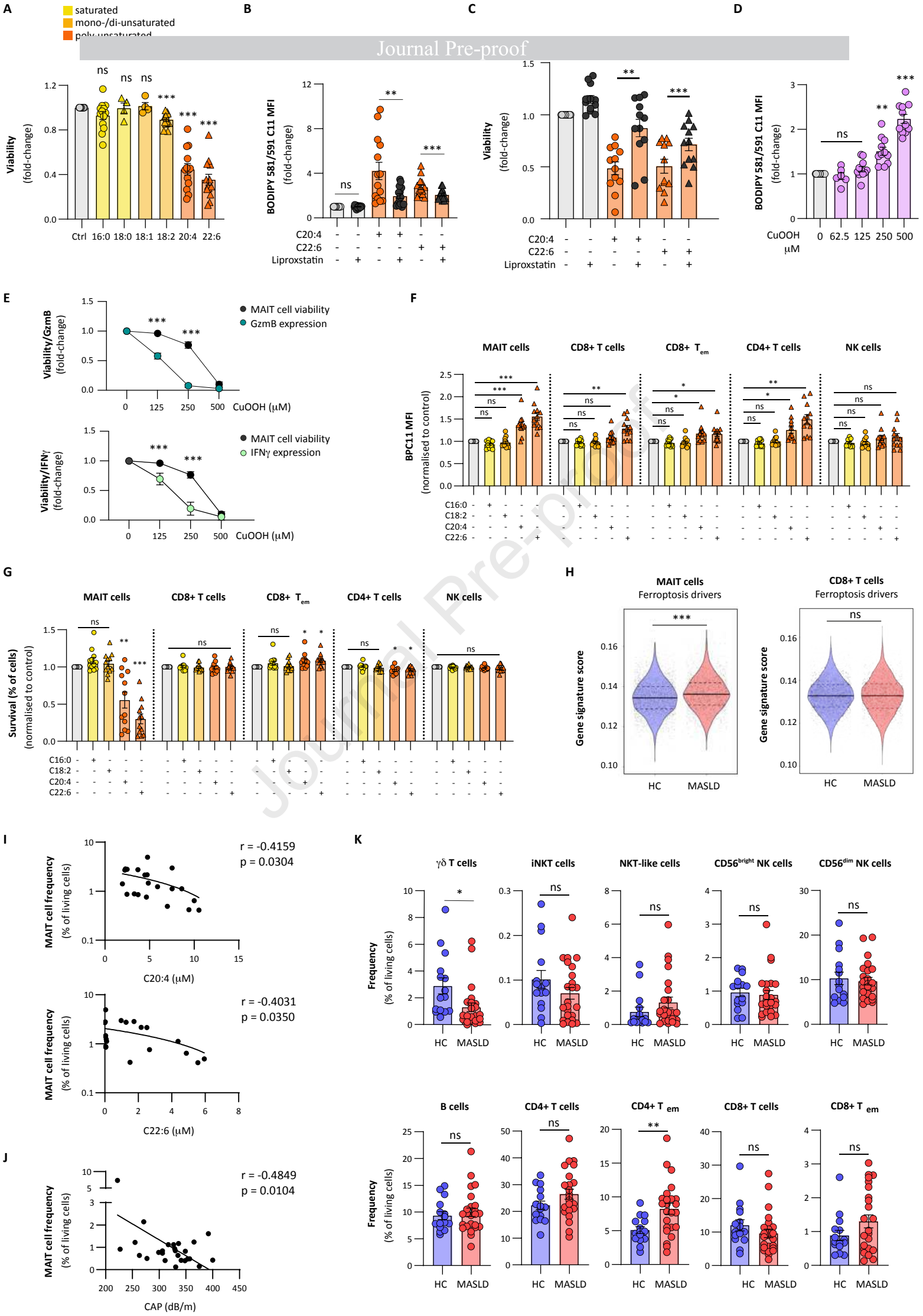


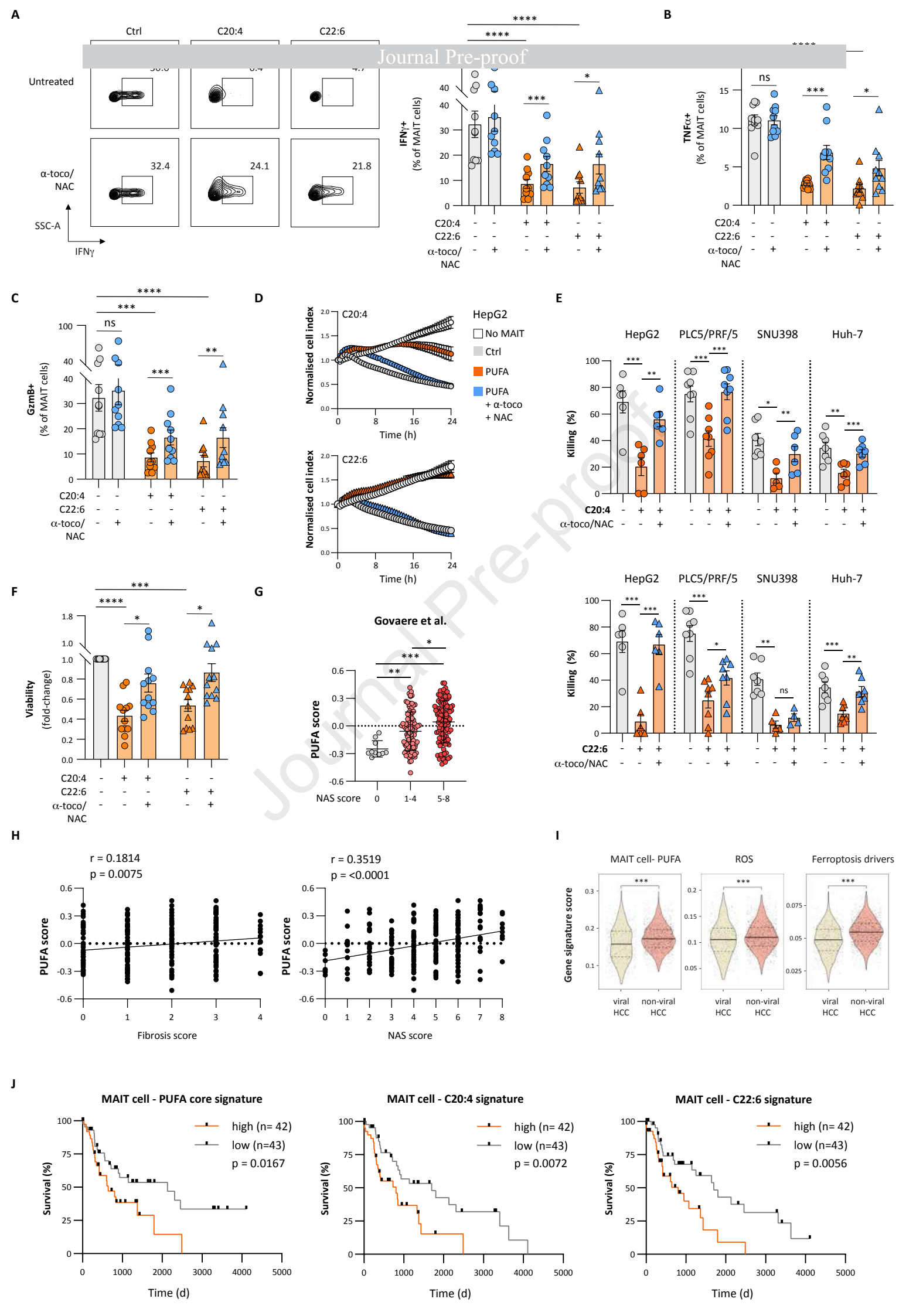












Highlights:

• MAIT cells lose their immune functions in patients with MASLD, leading to impaired tumour immune control

• MASLD-associated polyunsaturated fatty acids (PUFAs) drive MAIT cell dysfunction

• PUFAs trigger intracellular lipid peroxidation in MAIT cells, pushing them into a state of metabolic exhaustion

• Excessive lipid peroxidation causes MAIT cell death through ferroptosis

• Antioxidants prevent PUFA-induced metabolic exhaustion, thereby enhancing MAIT cell mediated anti-cancer immunity