Εθνικό Μετσόβιο Πολυτεχνείο
Σχολή Μηχανολόγων Μηχανικών
Τομέας Μηχανολογικών Κατασκευών & Αυτομάτου Ελέγχου

Systematic modelling of multi-omic data for deciphering mechanisms of human diseases

Διδακτορική Διατριβή

Δανάη Στέλλα Ζαρείφη

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Αθήνα

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PROLOGUE

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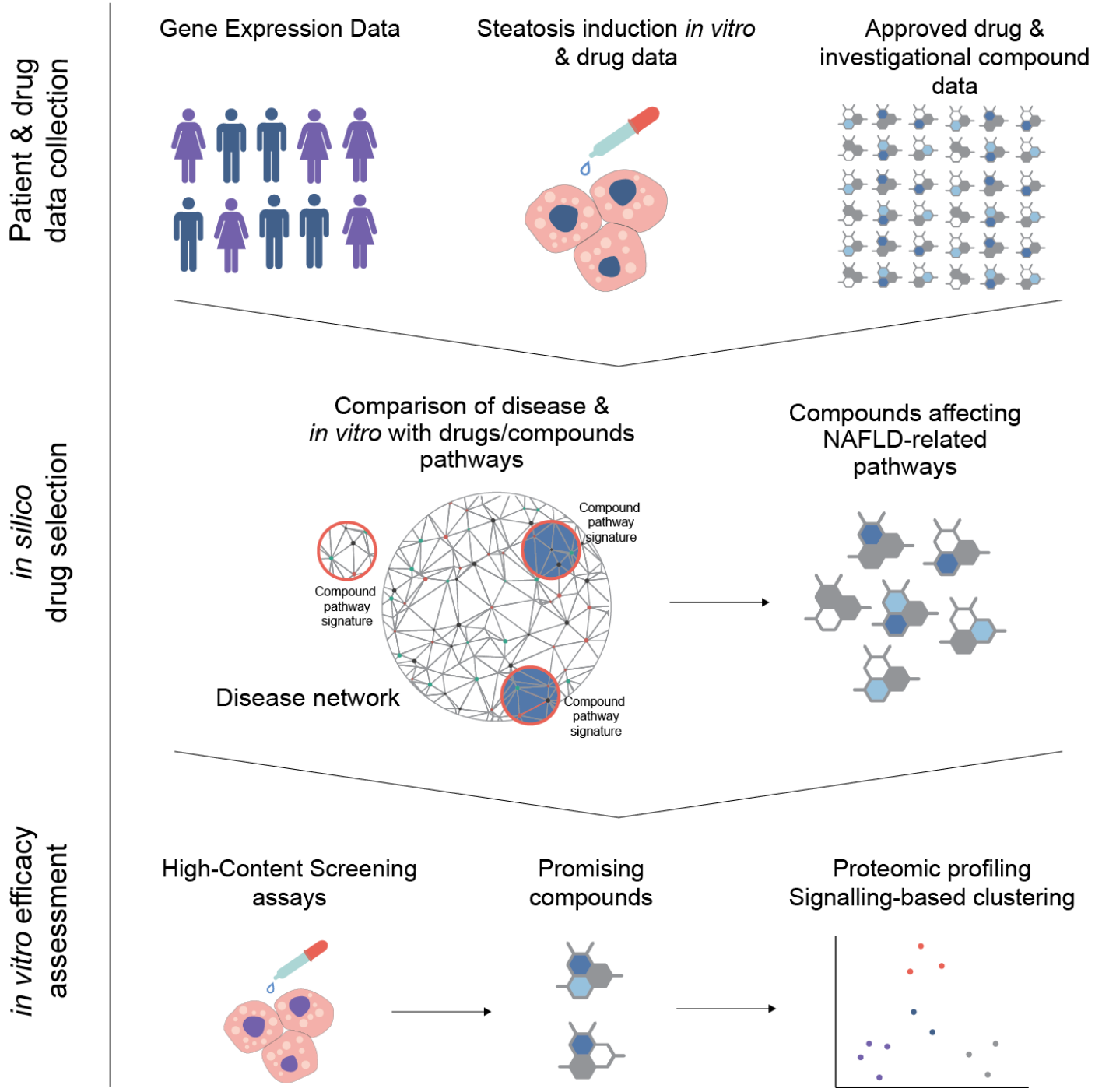
Danae Zareifi

Athens, September 2021

SUMMARY

The omics area that, was developed the past decades, has offered a basis for the development of systems biology technologies that include the integration of several omics data types to uncover molecular patterns linked with disease. Non-Alcoholic Fatty Liver Disease (NAFLD) is among the most common liver pathologies, however, none approved condition-specific therapy yet exists. The present study introduces a drug repositioning (DR) approach that combines *in vitro* steatosis models with a network-based computational platform, constructed upon genomic data from diseased liver biopsies and compound-treated cell lines, to propose effectively repositioned therapeutic compounds. The introduced *in silico* approach screened 20'000 compounds, whilst complementary *in vitro* and proteomic assays were developed to test the efficacy of the 46 in silico predictions. This approach successfully identified 6 compounds, including the known anti-steatogenic drugs Resveratrol and Sirolimus. In short, Gallamine triethiotide, Diflorasone, Fenoterol and Pralidoxime ameliorate steatosis similarly to Resveratrol/Sirolimus. The implementation holds great potential in reducing screening time in the early drug discovery stages and in delivering promising compounds for in vivo testing.

GRAPHICAL ABSTRACT



EXTENDED SUMMARY

Background and Aims:

Its escalating prevalence in the population (>25% in 2018) is establishing NAFLD as the most common cause of chronic liver disease. Many pharmacological agents have been tested for the management of the disease, but there is no therapy approved specifically for NAFLD/NASH by the US Food and Drug Administration (FDA) or the European Medicines Agency (EMA). On this front, drug repositioning (DR) offers an accelerated route for drug discovery. In drug discovery, drug repositioning (DR) grows popular as it is limiting failure, approval time and cost.

The present study introduces a multi-omic drug repositioning (DR) approach that combines *in vitro* steatosis models with a network-based computational platform, constructed upon genomic data from diseased liver biopsies and compound-treated cell lines, to propose effectively repositioned therapeutic compounds.

Methods:

For the in vitro induction of NAFLD, primary human hepatocytes (pHH) were exposed to free fatty acids (FFAs – palmitic acid, oleic acid) and to the steatogenic compounds amiodarone (AMI), tamoxifen (TMX), tetracycline (TET) and valproic acid (VPA).

A network-based computational approach was employed to suggest compounds for NAFLD. Briefly, gene expression networks derived from NAFLD patients were matched with drug-induced networks in an effort to identify drugs that affect the NAFLD-mechanisms. NAFLD-related networks were identified through gene set analysis (GSA) of microarray datasets of biopsy proven NAFLD/NASH patients from GEO (NCBI). Several common pathways with the aforementioned steatogenic compounds, used to induce NAFLD in vitro, were found through Drugbank database and MSig database. To suggest compounds that reverse the disease mechanism, the steatogenic compounds were used with the Connectivity Map database. The most promising compounds for drug repositioning are considered to belong in the intersection of GSA-derived and drug-derived networks.

For the DR in vitro screening, hepatocellular cell lines (HuH7,HepG2,Hep3B,FOCUS) were seeded on 384-well plates and exposed to free fatty acids (FFAs–palmitic and oleic acid) 200uM, DR compounds 10uM and FFAs - DR compounds co-treatment for 24h. Intracellular lipid droplets were verified via high-content screening that employed Nile Red fluorescent probe and Hoechst 33342 for nuclei counterstaining. JuLI™Stage (NanoEntek) automatically acquired images to undergo

MATLAB analysis. Compounds were found reduce or induce lipid droplet accumulation were examined for their ability to reduce/induce intracellular ROS production using the fluorescent substrate CM-H2DCFDA. Luminex xMAP assays were developed to measure phosphoproteins & cytokine release, while PCA analysis & k-means clustering classification was performed.

Results:

Initially, steatosis induction was achieved in the aforementioned hepatocyte lines as confirmed by the presence of lipid droplets and the increase in oxidative stress intracellularly. These models were integrated into the drug repositioning platform and used to validate the computational results.

In the first steps of computational analysis of gene expression data, as well as networks of steatogenic drugs, genes and pathways were identified which are differentially expressed in NAFLD. These networks were compared to approved-drug networks by a series of statistical tests and 46 drugs targeting differentially expressed genes and pathways were identified. All in all, the introduced in silico approach screened 20'000 compounds, whilst complementary in vitro and proteomic assays were developed to test the efficacy of the 46 in silico predictions. Of these, 25 were rejected as hepatotoxic, 5 have been used successfully or are in clinical trials (Resveratrol, Sirolimus), and 21 were further investigated. From their experimental study, 4 were identified as potentially therapeutic (Gallamine, Fenoterol, Pralidoxime, Diflorasone).

The four potentially therapeutic drugs, as well as Resveratrol and Sirolimus (positive control group), were further analyzed as the molecular signaling profile by measurements of phosphorylated proteins and secreted cytokines. Statistically significant differences were observed in the phosphorylation levels of STAT3, CREB1 in all treatments, while in some cases differences were also observed in the proteins MEK, PTN11, RS6, ERK, AKTS1, NFKB, IL8, IL12, CCL2 and CCL3. The application of clustering algorithms to these data has highlighted Diflorasone as the most promising drug for drug repurposing.

Conclusion:

In this study, an *in silico* and *in vitro* drug repurposing platform for NAFL/NASH was successfully developed. *In vitro* steatosis models were successfully developed in four hepatocyte lines using free fatty acids and four known steatogenic drugs. These models were integrated into the drug repositioning platform and used to validate the computational results. Of all the substances proposed by the drug repositioning platform, 2 have already been proven effective in vitro or in vivo and were used as a positive control group in the experimental validation of the platform.

Further experiments showed 4 new drugs that statistically significantly reduce steatosis and oxidative stress *in vitro*. Finally, proteomic analysis of the effect of promising drugs revealed statistically significant differences in phosphorylation and protein secretion patterns. To our knowledge, it is the first approach that combines computational drug repositioning platform with *in vitro* results verification, while the in silico approach can also be used for the EC of other metabolic diseases.

The implementation holds great potential in reducing screening time in the early drug discovery stages and in delivering promising compounds for in vivo testing. , this framework allows for the evaluation of a great number of compounds, at the early stages of drug discovery, by combining the large compound-examination capacity, offered by the in silico models, with the rigour of the in vitro validation. The attempted implementation actively saves up screening time, as several candidates were eliminated in silico, long before their verification in vitro.

ΠΕΡΙΛΗΨΗ

Οι omic τεχνολογίες που έχουν αναπτυχθεί τις τελευταίες δεκαετίες, έχουν προσφέρει τη βάση για την ανάπτυξη μεθοδολογιών βιολογίας συστημάτων, που περιλαμβάνει το συνδιασμό διαφόρων τύπων omic δεδομένων με σκοπό την ανακάλυψη μοριακών μοτίβων που σχετίζονται με ασθένειες. Η Μη Αλκοολική Λιπώδης Νόσος του Ήπατος (ΜΑΛΝΗ) συγκαταλέγεται ανάμεσα στις πιο κοινές παθολογίες του ήπατος, ωστόσο κανένα φάρμακο δεν έχει εγκριθεί για τη θεραπεία της. Η παρούσα έρευνα παρουσιάζει μια μεθοδολογία επαναστόχευσης φαρμάκων που συνδιάζει *in vitro* μοντέλα της νόσου με μια υπολογιστική πλατφόρμα βασιζόμενη σε βιολογικά δίκτυα, με σκοπό να προτείνει επιτυχώς εποαναστοχευμένες ουσίες για τη ΜΑΛΝΗ. Η *in silico* πλατφόρμα εξέτασε 20’000 ενώσεις, ενώ συμπληρωματικές *in vitro* και πρωτεομικές δοκιμασίες αναπτύχθηκαν για να δοκιμαστεί η αποτελεσματικότητα των 46 in silico προβλέψεων. Αυτή η προσέγγιση αναγνώρισε με επιτυχία 6 ενώσεις, συμπεριλαμβανομένων των γνωστών αντι-στεατογόνων φαρμάκων Resveratrol και Sirolimus. Εν ολίγοις, η Gallamine triethiodide, η Diflorasone, η Fenoterol και η Pralidoxime βελτιώνουν τη στεάτωση παρόμοια με τα Resveratrol/Sirolimus. Η εφαρμογή έχει μεγάλες δυνατότητες στη μείωση του χρόνου διαλογής στα πρώτα στάδια της ανακάλυψης φαρμάκων και στην παροχή πολλά υποσχόμενων ενώσεων για in vivo δοκιμές.

ΕΚΤΕΝΗΣ ΠΕΡΙΛΗΨΗ

Εισαγωγή:

Η Μη Αλκοολική Λιπώδης Νόσος του Ήπατος (ΜΑΛΝΗ) χαρακτηρίζεται από συσσώρευση λίπους στο ήπαρ απουσία αυξημένης κατανάλωσης αλκοόλ. Η παθογένεσή της είναι πολυπαραγοντική και οδηγεί σε ένα ευρύ φάσμα κλινικών εικόνων, από στεάτωση έως Μη Αλκοολική Στεατοηπατίτιδα (ΜΑΣ), ηπατοκυτταρικό καρκίνωμα και κίρρωση του ήπατος. Σχετίζεται με ποικίλες μεταβολικές παθήσεις, συμπεριλαμβανομένου του διαβήτη τύπου ΙΙ και του μεταβολικού συνδρόμου (Younossi, 2019). Ο μεγάλος επιπολασμός της νόσου (>25% το 2018), η απουσία εγκεκριμένων φαρμάκων από των Ευρωπαϊκό ή Αμερικανικό Οργανισμό Φαρμάκων, σε συνδυασμό με τα πολλά ασυμπτωματικά στάδια, υπογραμμίζουν την αναγκαιότητα ύπαρξης θεραπείας και πρόγνωσης (Rinella, 2019).

Η επαναστόχευση φαρμάκων (ΕΦ) χρησιμοποιεί σύγχρονες πειραματικές και υπολογιστικές προσεγγίσεις, με σκοπό την απόδοση νέων ενδείξεων σε ήδη εγκεκριμένα φάρμακα ελαχιστοποιώντας το κόστος, το χρόνο και το ρίσκο αποτυχίας της κλασσικής de novo ανακάλυψης νέων φαρμάκων. Σκοπός της παρούσας έρευνας ήταν η δημιουργία μια υπολογιστικής και in vitro πλατφόρμας ΕΦ για τη ΜΑΛΝΗ/ΜΑΣ.

Μέθοδοι:

Για την in vitro επαγωγή της ΜΑΛΝΗ, οι ηπατοκυτταρικές σειρές HuH7, HepG2, Hep3B και FOCUS εκτέθηκαν για 24 ώρες σε μίγμα ελεύθερων λιπαρών οξέων και στις στεατογόνες ουσίες amiodarone, tamoxifen, tetracycline και valproic acid. Η δημιουργία λιποσταγονιδίων παρατηρήθηκε μέσω μικροσκοπίας φθορισμού υψηλής απόδοσης, μετά από χρώση των κυττάρων με Nile Red και Hoechst 33342, και η ανάλυση των εικόνων έγινε σε MATLAB. Το οξειδωτικό στρες ποσοτικοποιήθηκε μέσω του φθορίζοντος υποστρώματος CM-H2DCFDA.

Πυρήνα της πλατφόρμας ΕΦ αποτέλεσε η σύγκριση δικτύων γονιδιακής έκφρασης από ασθενείς με ΜΑΛΝΗ, με αντίστοιχα δίκτυα φαρμακολογικών ουσιών. Τα δίκτυα της ΜΑΛΝΗ κατασκευάστηκαν βάσει ανάλυσης γονιδιακών δεδομένων από βιοψίες ασθενών από το GEO-NCBI. Παράλληλα, μέσω των βάσεων δεδομένων DrugBank, MSigDB και ConnectivityMap δημιουργήθηκαν δίκτυα φαρμακολογικών ουσιών με σκοπό την αναγνώριση αυτών που επηρεάζουν θετικά ή αρνητικά το μηχανισμό της νόσου.

Η επίδραση των ουσιών ως προς την ικανότητα μείωσης της στεάτωσης και του οξειδωτικούς στρες ελέγχθηκε in vitro με συγχορήγηση ελεύθερων λιπαρών οξέων με μεθόδους υψηλής απόδοσης.

Πολυπλεκτική ELISA (Luminex xMAP) χρησιμοποιήθηκε για την ποσοτικοποίηση πρωτεϊνών σηματοδότησης που σχετίζονται με τη ΜΑΛΝΗ ώστε να μελετηθούν τα σηματοδοτικά προφίλ των φαρμάκων. Η ανάλυση εικόνων, οξειδωτικού στρες και πρωτεϊνικής έκφρασης οδήγησε στην ομαδοποίηση των φαρμάκων βάσει ομοιότητας σε στεατογόνα, θεραπευτικά ή μη, με χρήση ανάλυσης PCA και k-means clustering.

Αποτελέσματα:

Αρχικά, επιτεύχθηκε η επαγωγή ΜΑΛΝΗ στις προαναφερθείσες ηπατοκυτταρικές σειρές όπως επιβεβαιώθηκε από την παρουσία λιποσταγονιδίων και την αύξηση του οξειδωτικού στρες ενδοκυτταρικά. Τα μοντέλα αυτά ενσωματώθηκαν στην πλατφόρμα ΕΦ και χρησιμοποιήθηκαν για την επικύρωση των υπολογιστικών αποτελεσμάτων.

Στα πρώτα βήματα υπολογιστικής ανάλυσης γονιδιακών δεδομένων, καθώς και δικτύων στεατογόνων φαρμάκων, αναγνωρίσθηκαν γονίδια και μονοπάτια τα οποία είναι διαφορικώς εκφρασμένα στη ΜΑΛΝΗ. Τα δίκτυα αυτά συγκρίθηκαν με δίκτυα εγκεκριμένων φαρμάκων με μια σειρά στατιστικών δοκιμών και αναγνωρίσθηκαν 46 φάρμακα που στοχεύουν τα διαφορικώς εκφρασμένα γονίδια και μονοπάτια. Από αυτά, 25 απορρίφθηκαν ως ηπατοτοξικές, 5 έχουν χρησιμοποιηθεί επιτυχώς ή είναι σε κλινικές δοκιμές (Resveratrol, Sirolimus), ενώ 21 διερευνήθηκαν περαιτέρω. Από την πειραματική μελέτη αυτών, 4 χαρακτηρίστηκαν ως εν δυνάμει θεραπευτικές (Gallamine, Fenoterol, Pralidoxime, Diflorasone).

Τα τέσσερα φάρμακα που χαρακτηρίστηκαν ως εν δυνάμει θεραπευτικά, καθώς και τα Resveratrol και Sirolimus (ομάδα θετικού ελέγχου), αναλύθηκαν περαιτέρω ως το προφίλ μοριακής σηματοδότησης μέσω μετρήσεων φωσφορυλιωμένων πρωτεϊνών και εκκρινόμενων κυτταροκινών. Παρατηρήθηκαν στατιστικά σημαντικές διαφορές στα επίπεδα φωσφορυλίωσης των STAT3, CREB1 σε όλες τις θεραπείες, ενώ κατά περιπτώσεις διαφοροποιήσεις παρατηρήθηκαν και στις πρωτεΐνες MEK, PTN11, RS6, ERK, AKTS1, NFKB, IL8, IL12, CCL2 και CCL3. Η εφαρμογή αλγορίθμων ομαδοποίησης στα δεδομένα αυτά ανέδειξε το Diflorasone ως το ποιο υποσχόμενο φάρμακο για ΕΦ.

Συμπεράσματα

Στην εργασία αυτή επιχειρήθηκε και αναπτύχθηκε επιτυχώς μια in silico και in vitro πλατφόρμα επαναστόχευσης φαρμάκων για τη ΜΑΛΝΗ/ΜΑΣ. Αναπτύχθηκαν επιτυχώς in vitro μοντέλα για τη ΜΑΛΝΗ σε τέσσερις ηπατοκυτταρικές σειρές με χρήση ελεύθερων λιπαρών οξέων και τεσσάρων γνωστών στεαοτογονικών φαρμάκων. Τα μοντέλα αυτά ενσωματώθηκαν στην πλατφόρμα ΕΦ και χρησιμοποιήθηκαν για την επικύρωση των υπολογιστικών αποτελεσμάτων. Από το σύνολο των ουσιών που απέδωσε η πλατφόρμα ΕΦ, τα εξ αυτών χρησιμοποιήθηκαν ως ομάδα θετικού ελέγχου

στην πειραματική διακρίβωση της πλατφόρμας καθώς είχαν επιτυχώς χρησιμοποιηθεί βάσει της βιβλιογραφίας σε *in vivo* και *in vitro* μελέτες. Περαιτέρω πειράματα ανέδειξαν 4 νέα φάρμακα που μειώνουν στατιστικά σημαντικά τη στεάτωση και το οξειδωτικό στρες in vitro. Τέλος, η πρωτεομική ανάλυση της επίδρασης των υποσχόμενων φαρμάκων φανέρωσε στατιστικά σημαντικές διαφορές στα μοτίβα φωσφορυλίωσης και έκκρισης πρωτεϊνών και είναι υπό διερεύνηση. Εν γνώσει μας, είναι η πρώτη προσέγγιση που συνδυάζει την υπολογιστική ΕΦ με την in vitro εξακρίβωση των αποτελεσμάτων, ενώ η in silico προσέγγιση μπορεί να χρησιμοποιηθεί και για την ΕΦ άλλων μεταβολικών ασθενειών.

Η εφαρμογή έχει μεγάλες δυνατότητες στη μείωση του χρόνου διαλογής στα πρώτα στάδια της ανακάλυψης φαρμάκων και στην παροχή πολλά υποσχόμενων ενώσεων για in vivo δοκιμές. , αυτό το πλαίσιο επιτρέπει την αξιολόγηση ενός μεγάλου αριθμού ενώσεων, στα πρώτα στάδια της ανακάλυψης φαρμάκων, συνδυάζοντας τη μεγάλη ικανότητα εξέτασης σύνθετων, που προσφέρουν τα μοντέλα πυριτίου, με την αυστηρότητα της in vitro επικύρωσης. Η απόπειρα εφαρμογής εξοικονομεί ενεργά χρόνο προβολής, καθώς αρκετοί υποψήφιοι εξαλείφθηκαν στο silico, πολύ πριν από την επαλήθευσή τους in vitro.

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1. INTRODUCTION

1.1. Omic data and multi-omic approaches in the study of human disease

The addition of the word "omics" to a molecular phrase denotes a complete, or global, evaluation of a collection of molecules. Genomics was the first omics field to emerge, focusing on the study of complete genomes as opposed to “genetics,” which investigated individual variations or single genes. Genomic research offers an extremely valuable framework for identifying and investigating particular genetic variations that contribute to both Mendelian and complex illnesses. The omics area has been primarily driven by technical developments that have enabled the low-cost, high-throughput study of biological molecules. The “expression array,” for example, was created in the late 1990s and is based on the hybridization of cDNA to arrays of oligonucleotide capture probes. With further development, array technology demonstrated the ability to measure the amounts of all protein-coding transcripts in a specific tissue. The capacity to scan global gene expression patterns finds immediate use in many disciplines of biology, including disease analysis. Array technology also enabled the mapping of loci that influence gene expression, known as expression quantitative trait loci (eQTL), which has proven helpful in the interpretation of genome-wide association studies (GWAS) and the modelling of biologic networks. Since then, numerous more omics tools that can interrogate whole pools of transcripts, proteins, and metabolites, as well as the genome, have been created.

High-throughput genotyping, in conjunction with the development of a high-quality reference map of the human genome, rigorous statistical tools, and large coordinated cohorts of thousands of patients, has enabled the mapping of thousands of genetic variants, both rare and common, that contribute to disease. However, as our ability to discover genetic variations linked to complicated diseases grew, many realizations emerged that impacted later methods of explaining disease aetiology. First, the loci that have so far been discovered explain just a small portion of the heritable component for certain illnesses. Second, although Mendelian diseases are caused by mutations in gene coding areas, common diseases are caused by changes in gene regulation. Third, depending on the environment and genetic background, the same genetic variations can often lead to various ultimate outcomes. These realizations, taken together, offered a basis for the development of systems biology technologies that include the integration of several omics data types to uncover molecular patterns linked with disease.

On its own, each form of omics data generally gives a list of differences linked with the illness. These data may be used as disease indicators as well as to determine whether biological pathways or processes differ between the illness and control groups. The study of only one data type, on the other hand, is restricted to correlations, which typically represent reactive processes rather than causal ones. Integration of various omics data types is frequently used to highlight probable causal alterations that lead to illness or therapeutic targets, which can subsequently be evaluated in additional molecular investigations (Hasin et al., 2017).

Multi-omics is defined as three or more omic datasets generated from distinct levels of biological regulation – not necessarily from the same level (exclusively derived from nucleic acid/DNA, i.e., epigenomics, transcriptomics, and genomics). More broadly, conducting multiple omics research frequently results in datasets with highly diverse data modalities emanating from several assay types and increasing dimensionality. The transcriptomics dataset generated by RNA-seq efforts can yield hundreds to thousands of transcripts in a multi-omics process (for example, when profiling RNA, protein, or metabolites) (and the isoforms). Individual researchers, on the other hand, can only profile a few thousand proteins (and their proteoforms) or a few hundred known metabolites (and features). As a result, transcriptomic information load can easily conceal more useful findings obtained from proteins or metabolites that are closer to the phenotype. If inadequate integrative frameworks for data processing are used, this might increase annotation bias and contribute to noise enrichment. The goal of multi-omics is to discover molecular markers linked with biological processes by identifying regulatory units across several omics layers. Multi-omics reveals molecular phenotypes by providing insights into the mechanisms underpinning biological processes including molecular functions, interactions, and cellular destiny, whether in vivo or in vitro. In the era of precision medicine, multi-omics can aid in the discovery of predictive or prognostic biomarkers, as well as potentially repurposed and new therapeutic targets. Thus, the ultimate goal of applied multi-omics is to enhance illness prediction, boost diagnostic yield, and create higher agricultural outputs through a thorough understanding of the genotype-to-phenotype connection (Krassowski et al., 2020).

Multi-omics interrogations, as opposed to single omics interrogations, can offer researchers with a better knowledge of the flow of information, from the primary cause of the disease (genetic, environmental, or developmental) to the functional implications or important interactions. Omics investigations, by definition, rely on a huge number of comparisons, customized statistical analyses, and a significant commitment of time, skilled labour, and money. As a result, meticulous preparation and execution are necessary (Civelek and Lusis, 2014).

The type of the disease is an essential factor in the design of multi-omic research. Simple illnesses caused by single-gene mutations have few etiological variables, and those factors generally play deterministic roles in disease formation, despite the fact that the severity or course of many diseases is modified by "modifier genes" or environmental factors. Thus, focused omics efforts at specific time points, concentrating on immediate molecular changes caused by the causal factor, are likely to yield enough information to enhance understanding of prospective treatment methods. It is important to note that the major etiological component does not have to be hereditary and might, for example, be an infectious agent.

Complicated disease aetiology is considerably more complex and is not based on a single cause. Various combinations of a number of variables might lead to phenotypically identical situations. Furthermore, in the absence of a clear deterministic factor that causes the disease, findings from a single layer of data are invariably associative and, because reactive effects exceed causal effects in biologic cascades, should be interpreted as such. Furthermore, because most common, complex diseases evolve over time and include both environmental and genetic variables, complete mechanistic understanding will need coordinated sets of many omics data gathered at numerous time periods from many disease-relevant tissues (Hasin et al., 2017).

On the other hand, omics methods produce data to offer biological understanding based on statistical inference from big datasets. As a result, the ability to identify connections or the flow of information is heavily dependent on effect magnitude, background noise heterogeneity, and sample size, with the latter, frequently being the only parameter controllable by researchers. Unfortunately, human studies are hampered by a plethora of confounding variables that are difficult or impossible to control (such as diet and lifestyle choices). Thus, the capacity of omics methods to provide significant insight into the human disease is very much reliant on available sample sizes, and in many circumstances, underpowered research may not only be a shot in the dark, missing real signals, but it is also more likely to create false-positive results. Another major hazard of omics methods is failing to pay adequate attention to data analysis needs both before and during data gathering. Although general analytical pipelines for each form of omics data are available, most omics areas have yet to create an agreed-upon gold standard. Furthermore, these datasets are frequently vast and complicated, necessitating customization of the basic statistical technique to the individual dataset (Hasin et al., 2017).

Omics research using both human and animal models give valuable information about diseases. Humans are the primary beneficiaries of medical research, therefore findings from human studies have higher translational potential than those from animal models. Numerous human-centric

consortia have generated a substantial corpus of transcriptomics and epigenomics data in different tissues, and several major biobanks have been established to collect, preserve, and analyze thousands of human illness samples. These samples may be analyzed using various omics techniques and utilized to discover molecular alterations that occur during illness or before it if prospective data is provided (Hasin et al., 2017).

While offering important information, human omics research has numerous drawbacks that can only be addressed in animal studies, provided the proper disease animal model is employed. One might argue that primary human cell lines are a good platform for studying a disease without using animal models, and cell lines have been utilized extensively to investigate specific individual molecular processes. However, their use is limited due to the complicated structure and convergence of many cell types that cause the majority of complex illnesses. The benefits of utilizing animal models include repeatability, control of environmental variables, accessibility of important tissues, precise phenotyping, the availability of a nearly infinite number of identical biological duplicates, and the capacity to test ideas experimentally. Animal studies have been critical for investigating the impacts of environmental stressors, such as responses to dietary change, which can give mechanistic insight into the connection between omics data and the response to a stressor. Furthermore, renewable populations of animal models, such as inbred strains of rats or mice, can be interrogated repeatedly, and omics studies of such populations have resulted in the development of powerful datasets containing detailed omic, physiological, and pathological data collected under a variety of conditions. The comparison of omics data across human and animal models can assist confirm the biological relevance of the model itself. However, animal models have drawbacks. Many gene-specific models are restricted to a single genetic background, mouse models may not accurately represent the human biology of complicated illness, and some symptoms of human disease may be difficult to test in mice (Krassowski et al., 2020).

1.1.1. Approaches to integrative analysis of multiple omics data

Multi-omics methods have been used to a wide range of biological issues and may be classified into three groups based on the initial emphasis of the investigation: "genome first," "phenotype first," and "environment first." As a result, the genome-first strategy aims to identify the mechanisms through which genetic loci contribute to illness. The phenotype first approach attempts to identify the pathways that contribute to illness without focusing on a specific gene. The environment first method looks at the environment as a key variable, examining how it affects pathways or interacts with genetic variation (Hasin et al., 2017).

1.1.1.1. The genome first approach

In the absence of somatic mutations, the fundamental DNA sequence remains constant throughout life and is unaffected by environment or development. As a result, for disease-associated genetic variations, it is thought that a specific variant contributes to disease rather than being a result of it. Such variations serve as a very strong anchor point for mechanistic investigations of disease aetiology and modelling interactions between different omics layers.

Initially, studies of causal changes at genetic loci focused on coding regions, but it has become clear that regulatory variation accounts for the bulk of the risk burden for many common diseases. Transcriptomics, whether employing expression arrays or RNA-Seq, has therefore proven to be extremely successful for identifying causal genes. For examining causality-based loci, several statistical methods, including conditional analysis and mediation analysis, have been developed, and large datasets for a variety of tissues in humans and animal models are now available (Hasin et al., 2017; Krassowski et al., 2020).

Identification of causative DNA variations impacting gene expression is difficult since a range of elements might contribute, both inside the gene and hundreds of kilobases distant from the gene. Once the causative variants or genes have been identified, further omics layers can aid in the identification of downstream interactions or pathways. Transcript levels frequently show a weak association with protein levels, therefore proteomics data are predicted to be closer to disease processes. Proteomics methods can also be utilized to discover interaction pathways that contribute to disease. Metabolomics can also be utilized to connect genetics to phenotype in some diseases (Hasin et al., 2017; Krassowski et al., 2020).

1.1.1.2. The phenotype first approach

Another method to use omics data to improve our knowledge of illness is to simply look for connections between disease or disease-related variables and omics-based data. Once distinct omics data items are discovered to correlate with a certain phenotype, they may be integrated into a logical framework that shows the impacted pathways and provides insight into the function of various variables in disease development (Hasin et al., 2017; Krassowski et al., 2020).

1.1.1.3. The environment first approach

In this approach, multi-omics analyses are employed to examine the mechanistic connections to disease utilizing an environmental component as the variable. Because it is difficult to accurately quantify environmental or control elements in humans, such as diet, animal models have proved very effective for investigating the impact of the environment on the studied disease. Several

applications have investigated various links between metabolome models and other layers of information, such as the transcriptome and proteome. Refinement of these approaches, followed by application to bigger population-wide datasets, will almost certainly lead to the identification of new important regulatory nodes in metabolite regulation (Hasin et al., 2017; Krassowski et al., 2020).

1.1.2. Integration of data across multi-omics layers

Depending on the research design, many methods may be employed to integrate data across various omics layers. Simple correlation and co-mapping are two often utilized techniques. As a result, if two omics components share a similar driver or if one perturbs the other, they will show correlation or connection. Several specialized statistical methods, many of which rely on conditioning, have been developed. In these techniques, a statistical model is utilized to determine if each model element contributes to the disease separately or whether one is a consequence of the other. More broadly, multi-layer omics may be represented as networks, either data-driven or with the help of existing knowledge about molecular networks. The connection of IDs of the same items across omics layers, known as ID conversion, is a practical concern in multi-omic investigations. This is accomplished through the use of route databases such as KEGG and cross-reference tables. Multi-omics datasets should ideally be gathered from the same set of samples, although this is not always achievable. Multi-omics datasets should ideally be gathered from the same set of samples, although this is not always achievable (Palsson and Zengler, 2010; Sun and Hu, 2016).

Genes and their products participate in complex, linked networks rather than linear routes, which is an important idea in contemporary biology. One method to represent such networks is as graphs comprised of components that interact with one another. Such networks were first built on metabolic pathways, with metabolites corresponding to nodes and enzymatic transformations corresponding to edges. Following that, networks were modelled using co-expression over a series of perturbations, with the genes encoding the transcripts belonging to the nodes and the correlations corresponding to the edges (Barabási and Bonabeau, 2003).

Co-expression networks may be built for illness research based on differences in gene expression between control and afflicted individuals. The comparison of network architectures between control and illness groups enables the identification of tightly linked nodes ("modules") that are most correlated with disease status. Co-expression of interaction networks, in general, is “undirected” in the sense that the causal nature of the connections is unclear. Although interaction networks

may be evaluated experimentally, the large number of suggested relationships found in each research makes indiscriminate testing costly (Palsson and Zengler, 2010; Sun and Hu, 2016).

1.2. Non-Alcoholic Fatty Liver Disease

Non-Alcoholic Fatty Liver Disease (NAFLD) is becoming one of the most common liver diseases in the world, reaching a global occurrence of almost 25% (Younossi, 2019) over the past four decades, and it is presently known to have a close, bidirectional association with components of the metabolic syndrome. As a result of its high prevalence, NAFLD is now the world's fastest-growing cause of liver-related mortality worldwide (Paik et al., 2020) and is emerging as a significant cause of end-stage liver disease (Estes et al., 2018), primary liver cancer (Singal et al., 2016), and liver transplantation with a considerable health economic burden.

1.2.1. Definition

NAFLD is defined as the presence of steatosis in more than 5% of hepatocytes and in the absence of excessive alcohol intake (30g/day for men and 20g/day for women) or other chronic liver diseases and in conjunction with metabolic risk factors (mostly obesity and type 2 diabetes) ("EASL–EASD–EASO Clinical Practice Guidelines for the management of non-alcoholic fatty liver disease," 2016). Individuals with NAFLD should not also present with other secondary causes of hepatic steatosis such as other liver diseases (e.g., lipodystrophy) or drugs (e.g., amiodarone, steroids, tamoxifen, valproic acid) (Chalasani et al., 2020).

NAFLD is considered as the hepatic manifestation of a group of metabolic disorders, comprising of two broadly recognised subtypes: Nonalcoholic fatty liver (NAFL), the mild form of simple steatosis, and non-alcoholic steatohepatitis (NASH), the more progressive form of NAFLD. Although fatty liver hepatitis leading to cirrhosis had been documented roughly 20 years prior (Thaler, 1962), Ludwig and colleagues coined the term non-alcoholic steatohepatitis (NASH) in 1980. (Ludwig et al., 1980)

NAFLD is an umbrella term for a variety of clinicopathological findings. Histologically, NAFLD encompasses a disease continuum that includes steatosis with or without mild inflammation (non-alcoholic fatty liver, NAFL) and a necroinflammatory subtype (NASH), which is further characterized by the presence of hepatocellular injury (steatosis, lobular inflammation, and ballooning with or without perisinusoidal fibrosis). A small percentage of individuals with NASH may advance to cirrhosis and HCC (Loomba et al., 2021; Powell et al., 2021).

Patients with NAFLD may have a wide range of disease drivers, while disease progression and treatment response are variable. To assess the severity of liver disease and provide accurate prognostic information, information about disease activity, particularly the extent of liver fibrosis,

is required. Novel insights from metabolomics, genomics, and other studies will enable disease phenotyping and potentially facilitate disease stratification (Powell et al., 2021).

Current nomenclature suggests that NAFLD is a diagnosis of exclusion rather than inclusion, meaning that it is based on the exclusion of significant alcohol intake and other causes of chronic liver disease, and there is an unceasing discussion over the terminology's and diagnostic criteria's limitations. In 2020, the alternative term metabolic dysfunction-associated fatty liver disease (MAFLD) was proposed in 2020 by an international panel of experts to emphasize the role of cardiometabolic risk factors in the development and progression of liver disease, including cases of individuals with other liver diseases. Instead of being an exclusionary diagnosis, MAFLD is diagnosed based on a set of positive criteria. MAFLD is diagnosed upon the presence of hepatic steatosis and type 2 diabetes mellitus, obesity, or signs of metabolic dysregulation. Furthermore, the term NAFLD does not link the disease to the metabolic syndrome. MAFLD is most likely the missing piece in the majority of cases of progressive liver disease despite treatment of the underlying cause of chronic liver disease, such as chronic hepatitis B or C infection. However, neither the American Association for the Study of Liver Diseases (AASLD) nor the European Association for the Study of Liver Diseases (EASL) does yet recognize MAFLD as an accepted nomenclature (Eslam et al., 2020; Tilg and Effenberger, 2020).

1.2.2. Clinical presentation

NAFLD is an asymptomatic disease and may go undetected until it has progressed to cirrhosis. (Spengler and Loomba, 2015). The most common symptoms observed in people with NAFLD at the time of initial referral are right upper quadrant pain and fatigue. These individuals may have an echogenic liver on ultrasound or evidence of liver fat based on an imaging test discovered incidentally or as part of a workup for right upper quadrant pain. (Chalasani et al., 2018). In liver-related serum tests, serum alanine aminotransferase (ALT) is typically higher than serum aspartate aminotransferase (AST), indicating a hepatocellular pattern of enzyme elevations (Torres et al., 2012). Platelet count decreases over time as advanced fibrosis, cirrhosis, and portal hypertension develop. Cirrhotic patients with evidence of synthetic dysfunction have lower serum albumin and a higher prothrombin time. (Loomba and Adams, 2019).

Over the last decade, lean or non-obese NAFLD has become more widely acknowledged and it has sparked a lot of attention. Five systematic reviews and meta-analyses were recently punished on the subject (4 of them in 2020). Lean NAFLD is referred to people with a normal BMI (23 kg/m^2 for Asians and 25 kg/m^2 for Caucasians whereas non-obese NAFLD is commonly described as

NAFLD in people with a BMI of less than 25 kg/m^2 for Asians and more than 30 kg/m^2 for Caucasians. (F. Chen et al., 2020).

1.2.3. Epidemiology

According to Younossi et al's (Younossi, 2019), systematic review and meta-analysis, the Middle East had the highest prevalence of NAFLD at 32%, followed by South America (30%), Asia (27%), North America (24%), Europe (24%), and Africa (13%). This evidence suggests that the prevalence of NAFLD in Asia has surpassed that of North America and Europe hence it should no longer be regarded as a Western disease. Due to a lack of epidemiologic data in Africa, the demonstrated lower prevalence may be an underestimation (Paruk et al., 2019). NAFLD is more prevalent in urban areas than in rural areas, most likely due to socioeconomic differences between the two (Younossi, 2019).

It has also been demonstrated that NAFLD's prevalence increases proportionally with the gross domestic product (GDP) value (Zhu et al., 2015). The high prevalence of NAFLD has eventually become a burden on a country's economy and will continue to do so. Medical costs to treat NAFLD patients in the United States are estimated to be $103 billion per year, whereas the cost to treat NAFLD patients in Europe-4 countries is estimated to be over 35 billion per year. As current projections demonstrate that the prevalence of NAFLD will continue to rise, as will the prevalence of more severe liver disease, medical costs associated with NAFLD will continue to rise and be a major burden to countries worldwide. (Younossi et al., 2018)

There is also a proportional relationship between NAFLD's prevalence and age and a dependency on gender. In detail, it was shown to rise from 22% in the age group 30-39 years to 34% in the age group 70-79 years. NAFLD's prevalence has consistently been demonstrated to be higher in male subjects compared to female subjects up to the age of 50 and the prevalence of NAFLD among female subjects rises dramatically, mirroring the rates observed in male subjects (Li et al., 2019; Younossi et al., 2018). This finding could be related to postmenopausal estrogen deficiency. Estrogen may be protective against NAFLD, and low estrogen levels may lead to visceral fat accumulation and metabolic dysregulation, increasing the risk of insulin resistance, cardiovascular disease, and NAFLD (Wong and Chan, 2021).

Obesity was independently associated with a 3.5-fold increased risk of NAFLD in a meta-analysis of 21 cohort studies by Li et al, and there was a dose-dependent relationship between body mass index (BMI) and NAFLD risk (Li et al., 2016). The prevalence of NAFLD increases with the number of criteria for the metabolic syndrome, rising from 5% among those who do not meet any of the

criteria to 80% among those who meet all five (Wong and Chan, 2021). NAFLD is more common in patients with type 2 diabetes mellitus (T2DM), with a prevalence ranging from 49.6 to 72.4 %. Furthermore, the presence of T2DM in NAFLD patients is linked to more severe liver diseases, such as steatohepatitis, advanced fibrosis, liver cirrhosis, hepatocellular carcinoma, and death (Younossi et al., 2004). Considering this finding, a reasonable approach would be targeting patients with type 2 diabetes mellitus to identify those with more severe liver disease. In this case, initial assessments should be taking the form of a fibrosis score based on readily available parameters, followed by referral of intermediate- and high-risk patients for further evaluation (Lim and Bernstein, 2018).

According to Ye et al's recent systematic review, the largest systematic review on lean NAFLD to date, the prevalence of lean and non-obese NAFLD in the general population was found to be 5.1% and 12.1%, respectively. Moreover, 19.2% of people with NAFLD were slim, and 40.8% were non-obese. NAFLD was diagnosed in 10.6% of slim people and 18.3% of non-obese people. Because the bulk of studies on lean NAFLD were conducted in Asia, the data may not correctly reflect global prevalence due to the underrepresentation of other populations. Over the last three decades, as the prevalence of NAFLD has increased, so has the prevalence of lean NAFLD. Lean patients are not always healthy, as some may be metabolically obese, normal-weight people. When compared to lean healthy control subjects, lean NAFLD patients are more likely to have metabolic syndrome, central obesity, hypertension, dyslipidemia, and type 2 diabetes mellitus. Lean NAFLD patients had a lower prevalence of metabolic syndrome and its components than obese NAFLD patients (Lu et al., 2020).

The majority of lean or non-obese NAFLD patients have visceral obesity, which is a major contributor to insulin resistance, which in turn plays an important role in the pathogenesis of NAFLD (Wong and Chan, 2021). As a result, because BMI does not measure body fat content and does not identify visceral obesity, it may not be a good predictor of the presence of NAFLD. Other suitable markers of visceral or central obesity have been investigated, including waist circumference, sagittal abdominal diameter, visceral adiposity index, and percentage of fat mass (Kim and Kim, 2017). Even though lean or non-obese NAFLD patients have less severe liver disease based on histology and non-invasive test results when compared to obese NAFLD patients, these patients can still develop NASH, liver fibrosis, cirrhosis, hepatocellular carcinoma, and incident diabetes, hypertension, and cardiovascular diseases. Indeed, some studies have found that lean or non-obese NAFLD patients have a higher prevalence of more severe liver disease or cirrhosis. Differences in genetics and pathophysiology could explain the poorer result, as well as

less aggressive care, which could be related to the notion that slim or non-obese NAFLD patients have less severe liver disease and metabolic burden (Wong and Chan, 2021).

Regarding childhood obesity, an essential risk factor for NAFLD, the number of cases is still rising. Namely, in the United States, the prevalence of obesity among children aged 2–5 years grew from 8.4% in 2011–12 to 13.9% in 2015–16. The pooled mean prevalence of NAFLD in children is 7.6% in the general population and 34.2 % in pediatric obesity clinics. Individuals who develop this condition in childhood are more likely to suffer liver-related events and other comorbidities associated with metabolic syndrome throughout the course of their lives than those who develop the disease as an adult (Powell et al., 2021; Vittorio and Lavine, 2020).

1.2.4. Risk factors

2. Age

The older population is recognized to have greater risk factors for NAFLD and NASH than younger individuals, such as hypertension, dyslipidemia, and diabetes. In elderly individuals, the prevalence of NASH, as well as the development of NASH-induced fibrosis and HCC, was shown to be greater. However, this direct relationship between age and NASH development and/or severity might be attributable to the disease's persistence rather than age (Vernon et al., 2011).

3. Gender

NAFLD and NASH were considered to be more frequent in women at first, but investigations have led to conflicting results. In several studies, the male gender was related to fibrosis and higher blood ALT and AST levels. Because the findings of existing research are ambiguous, large-scale population studies are required to address the gender question (Vernon et al., 2011).

4. Ethnicity and race

Numerous studies have shown that the prevalence of NAFLD differs by ethnicity. The cause for this variance is unknown. Environmental variables and genetic variations, on the other hand, may be implicated (Vernon et al., 2011).

5. Metabolic syndrome

As previously stated, NAFLD is regarded as the hepatic component of the metabolic syndrome, which is a combination of metabolic disorders that mostly include T2D, obesity, hypertension, and dyslipidemia. As a result, the frequency of NAFLD is inherently higher among individuals who have a pre-existing metabolic syndrome. T2D is generally regarded as an independent risk factor for liver-related mortality. Furthermore, NASH may develop more rapidly in T2D patients. Previous

research has also found an increased incidence of NASH in women with polycystic ovarian syndrome (PCOS). Obesity is also associated with NAFLD; the prevalence of NAFLD in the obese population is about 35%, particularly in cases of visceral adiposity, which is characterized by increased waist circumference. NASH not only develops more frequently in individuals with metabolic syndrome, but it also progresses more frequently. Patients with diabetes, obesity, and hypertension, all of which are components of the metabolic syndrome, had higher rates of fibrosis development (Gerges et al., 2021; Younossi et al., 2016).

6. Diet composition

A variety of nutrients have been implicated in the development of NAFLD. Sugars in glucose and fructose beverages increase de novo lipogenesis (DNL), the production of free fatty acids (FFAs) and triglycerides (TG), resulting in inflammation. Furthermore, eating high-fat meals causes extra FFAs to be transported from the stomach to the liver via the portal circulation, where they are uptaken by the liver via fatty acid transporters, increasing the chance of developing steatosis (Boden, 2006). Meals high in carbohydrates, lipids, and proteins cause higher postprandial blood glucose levels and quicker insulin release (Petta et al., 2016). Insulin further raises DNL, leading to the development of steatosis and TG accumulation in the liver (Sanders et al., 2018).

7. The gut-liver axis

The gut-liver axis has been identified as a key component in the development of NAFLD (Zhou and Zhong, 2017). The portal circulation transports nutrients and other signals from the gut to the liver, and due to the slow rate of blood flow in the hepatic sinusoids, contact between gut-derived signals, hepatic cells and hepatic immune cells is possible. Gut barrier failure, meaning increased gut permeability, has been reported to be substantially related to NAFLD, since it promotes inflammation and potentially fibrogenesis by increasing the rush of bacterial lipopolysaccharide (LPS) into the portal circulation and to the liver (Szabo, 2015). In addition, animal studies have indicated that changes in the gut microbiota (dysbiosis) are a risk factor for NAFLD development (Dumas et al., 2006).

8. Chronic infections

NAFLD has been linked to diseases such as hepatitis C, which aggravates fatty liver by increasing insulin resistance (IR) (Adinolfi et al., 2016). NAFLD frequency was shown to be significant in patients with human immunodeficiency virus. However, it is unclear if the steatosis is caused by the virus, the antiviral medications employed, or both (Vernon et al., 2011). Furthermore,

additional illnesses such as hypothyroidism and hypopituitarism are thought to be linked to NAFLD (Chalasani et al., 2018).

9. Genetic predisposition

Several studies have found that genetic factors play a significant role in the development of NASH. One of the most often associated genetic variants with NAFLD is a missense mutation in the patatin-like phospholipase domain-containing 3 (PNPLA3) gene. PNPLA3 has recently been shown to have a significant impact on hepatic fat accumulation as well as the histological severity of hepatic damage. This gene encodes a protein that is present on the surface of lipid droplets in the liver and adipose tissue (AT) cells, as well as in the endoplasmic reticulum (ER). This protein's expression rises after meals and in IR environments. The mutated protein seems to disrupt lipid metabolism while boosting phospholipid synthesis (Anstee and Day, 2015). NAFLD has also been associated with single nucleotide polymorphisms in the neurocan, glucokinase regulator, and lysophospholipase-like 1 gene. These changes are linked to increased hepatic glucose absorption and fat accumulation (Speliotes et al., 2011).

10. Pharmacological agents

Several medicines, including amiodarone, sodium valproate, methotrexate, and several anticancer and antiviral medications, have been reported to cause hepatic steatosis (Amacher and Chalasani, 2014). The majority of medications that cause hepatic steatosis or steatohepatitis act by inhibiting mitochondrial fatty acid -oxidation, which results in elevated levels of fatty acyl-CoA and non-esterified fatty acids, culminating in the production of TG and, ultimately, hepatic steatosis (Pessayre et al., 2012). Furthermore, inhibiting -oxidation causes an increase in reactive oxygen species (ROS), which aggravates hepatic damage and leads to steatohepatitis and necrosis (Koek et al., 2011). Some medicines, such as protease inhibitors, could enhance hepatic TG accumulation by either boosting synthesis or reducing secretion, whereas others, such as antipsychotics, may increase DNL (Amacher and Chalasani, 2014).

1.2.5. Pathogenesis

Although the pathogenesis of NAFLD and NASH is not fully understood, it is known to be complex and multifaceted, including the interplay of several genetic, metabolic, environmental, and dietary variables.

1.2.5.1. The two-hit hypothesis

Several hypotheses have been proposed to explain the pathophysiology of NAFLD. First, a two-hit theory was presented. According to this hypothesis, the first hit is an excessive buildup of lipids in the liver, which is produced by a variety of variables such as a high-fat diet, a sedentary lifestyle, obesity, and IR. This first hit sensitizes the liver to the second hit, which is likewise produced by several metabolic insults that induce inflammation and fibrogenesis. This two-hit concept was later considered to be an unduly simplistic explanation of NASH aetiology. As a result, a multiple-hit hypothesis was developed (Buzzetti et al., 2016; Peverill et al., 2014).

1.2.5.2. The multiple-hit hypothesis

Poor dietary habits, excessive consumption of high-calorie diets, along with genetic predisposition, are environmental factors that may contribute to the development of insulin resistance (IR), obesity, adipose tissue (AT) dysfunction, and changes in the intestinal microbiota, all of which are factors involved in NAFLD development and progression (Buzzetti et al., 2016). In addition, intracellular processes and interplay between hepatocytes, Kupffer cells, and hepatic stellate cells (HSCs) play a part in the development of NASH (Giraudi et al., 2015; Hardy et al., 2016).

A) Development of hepatic steatosis

Hepatic and peripheral insulin resistance (IR)

Insulin is a hormone generated and released by pancreatic beta cells that regulates blood glucose levels and affects a number of metabolic tissues, including the liver, muscles, and AT (Röder et al., 2016). Insulin promotes glucose absorption in the liver and skeletal muscles, where glucose is either oxidized or stored as glycogen, as well as in the AT, where glucose is used in TG synthesis. Furthermore, it promotes TG synthesis in the AT and liver via DNL while blocking AT lipolysis. Insulin also suppresses hepatic glucose production. It works by tyrosine-phosphorylating and activating downstream targets such as insulin receptor substrate (IRS), phosphatidyl inositol 3 kinase (PI3K), and protein kinase B. (or AKT). As a consequence, active AKT mediates the effects of insulin on glucose and lipid metabolism, increasing glycogen synthesis and lipogenesis while suppressing gluconeogenesis (Huang et al., 2018; Matsuda et al., 2013).

IR is defined as a poor physiologic response to insulin, especially insulin-mediated glucose absorption, despite normal or high insulin levels. In IR circumstances, the pancreas is prompted to generate more insulin to compensate for deficits in peripheral glucose absorption and to limit hepatic glucose production. The portal vein transports insulin from the pancreas to the liver, where insulin stimulates glycogenesis, inhibits gluconeogenesis, and promotes DNL (Edgerton et al.,

2006). Insulin stimulates lipogenesis and inhibits lipolysis in adipocytes by esterifying fatty acids. As a result, systemic IR in NASH disrupts insulin-mediated lipolysis control in the AT, resulting in increased adipocyte lipolysis and FFA release into the serum. The liver absorbs large quantities of FFAs in the blood (E. et al., 2010; Luef et al., 2009).

B) Obesity and adipose tissue dysfunction.

Because it secretes hormones and adipokines such as adiponectin and leptin, the adipose tissue (AT) serves an essential endocrine role. Obesity causes excessive lipid buildup in adipocytes as well as an increase in AT size. This activates the c-JNK and IK inflammatory pathways, resulting in decreased insulin sensitivity in the AT. As a result, obesity is regarded as a risk factor for both IR and NAFLD. AT IR reduces the anti-lipolytic action of insulin in the AT, resulting in enhanced lipolysis. Furthermore, hyperinsulinemia and hepatic IR enhance hepatic DNL and TG production as very-low-density lipoprotein from the liver, resulting in a high circulating lipid load that is transported to the AT, exacerbating adipocytes' inability to deposit them as lipid droplets (Guilherme et al., 2008; Tsochatzis et al., 2006).

AT expresses and releases the following adipokines, which play key roles in the genesis and progression of NAFLD and NASH:

Adiponectin. Adiponectin is a cytokine that is mostly generated by adipocytes and has a variety of positive and hepatoprotective properties. Adiponectin has been shown to reduce hepatic IR by inhibiting glycogenolysis and lipogenesis and increasing glucose consumption. Adiponectin has also been shown to have antifibrotic properties. Adiponectin also possesses anti-inflammatory, antioxidant, and anti-obesity properties. As a result, its shortage may play a role in the development of mitochondrial dysfunction, IR, and obesity. Adiponectin expression reduces with increasing adipocyte cell size and IR. Chronic metabolic disorders such as obesity and diabetes were shown to have lower levels of adiponectin. Furthermore, NAFLD patients have low plasma adiponectin levels, which indicate NASH risk (Osborn and Olefsky, 2012; Tsochatzis et al., 2006). Furthermore, after therapy with thiazolidinediones, adiponectin levels were shown to be increased in NASH patients, and injection of recombinant adiponectin was found to dramatically ameliorate steatohepatitis in mice (Gastaldelli et al., 2010).

Leptin. Leptin, similar to adiponectin, is a cytokine produced mostly by the AT and is a hormone that regulates body weight and fat content. Leptin primarily functions in the central nervous system to restrict food intake and improve energy utilization, and also prevents lipid buildup in organs other than the AT, such as the liver (Petta et al., 2016). Obese and NASH patients develop leptin resistance, which causes leptin levels to rise. Elevated leptin levels have been related to

decreased glucose absorption and increased gluconeogenesis, which results in hyperglycemia and contributes to the development of IR. Furthermore, leptin has anti-inflammatory effects and it was shown to have critical roles in animal models of NASH. Increased leptin levels, for example, were found to enhance Kupffer cell hyperreactivity to LPS in a mouse model of NASH, leading to accelerated fibrosis. In another model of NASH cirrhosis, hyperleptinemia was found to aggravate hepatic IR and contribute to portal hypertension. (Adolph et al., 2017; Osborn and Olefsky, 2012; Tsochatzis et al., 2006). Additionally, a 2016 meta-analysis revealed that NAFLD patients had higher plasma leptin levels than controls, with higher leptin levels indicating more severe NAFLD cases (Polyzos et al., 2016).

C) Hepatic fat accumulation

Multiple metabolic, environmental, and genetic variables, such as the previously described hepatic and peripheral IR, obesity and AT dysfunction, gut barrier dysfunction, and poor eating choices, all contribute to hepatic steatosis. Circulating FFAs are the primary cause of hepatic fat accumulation in NAFLD. Excess FFAs from the AT or the gut creates fatty acyl-CoAs, which are either oxidized by mitochondrial fatty acid -oxidation or retained inside cytoplasmic lipid droplets as TG and diacyl-glycerol or other lipid metabolites such as ceramides (Ferramosca and Zara, 2014).

As previously stated, systemic IR causes excessive lipolysis in the AT, resulting in the release of large amounts of FFAs, which are then transported to the liver (E. et al., 2010). Furthermore, hepatic IR promotes hyperinsulinemia and increases hepatic fat accumulation by increasing DNL and inhibiting FFA-oxidation, worsening steatosis. Hepatic DNL was shown to be higher in NAFLD patients than in controls, and it is not reduced by fasting. Insulin binding to IRS activates sterol regulatory element-binding protein-1 (SREBP-1), which promotes hepatic DNL. IR causes SREBP-1 overexpression and, as a result, DNL upregulation (Lambert et al., 2014). Furthermore, consuming high-fat and high-sugar meals enhances hepatic fat delivery and storage, as well as hepatic DNL (Boden, 2006).

1.2.5.3. Progression from simple steatosis to NASH

Lipotoxicity

All lipid metabolites that accumulate in the liver contribute to the formation of lipotoxicity, which is a term used to characterize the toxic effects of FFAs on cells as ROS (Petta et al., 2016). According to current research, lipotoxicity is the hit that causes hepatocyte damage and the development of NASH (Peverill et al., 2014).

Lipotoxicity appears in a variety of ways. Several studies, for example, have discovered that non-esterified FFAs that are not maintained in lipid droplets might induce persistent cellular damage and inflammation. Furthermore, elevated DNL is associated with an increased risk of lipotoxicity and cellular damage. (Saponaro et al., 2015). Ceramides have also been identified to have a role in the development of hepatic IR (Ussher et al., 2010). The accumulation of TG in the liver is not hepatotoxic in and of itself. In fact, it can function as a preventive mechanism, balancing excessive FFA accumulation (Listenberger et al., 2003). Increased hepatic TG level, on the other hand, occurs in conjunction with lipotoxicity and liver damage (Neuschwander-Tetri, 2010). Cholesterol is also a key lipotoxic molecule in the development of a number of metabolic disorders, including NAFLD. Aside from TG, research has shown that cholesterol builds up in the livers of NAFLD and NASH patients (Puri et al., 2007). Accumulation of cholesterol in the cytoplasm as well as in the intercellular space leads to the formation of cholesterol crystals, along with the expression and secretion of inflammatory cytokines (e.g. TNFa and IL-6), leading to cholesterol-mediated hepatocellular injury and cell death (Ioannou, 2016).

Inflammation

Several mechanisms contribute to increased levels of inflammatory cytokines in NAFLD, both in the liver and systemically. As previously stated, elevated circulating and hepatic FFA levels contribute to the formation of hepatic IR by activating the c-JNK and IKB serine/threonine kinases in the liver (Dongiovanni et al., 2010). Both c-JNK and IK increase chronic inflammation in addition to affecting insulin signalling; c-JNK belongs to the family of mitogen-activated protein kinases, while IK is required for the activation of nuclear factor kappa-B (NF-B) during inflammation. The NF-B pathway is persistently activated in NAFLD animal models and NASH patients, resulting in increased production of inflammatory cytokines such as TNF- and IL-6 (Gerges et al., 2021).

This increased hepatic production of inflammatory cytokines causes histological changes that are characteristic of NASH, such as hepatocellular necrosis, neutrophil infiltration, and the formation of Mallory bodies. This plays a significant role in the disease's development from basic steatosis to NASH and perhaps fibrosis. TNF- levels in the blood and liver were shown to be higher in NASH than in simple steatosis, and to correspond with the amount of histological damage (Hadizadeh et al., 2017). Unfortunately, persistent inflammation may promote carcinogenesis and hence contribute to the development of the disease to HCC (Gerges et al., 2021).

The activation of c-JNK and NF-B inflammatory pathways in the AT leads to increased production of inflammatory cytokines such TNF- and IL-6 and, in turn, higher plasma levels that impact other organs such as the liver and muscles (Shoelson et al., 2006). Furthermore, their serum levels

drop following weight reduction and are associated with improved insulin sensitivity. Obesity and AT dysfunction also results in lower levels of adiponectin, which has an anti-inflammatory impact by inhibiting NF-B activation and reducing the production of several inflammatory cytokines such as TNF- and IL-6. As previously stated, adiponectin levels are lower in NAFLD and NASH patients (Tsochatzis et al., 2006). Leptin levels, on the other hand, are increased owing to leptin resistance, and leptin has pro-inflammatory properties (Adolph et al., 2017).

Inflammation exacerbates hepatic IR. Exposure of the liver to TNF- and IL-6 produced by the AT, for example, leads to the development of hepatic IR. TNF- was discovered to increase IR by promoting adipocyte lipolysis and increasing IRS serine/threonine phosphorylation via c-JNK activation (Shoelson et al., 2006). Furthermore, IL-6 has been shown to induce IR by inhibiting the expression of IRS and glucose transporter-4, as well as by downregulating the PI3K pathway (Matsuda et al., 2013).

Oxidative stress

Endoplasmic reticulum (ER) stress

In NAFLD and NASH patients, high levels of saturated fatty acids in liver cells were reported to induce ER stress. ER stress, in turn, activates c- JNK, exacerbating hepatic IR and inflammation (Özcan et al., 2004).

Mitochondrial dysfunction

NAFLD is characterized by changes in mitochondrial structure and function (Pessayre and Fromenty, 2005). Excess FFA buildup in the liver leads to mitochondrial dysfunction by increasing the permeability of the inner mitochondrial membrane, resulting in ROS generation. Increased mitochondrial-oxidation for FFA overload causes electron leakage, boosting ROS generation and aggravating oxidative stress (Li et al., 2008). Furthermore, when lipid flow exceeds mitochondrial capacity, cellular respiration may be disrupted, resulting in disturbed fat homeostasis and excessive production of harmful lipid metabolites and ROS. ROS stimulate several inflammatory pathways, resulting in hepatic inflammation, which aggravates mitochondrial damage (Leamy et al., 2013). For example, ROS production causes NF-B activation, increasing inflammation and ROS production (Morgan and Liu, 2011).

Progression to fibrosis

Hepatic fibrosis is a normal result of chronic liver damage, which is mostly caused by chronic inflammation (Friedman, 2008). Through the stimulation of HSCs, inflammatory cytokines, ROS, and dead cells play a key role in the development of NASH to fibrosis (Tilg and Moschen, 2010).

Kupffer cells are hepatic tissue macrophages that reside in close proximity to collagen-releasing HSCs and can activate them directly. Damage-associated molecular patterns and ROS produced from dead liver cells all contribute to Kupffer cell activation, leading them to generate profibrogenic cytokines like TGF- and inflammatory cytokines like IL-6. HSCs are the primary myofibroblastic cells in charge of collagen deposition and scar formation. These cells are normally inactive. In situations of persistent damage and/or inflammation, however, they become triggered by signals produced by neighbouring cells such as Kupffer cells (Holt et al., 2008). Upon activation, HSCs multiply into a contractile matrix releasing form that secretes massive amounts of extracellular matrix (ECM), mostly collagen, resulting in fibrosis (Peverill et al., 2014). Furthermore, activated HSCs have enhanced alpha-smooth muscle actin expression and produce cytokines and chemokines that exacerbate inflammation and, as a result, fibrosis (Friedman, 2008).

It has been proposed that leptin induces Kupffer cells to generate TGF-, which activates HSCs (J. Wang et al., 2009). Leptin has also been shown to stimulate HSCs via several routes (Choi et al., 2010). By activating HSCs, high insulin levels also promote fibrogenesis ("Induction of proliferation and activation of rat hepatic stellate cells via high glucose and high insulin," 2017). Lastly, accumulation of FFAs and hazardous lipid metabolites in the liver was shown to promote TGF- signaling, indicating that FFAs play a key role in disease progression to fibrosis (Wanninger et al., 2011). Adiponectin, on the other hand, is known to relieve NASH and fibrosis by suppressing Kupffer cells and HSC activation (Y. Wang et al., 2009).

1.2.5.4. Role of the immune system in NASH

Both innate and adaptive immune responses are involved in the development of NASH.

Innate immunity

In NASH and other liver disorders, the innate immune system has a major influence on hepatic inflammation. Different cells, such as neutrophils, natural killer (NK) cells, and Kupffer cells, control this innate immune response. Neutrophils take part in the first inflammatory response to tissue injury. NK cells are numerous in the liver and may have a role in the development of NAFLD (Zhan and An, 2010). Although the number of circulating NK cells was shown to be reduced in obesity, their quantity in the liver is likely increased in NASH (O'Shea et al., 2010). HSCs also contribute to the innate immune response by expressing chemokines that promote leukocyte recruitment. HSCs continue to attract leukocytes after activation even in the absence of additional pro-inflammatory cytokines (Holt et al., 2008).

The adaptive immune system is also involved in the pathophysiology of NASH, particularly the development of fibrosis. The mechanisms behind this relationship are unknown, although T cells are likely to interact with fibroblasts and macrophages or to enhance HSC activation either directly or indirectly. Thus, cytokines produced by T cells activate HSCs, which then behave as antigen-presenting cells, stimulating T cells (Holt et al., 2008). Furthermore, pro-fibrotic cytokines such as IL-6 were discovered to be released by B cells (Mosmann, 2000).

1.2.6. Management and treatment

Current treatment strategies are still limited to provisional changes in diet and lifestyle ("EASL–EASD–EASO Clinical Practice Guidelines for the management of non-alcoholic fatty liver disease," 2016, p.), while the diagnosis and long-term therapy of chronic patients impose an enormous economic burden (Younossi et al., 2018).

The treatment of NAFLD focuses on four main objectives:

1. Weight loss via lifestyle changes
2. Control of cardiometabolic risk factors
3. Correction of all modifiable risk factors that contribute to the development and progression of advanced forms of NAFLD
4. Prevention of hepatic and extra-hepatic complications.

In principle, an optimal NAFLD treatment should reduce severe liver-related effects:

1. Reduction of hepatic steatosis, inflammation, and if possible fibrosis
2. Improvement of the metabolic background to avoid the manifestation of T2DM, cardiovascular disease, and other no-liver malignancies.

Given that, it is possible that no single pharmacological substance or therapeutic method will be able to achieve these goals and a combination of therapeutic approaches might be needed. (Mantovani and Dalbeni, 2021)

Several treatment methods exist and are being explored by many research groups. Drug discovery is stepping up, with several compounds now reaching clinical trials II and III (i.e. Obeticholic Acid, Resmetirom). However, none has been yet approved by the European Medicines Agency (EMA) nor the US Food Drug Administration (FDA) (Rau and Geier, 2021).

1.2.6.1. Lifestyle Modifications

A substantial amount of data supports the importance of lifestyle modification as the major method for the management of NAFLD and its advanced stages. Weight loss can reverse liver disease while also lowering the risk of cardiovascular disease and T2DM [18]. Weight loss of about 10% can result in NASH resolution as well as fibrosis improvement by at least one stage. Modest weight reduction (from 5% to 10%) can improve several components of the NAFLD activity score (NAS). (Chalasani et al., 2018; "EASL–EASD–EASO Clinical Practice Guidelines for the management of non-alcoholic fatty liver disease," 2016; Glen et al., 2016; Petroni et al., 2021; Romero-Gómez et al., 2017)

For these reasons, the EASL-EASO-EASD and American Association for the Study of Liver Diseases (AASLD) treatment recommendations for the management of NAFLD state that a 5–10% weight loss is the primary objective of most lifestyle therapies in overweight/obese NAFLD patients (Chalasani et al., 2018; "EASL–EASD–EASO Clinical Practice Guidelines for the management of non-alcoholic fatty liver disease," 2016). Guidelines from the National Institute for Health and Care Excellence (NICE) made similar suggestions (Glen et al., 2016). Some data also suggest that non-obese NAFLD patients can achieve NAFLD remission with a 3–10% weight loss and are more likely than obese NAFLD patients to sustain weight loss and normal liver enzymes over time (Wong et al., 2018).

The effect of weight reduction on histological improvement appears to be related to the quantity lost rather than the method used to attain it. Indeed, lifestyle treatments such as a low-calorie diet and physical exercise, drug-induced weight loss (e.g., orlistat), and weight loss after bariatric surgery appear to have a comparable positive effect on NASH resolution and fibrosis regression. (Romero-Gómez et al., 2017). All individuals with NAFLD, regardless of T2DM, should avoid moderate alcohol intake and, where feasible, hepatotoxic medications. Clinicians should additionally advise patients to avoid smoking as well as fructose-containing drinks and meals (Chalasani et al., 2018; "EASL–EASD–EASO Clinical Practice Guidelines for the management of non-alcoholic fatty liver disease," 2016; Glen et al., 2016).

A variety of dietary patterns have been investigated for the management of NAFLD (Romero-Gómez et al., 2017; Zelber-Sagi et al., 2017). Mediterranean diet has been repeatedly proven, through observational studies and small randomized controlled trials (RCTs), to have a positive effect on NAFLD patients. In fact, the Mediterranean diet has been proven to reduce liver fat and improve metabolic profile regardless of weight reduction (Meir et al., 2021; Parra-Vargas et al., 2020; Zadeh et al., 2021; Zelber-Sagi et al., 2017). Hence, at present, the Mediterranean diet is

the recommended dietary pattern in NAFLD patients by most published guidelines (Chalasani et al., 2018; "EASL–EASD–EASO Clinical Practice Guidelines for the management of non-alcoholic fatty liver disease," 2016; Zelber-Sagi et al., 2017). In summary, the Mediterranean diet emphasizes vegetables, legumes, whole grains, olive oil (as the primary source of added fat), fish, shellfish, nuts, and fruits, while limiting red meat, processed meats, and sweets (Romero-Gómez et al., 2017; Zelber-Sagi et al., 2017). Polyphenols, carotenoids, oleic acid, polyunsaturated fatty acids (PUFAs), and fibre are all potential molecular pathways for the Mediterranean diet's beneficial impact (Parra-Vargas et al., 2020; Zelber-Sagi et al., 2017). These components may have advantageous effects on certain pathways involving the gut, adipose tissue, and the liver (Parra-Vargas et al., 2020; Targher and Byrne, 2018; Wang et al., 2021). While there is considerable evidence supporting the significance of dietary modifications as the main therapy in NAFLD treatment, patient adherence to favourable dietary changes is a possible clinical issue (Targher and Byrne, 2018). It is also worth mentioning that the Mediterranean diet may not be suitable for every country or demography, and as a result, some authors have proposed a "wise choices" approach in which a patient selects the best nutritional options while adhering to at least some of the Mediterranean diet principles. Given that every component of the Mediterranean diet is beneficial to NAFLD, this may be an appropriate therapeutic compromise (Targher and Byrne, 2018; Zelber-Sagi et al., 2017).

Physical activity of various types appears to have similar effects on liver fat content (Bacchi et al., 2013; Marchesini et al., 2016; Petroni et al., 2021; Romero-Gómez et al., 2017). In Oh et al.'s study, resistance training, high-intensity interval aerobic training, and moderate-intensity continuous aerobic training were all equally effective in reducing hepatic fat content, but only high-intensity interval aerobic training improved hepatic stiffness and restored Kupffer cell function. Notably, these benefits did not appear to be dependent on weight loss or visceral fat removal. (Oh et al., 2017). The advantages, however, are lost if patients do not continue to exercise (Romero-Gómez et al., 2017). Interestingly, in NAFLD patients, genetic background (e.g., PNPLA3 rs738409 variation) may impact their response to physical exercise. Notably, these benefits did not appear to be dependent on weight loss or visceral fat loss (Oh et al., 2017). For example, growing data suggest that NAFLD patients with the rs738409 G/G genotype react better to lifestyle modification than those with the rs738409 G/G CC or C/G genotype (Romero-Gómez et al., 2017; Shen et al., 2015). Furthermore, the rate of NASH resolution and fibrosis regression following lifestyle management appears to be decreased in other subsets of individuals, such as the elderly, T2DM patients, and those with greater histological activity in liver biopsy (NAS > 5) (Romero-Gómez et al., 2017).

Changes in energy balance, insulin sensitivity, and circulatory lipids are among the processes behind the change in liver fat content caused by exercise (Golabi et al., 2016; Petroni et al., 2021; Romero-Gómez et al., 2017; Thyfault and Bergouignan, 2020). Exercise increases hepatic and systemic insulin sensitivity, improving insulin action and decreasing de novo lipogenesis. Exercise also has a direct influence on glycemic management and lipid flux, with an increase in very-low-density lipoprotein (VLDL) clearance, supporting liver fat reduction. Exercise also causes a significant decrease in visceral fat tissue. Visceral fat is linked to liver inflammation and fibrosis, irrespective of insulin resistance and hepatic steatosis (Golabi et al., 2016; Romero-Gómez et al., 2017; Thyfault and Bergouignan, 2020). Finally, because patients with NAFLD are more likely to develop cardiovascular disease than those who do not, it is critical to remember that the positive effects of exercise extend to the entire cardiovascular system (Moreira et al., 2020; Romero-Gómez et al., 2017).

1.2.6.2. Bariatric Surgery

Bariatric surgery refers to surgical treatments that cause weight reduction by limiting the quantity of food the stomach can contain and/or creating nutritional malabsorption. Laparoscopic sleeve gastrectomy, laparoscopic Roux-en-Y gastric bypass, laparoscopic adjustable gastric banding, and duodenal switch are the most frequent bariatric surgery techniques performed worldwide. Bariatric surgery can improve insulin resistance, obesity, T2DM, hypertension, dyslipidemia, and obstructive sleep apnea in addition to weight loss (Nguyen and Varela, 2017). Importantly, all histological characteristics of NAFLD, including fibrosis, can be significantly improved by bariatric surgery (Chalasani et al., 2018; "EASL–EASD–EASO Clinical Practice Guidelines for the management of non-alcoholic fatty liver disease," 2016; Nguyen and Varela, 2017).

The possible liver-related advantages of bariatric surgery may go beyond weight loss. Indeed, bariatric surgery can raise circulation levels of glucagon-like peptide-1 (GLP-1) (Brunt et al., 2015), which reduces hunger, delays stomach emptying, and improves insulin sensitivity. Furthermore, GLP-1 affects bile acid signalling via the farnesoid X receptor (FXR), which can change the gut microbiota and induce NAFLD (Chu et al., 2019; Jiang et al., 2020). Based on these assumptions, current guidelines suggest that bariatric surgery may be a viable choice for individuals with T2DM or extreme obesity (i.e., BMI >35 kg/m2) (Chalasani et al., 2018; "EASL–EASD–EASO Clinical Practice Guidelines for the management of non-alcoholic fatty liver disease," 2016). When T2DM is not effectively managed by an appropriate medication regimen, bariatric surgery should be explored as an alternate option in patients with a BMI between 30 and 35 kg/m2, particularly in the presence of cardiovascular disease (CVD) risk factors (Chalasani et al.,

2018; "EASL–EASD–EASO Clinical Practice Guidelines for the management of non-alcoholic fatty liver disease," 2016). While bariatric surgery is effective, there are significant limitations, such as complications (e.g., bleeding, infection, adverse anesthesia reactions, blood clots, leaks in the gastrointestinal system, bowel obstruction, dumping syndrome, flushing, nausea, vomiting, diarrhea, gallstones, hernias, malnutrition, acid reflux, and even death), patient acceptability, service availability, and costs (Nguyen and Varela, 2017). For these reasons, the possible long-term benefits and adverse effects of bariatric surgery should be carefully examined. Although the positive effects of bariatric surgery are therapeutically important, long-term prospective studies (including RCTs) are needed to evaluate if NAFLD remission is durable and the exact incidence of NAFLD recurrence in individual patients. Furthermore, no consistent information on the comparative effects of different bariatric operations on liver fat levels is currently available (Chalasani et al., 2018; "EASL–EASD–EASO Clinical Practice Guidelines for the management of non-alcoholic fatty liver disease," 2016).

1.2.6.3. Liver Transplantation

NASH-associated cirrhosis is currently one of the top three indications for liver transplantation in the majority of high-income nations, with a worrisome trend toward becoming the most prevalent (Chalasani et al., 2018; "EASL–EASD–EASO Clinical Practice Guidelines for the management of non-alcoholic fatty liver disease," 2016). Patients having a liver transplant for NASH-associated cirrhosis had outcomes that are significantly comparable to those for other reasons at 1, 3, and 5 years (Burra et al., 2020; M. R. Charlton et al., 2011; X. Wang et al., 2014). Overall mortality in patients with NASH-related cirrhosis receiving liver transplant appears to be more commonly associated with the recipient's age (i.e., >60 years), obesity (i.e., BMI > 30 kg/m2), T2DM, or post-transplant MetS. NAFLD recurrence is also frequent following liver transplantation, ranging from 20% to 40% of patients depending on the methods employed for diagnosis. The following risk variables have been linked to NAFLD recurrence: (a) post-transplant weight increase, (b) steroid usage, (c) MetS presence, and (d) PNPLA3 rs738409 in the recipient. Furthermore, around 30% of patients transplanted for causes other than NASH-associated cirrhosis might develop de novo NAFLD, generally within 3 years following the transplant. The most prominent risk factors for de novo NAFLD include T2DM, obesity, arterial hypertension, and liver transplant steatosis. As a result of these findings, careful treatment of the MetS components is important for long-term survival in these individuals. Furthermore, patients with NASH-related cirrhosis have a poor performance status, which has been linked to worse graft survival and overall patient 5-year

survival rates (Burra et al., 2020; M. R. Charlton et al., 2011; Gitto et al., 2016; Samji et al., 2020).

Patients undergoing liver transplantation who have NASH-associated cirrhosis encounter several difficulties. First, the rising incidence of obesity and type 2 diabetes has considerably increased the occurrence of hepatic steatosis in potential donors, decreasing the number of transplantable livers. Donor livers with more than 60% steatosis are now deemed un-transplantable, but those with 30% steatosis are regarded usable. Livers with 30–60% steatosis may be utilized for particular patients, although they are linked with poor outcomes. Second, because NAFLD is the hepatic manifestation of MetS, patients with NASH-associated cirrhosis typically have several comorbidities, including obesity, cardiovascular disease, type 2 diabetes, and chronic kidney disease (Samji et al., 2020, p.). In this regard, AASLD practice guidelines consider severe obesity (BMI 40 kg/m2) to be a relative contraindication for liver transplantation (Chalasani et al., 2018), whereas EASL-EASD-EASO practice guidelines state that patients with a BMI > 35 kg/m2 should always be evaluated by a multidisciplinary team before being placed on the waiting list ("EASL–EASD–EASO Clinical Practice Guidelines for the management of non-alcoholic fatty liver disease," 2016). Furthermore, AASLD practice recommendations suggest that patients with NASH-associated cirrhosis be thoroughly assessed for CVD during the transplant assessment process (Chalasani et al., 2018). Third, due to lower Model for End-Stage Liver Disease (MELD) scores as compared to other etiologies of chronic liver disease, individuals with NASH-associated cirrhosis who are on the waiting list for liver transplantation may compete for liver allograft allocations. Individuals on the waiting list for liver transplantation who have end-stage liver disease owing to NASH may have improved liver functioning and, as a result, lower MELD scores when compared to other etiologies of liver cirrhosis (Mikolasevic et al., 2018).

However, additional research is required to improve the health care of this growing patient group.

1.2.6.4. Pharmacological Approach

Although no specific agents are currently approved for the treatment of NAFLD and its advanced forms, several potential agents, such as glucose-lowering drugs (such as metformin, pioglitazone, GLP-1 receptor agonists, and sodium-glucose co-transporter-2 (SGLT-2) inhibitors), statins, and other lipid-lowering therapies, anti-hypertensive drugs, and other possible agents, such as obeticholic acid, have been widely investigated in recent decades.

Metformin is a biguanide that is widely suggested as the initial medication in the majority of T2DM patients at the time of diagnosis. (Association, 2020; Raschi et al., 2018). Metformin is considered safe and effective in T2DM patients with no contraindications such as chronic renal disease stage 4 or 5, severe heart failure, advanced pulmonary sickness, or a history of lactic acidosis, with a 0.5 to 1% drop in HbA1c levels. (Association, 2020). Metformin lowers blood glucose levels experimentally via processes involving an AMP-activated protein kinase (AMPK)-dependent improvement in hepatic glucose metabolism and enhanced glucose absorption into muscle cells (Association, 2020; Foretz et al., 2014). Other mechanisms involving changes in cellular energy charge, fructose-1,6-bisphosphatase 1, and modulation of the cellular redox state via direct inhibition of mitochondrial glycerol-3-phosphate dehydrogenase have been proposed as potential factors of this glucose-lowering agent's inhibition of gluconeogenesis (Foretz et al., 2014). Metformin can also lower the risk of cardiovascular events and mortality in overweight or obese T2DM patients (Association, 2020; Raschi et al., 2018).

Despite its favourable effects on liver enzymes and HbA1c levels in RCTs involving individuals with biopsy-proven NAFLD, metformin exhibited minor beneficial effects on liver steatosis or inflammation but no benefits on the resolution of NASH and liver fibrosis (Mantovani and Dalbeni, 2021). Furthermore, in the Treatment of NAFLD in Children (TONIC) study, which included obese children/adolescents with biopsy-proven NASH, metformin had no positive impact on different aspects of liver histology (Lavine et al., 2011). Although metformin is typically well taken, some T2DM patients may experience gastrointestinal symptoms (such as diarrhoea, bloating, or stomach discomfort). Long-term metformin usage has also been linked to vitamin B12 insufficiency (Association, 2020). The EASL-EASO-EASD and AASLD practice recommendations for NAFLD management, as well as the NICE guidelines, do not currently support the use of metformin for the treatment of NAFLD (Chalasani et al., 2018; "EASL–EASD–EASO Clinical Practice Guidelines for the management of non-alcoholic fatty liver disease," 2016; Glen et al., 2016). However, metformin's potential advantages in chronic liver disease appear to be confined to lowering the risk of cirrhosis and HCC. Indeed, several case-control and cohort studies, as well as meta-analyses, have discovered an independent connection between metformin and a reduced risk of HCC in T2DM patients with chronic liver disease (Kaplan et al., 2020; Schulte et al., 2019; Singh et al., 2013; Vilar-Gomez et al., 2019; Zhou et al., 2020). A retrospective cohort study involving T2DM patients with biopsy-proven NASH, bridging fibrosis or compensated cirrhosis, showed that long-term metformin use was independently associated with a lower risk of all-cause mortality and HCC incidence over a 7-year follow-up. (Vilar-Gomez et al., 2019). A meta-analysis of six

retrospective cohort studies published in 2020, included four studies with curative treatment with metformin and other anti-hyperglycemic agents for HCC, and two studies with non-curative treatment with metformin other glucose-lowering agents for HCC. When compared to other glucose-lowering agents, metformin treatment was associated with significantly longer overall survival and recurrence-free survival rates in HCC patients (Zhou et al., 2020). Metformin may suppress cancer invasion and metastasis via the AMPK (AMP-activated protein kinase) signalling route, the EMT (epithelial-mesenchymal transition) signalling pathway, and certain epigenetic changes improving the prognosis of cancer patients (Y. C. Chen et al., 2020). However, while this data suggests that metformin may be related to a decrease in cancer frequency and incidence in T2DM patients, specific randomized controlled trials are required to confirm the findings obtained by case-control and cohort studies.

PPAR-Gamma Agonists | Rosiglitazone, Pioglitazone, Elafibranor, Saroglitazar

Rosiglitazone and pioglitazoneare are selective PPAR-activated receptor γ (PPAR) ligands (Association, 2020; Raschi et al., 2018). Peroxisome proliferator-activated receptors (PPARs), present with three distinct isotypes (i.e. α, β/δ, and γ) and are nuclear regulatory factors modulating critical elements of glucose and fat metabolism, as well as inflammatory cell activation and fibrotic processes (Francque et al., 2021). Glitazones that bind to the PPAR-γ isotype modulate insulin, glucose, and lipid metabolism, as well as inflammatory and adipose tissue pathways. The PPAR-γ receptor-2 isoform is mostly expressed in the AT, where it plays an important role in the redistribution of intra-abdominal and subcutaneous adipose tissue by enhancing TG accumulation in peripheral adipose tissue depots. PPAR-γ is also expressed in Kupffer cells, which are involved in fibrosis. (Association, 2020; Francque et al., 2021; Raschi et al., 2018). Finally, PPAR-γ may decrease portal pressure, inflammation, angiogenesis, and portosystemic shunts in the setting of cirrhosis (Francque et al., 2021).

Several studies (Aithal et al., 2008; Belfort et al., 2006; Cusi et al., 2016; Ratziu et al., 2008; Sanyal et al., 2010) have been conducted to investigate the possible therapeutic benefits of glitazones in NAFLD/NASH patients based on these pre-clinical results. A recent comprehensive review, for example, found that using pioglitazone in patients with biopsy-confirmed NASH can have significant advantages on liver function, hepatic fat content, and NASH resolution in individuals with and without T2DM. In contrast to its favourable effects on NASH, the effect of pioglitazone on liver fibrosis appears to be limited (Mantovani and Dalbeni, 2021). However, in a placebo-controlled randomized trial of biopsy-proven NASH and T2DM patients randomly assigned to pioglitazone or placebo for 18 months, pioglitazone was found to be more beneficial than placebo. It was claimed that over 60% of patients in the pioglitazone group met the primary

objective, which was defined as a reduction of at least 2 points in the NAFLD activity score without increasing fibrosis, and nearly 50% had full NASH remission. Long-term pioglitazone treatment improved individual histologic scores of NASH, including fibrosis scores (Cusi et al., 2016). Musso et al. demonstrated that pioglitazone reduced advanced fibrosis in NASH patients independent of T2DM in a meta-analysis of eight RCTs enrolling approximately 500 individuals with biopsy-confirmed NASH followed up to 24 months (Musso et al., 2017). Rosiglitazone data, on the other hand, are more restricted. The FLIRT (Fatty Liver Improvement with Rosiglitazone Therapy) experiment found that steatosis improved considerably in individuals receiving rosiglitazone compared to those getting placebo (47 % vs. 16 %, p 0.05). In contrast, there were no substantial alterations in liver fibrosis (Ratziu et al., 2008). However, rosiglitazone studies were halted due to an elevated cardiovascular risk (Raschi et al., 2018).

Based on these findings, the EASL-EASO-EASD, AASLD, and NICE practice recommendations for NAFLD treatment advocate the use of pioglitazone in NASH patients (Chalasani et al., 2018; "EASL–EASD–EASO Clinical Practice Guidelines for the management of non-alcoholic fatty liver disease," 2016; Glen et al., 2016). However, pioglitazone has not yet been licensed by most national pharmaceuticals regulators for usage other than the treatment of T2DM. As a result, the off-label use of pioglitazone for NAFLD/NASH therapy now needs patient permission (Raschi et al., 2018). Concerns about weight gain (most RCTs showed an increase of 2–4% of body weight after 6–36 months of pioglitazone medication), fluid retention, and the risk of bone fractures (mainly in women) or bladder cancer may limit the use of pioglitazone in NAFLD patients. Finally, pioglitazone has significant cardiovascular advantages, lowering the incidence of myocardial infarction and stroke in individuals with type 2 diabetes or prediabetes (Dormandy et al., 2005; Spence et al., 2019). Given that individuals with NAFLD are at a greater risk of developing cardiovascular disease than those without liver involvement, regardless of the presence of T2DM, this cardiovascular-protective medication should be evaluated in NASH patients. Interestingly, growing data suggests that particular genetic polymorphisms may account for some of the inter-individual heterogeneity in responsiveness to pioglitazone therapy in NASH patients. For example, Kawaguchi-Suzuki et al. reported that ADORA1 (Adenosine A1 Receptor) rs903361 was associated with resolution of NASH and improvement in the ballooning score in patients receiving pioglitazone in a small pilot study of 55 participants from a randomized controlled trial designed to determine the efficacy of long-term pioglitazone treatment in patients with NASH (NCT00994682).

Agents capable of promoting the safe disposal of different metabolic substrates, such as PPARα/δ (i.e., dual action on both PPAR-α and PPAR-δ receptors) and PPARβ/δ agonists, are being studied (Francque et al., 2021; Friedman et al., 2018a). Experimentally, the PPARα component increases

fatty acid oxidation, whereas the PPARδ component has significant anti-inflammatory benefits. Elafibrinor is a PPARα/δ agonist prototype that can reduce insulin resistance and inflammation (Friedman et al., 2018a). In a phase 2b study of patients with biopsy-proven NASH who were randomly allocated to receive elafibranor or placebo, elafibranor was superior in reversing NASH without increasing fibrosis (20% in the elafibranor group vs. 11% in the placebo group). A post-hoc analysis of this study, based on new criteria for NASH resolution, confirmed these findings (Ratziu et al., 2008). In a phase 3 study, RESOLVE-IT (NCT02704403), the effectiveness and safety of this drug in patients with NASH and different degrees of fibrosis were also assessed (Francque et al., 2021). However, the interim analysis failed to demonstrate accomplishment of the primary histological end objective of NASH remission without fibrosis worsening, and therefore the study was halted (Armstrong et al., 2016).

The PPARα/δ dual agonist saroglitazar has recently been shown in animal models of NASH to have positive effects on the liver (Francque et al., 2021). A meta-analysis of 18 trials found that saroglitazar might lower blood ALT levels and improve cardiometabolic profiles in individuals with diabetic dyslipidaemia (Kaul et al., 2019). A preliminary review of a randomized, double-blind, phase 2 study with non-invasive liver end goals yielded encouraging results on the potential function of saroglitazar in NASH patients (EVIDENCES II; NCT03061721). Ongoing clinical studies suggest that dual- and pan-PPAR agonists may benefit NASH via interconnected pathways (Francque et al., 2021).

SGLT-2 Inhibitors | Dapagliflozin, Empagliflozin, Ipragliflozin, Canagliflozin

Sodium-glucose cotransporter-2 (SGLT-2) inhibitors are a relatively new family of glucose-lowering medicines that promote reabsorption of glucose by the kidneys, as well as the gut and heart. SGLT-2 is found on the renal epithelial cells bordering the S1 section of the proximal convoluted tubule and promotes glycosuria. In this regard, insulin synthesis has little effect on blood glucose control (Association, 2020; Raschi et al., 2018). Due to a combination of negative energy balance induced by increased glycosuria and substrate shift toward lipids as a source of energy expenditure, SGLT-2 inhibitors were found to be helpful in animal NASH models for liver steatosis, inflammation, and fibrosis (Katsiki et al., 2019). For these reasons, SGLT-2 inhibitors have been studied as a potential treatment for NASH in humans. Mantovani et al. reported in a 2021 meta-analysis of 12 RCTs evaluating the efficacy of dapagliflozin, empagliflozin, ipragliflozin, or canagliflozin to treat NAFLD for a median period of 24 weeks with a total of 850 middle-aged overweight or obese individuals with NAFLD that, compared to placebo or reference therapy, treatment with SGLT-2 inhibitors decreased serum ALT and GGT levels, as well as the absolute

percentage of liver fat content on magnetic resonance imaging (Mantovani et al., 2021). A similar set of findings was found in another meta-analysis (Coelho et al., n.d.). It is important to highlight, however, that the bulk of the RCTs currently available are small and do not investigate the effect of SGLT-2 inhibitors on liver histology. In published RCTs, SGLT-2 inhibitors had a similar adverse event profile to placebo (or reference treatment), with the exception of a higher frequency of genitourinary infections (Mantovani et al., 2020). Because SGLT-2 inhibitors have proven significant cardio-renal benefits in large RCTs involving people with and without T2DM, they are tempting medications for NAFLD patients. Other RCTs are currently being conducted in these patients to examine the impact of SGLT-2 inhibitors (Neuen et al., 2019; Zelniker et al., 2019).

Statins and Other Lipid-Lowering Agents

Because NAFLD is linked with particular MetS characteristics such as T2DM, hypertension, obesity, and (atherogenic) dyslipidemia, physicians must regularly address these diseases in NAFLD patients. Statins, which are known to block 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCoA), a major enzyme implicated in cholesterol production, can be used to reduce abnormal blood cholesterol levels. Statins, in addition to their lipid-lowering action, have pleiotropic characteristics such as antioxidative and anti-inflammatory effects, neoangiogenesis, and improved endothelial functioning (Pose et al., 2019). Although statins may increase aminotransferase levels, liver injury caused by this lipid-lowering medication is uncommon in clinical practice (Ekstedt et al., 2007; Pose et al., 2019). In this manner, it is predicted that an increase in liver enzymes greater than three times the upper limit of normal can be detected in 1% of individuals treated with statins (Cohen et al., 2006; Pose et al., 2019). As a result, frequent monitoring of transaminase levels is no longer suggested (Pose et al., 2019). Furthermore, NAFLD patients are at an increased risk of cardiovascular morbidity and death (Lonardo et al., 2018, 2017; Targher et al., 2010). In a post hoc analysis of the Greek Atorvastatin and Coronary Heart Disease Evaluation (GREACE) study, which enrolled patients with moderately abnormal liver tests at baseline of NAFLD presence it was reported that NAFLD patients who received statins had significantly lower cardiovascular morbidity, without significant differences in mortality compared to those who didn’t. Interestingly, growing data shows that statin therapy is linked with a considerable improvement in liver steatosis, inflammation, and even fibrosis in individuals with NAFLD (Dongiovanni et al., 2015; Lee et al., 2021; Nascimbeni et al., 2016). For example, a recent observational study of more than 11 million people from the Republic of Korea's National Health Information Database found that statin use was associated with a lower risk of NAFLD as well as a lower risk of significant liver fibrosis, as measured by BARD score, even after controlling for several metabolic risk factors (Lee et al., 2021). Animal model studies have provided insight into

the potential pathways by which statins may enhance the improvement of liver histology in NAFLD patients. Statins, for example, impact the paracrine signaling (including the RhoA/Rho-kinase pathway) of hepatocytes on hepatic stellate cells in experimental NASH, therefore inhibiting hepatic stellate cell activation and, as a result, fibrosis processes. Statins' antifibrotic action in a bile duct ligated mouse model was attributed to a decrease in serum bile acid levels. Statins reduced fibrosis in several models, including angiotensin-II-induced liver fibrosis, by decreasing inflammatory activity (Pose et al., 2019).

All chronic liver disease etiologies have a similar end-stage defined by portal hypertension and liver remodelling. Statins may even influence the pathways (e.g., RhoA/Rho-kinase and nitric oxide (NO)) implicated in poor intrahepatic resistance and vascular tone control, resulting in portal hypertension. Based on these findings, statins appear to be capable of modulating the dynamic and structural components of chronic liver disorders (including fibrosis), making them beneficial in the treatment of patients with and without T2DM who have cirrhosis and portal hypertension (Athyros et al., 2017; Pose et al., 2019).

There is also information available on the impact of various lipid-lowering medications on liver histology in NAFLD patients. Ezetimibe is a lipid-lowering medication that works by reducing cholesterol absorption in the intestines. It specifically inhibits the Niemann–Pick C1–like 1 (NPC1L1) protein, a key mediator of cholesterol absorption, in gastrointestinal tract epithelial cells as well as hepatocytes. A modest meta-analysis of six studies (two RCTs and four single-arm trials) with NAFLD patients with and without T2DM found that ezetimibe significantly decreased blood liver enzyme levels while also improving hepatic steatosis and hepatocyte ballooning. However, ezetimibe did not ameliorate liver fibrosis in that trial (Nakade et al., 2017).

Fenofibrate, a PPAR-α agonist, does not appear to decrease the liver fat content in NAFLD patients with or without T2DM (Dewidar et al., 2020).

A-linolenic acid (α-ALA), stearidonic acid (SDA), eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA), and docosahexaenoic acid (DHA) are all long-chain fatty acids found in omega-3 polyunsaturated fatty acids (n-3 PUFAs) (DHA). EPA and DHA lower triglyceride and very-low-density lipoprotein levels, which are then transformed to low-density lipoprotein and intermediate-density lipoprotein levels. A growing body of data shows that dietary omega-3 PUFAs may reduce insulin resistance via modulating mitochondrial activity and mediating anti-inflammatory effects (Lee et al., 2020, p. 3). Several preclinical investigations utilizing animal NASH models have shown that n-3 PUFA supplementation may have a beneficial effect on NAFLD by reducing hepatic fat accumulation and avoiding the proinflammatory state (Valenzuela and Videla, 2020). For these

reasons, n-3 PUFAs have been studied as a potential treatment for NASH in humans. Lee et al. found in a 2020 meta-analysis of 22 RCTs including patients with and without T2DM that n-3 PUFA supplementation substantially decreased liver fat when compared to placebo. Furthermore, in that research, n-3 PUFA supplementation substantially reduced triglyceride, total cholesterol, high-density lipoprotein, and BMI levels (Lee et al., 2020, p. 3). A similar pattern was seen in another meta-analysis. However, it is essential to note that the magnitude of the impact of n-3 PUFAs is rather minor, and the appropriate dose and duration of therapy with them have yet to be determined (Parker et al., 2012). As a result, further well-designed randomized clinical studies are required before we can propose omega-3 PUFA supplementation for the treatment of NAFLD in individuals with and without T2DM. In this regard, NICE guidelines do not suggest n-3 PUFA supplementation in NAFLD patients based on existing data (Glen et al., 2016).

PCSK-9 (proprotein convertase subtilisin kexin type-9) is a critical regulator of cholesterol homeostasis and a strong inhibitor of the LDL receptor (LDLR) pathway. Circulating PCSK-9 binds to the extracellular EGF(A) domain of the LDLR and promotes its lysosomal degradation once released by the hepatocyte (Wargny et al., 2018). Data shows that high intrahepatic or circulating PCSK-9 levels promote the development of NAFLD by increasing muscle and liver lipid storage, adipose energy storage, hepatic fatty acid and triglyceride storage. Preliminary data suggest that antisense particles targeting PCSK-9 mRNA or anti-PCSK-9 antibodies capable of lowering circulation PCSK-9 levels may ameliorate NAFLD independently of LDL cholesterol decrease (Eleni et al., 2018).

Anti-Hypertensive Agents | Rosiglitazone, Losartan

Although not all studies have shown it, angiotensin-converting enzyme inhibitors (ACEi) or angiotensin II receptor blockers (ARBs) may have an anti-fibrotic impact on the liver. In animal NASH models, for example, therapy with ACEi and ARBs can turn off the profibrogenic state and lead to fibrosis regression (Hirose et al., 2007; Jonsson et al., 2001; Moreno et al., 2010; Park et al., 2019). Clinical trials to investigate the potential anti-fibrotic benefits of these medicines in NAFLD patients have yielded inconclusive findings (Georgescu et al., 2009; McPherson et al., 2017; Torres et al., 2011; Yokohama et al., 2004). Furthermore, trials recruiting only T2DM patients are still lacking. In a randomized, open-label trial of individuals with biopsy-proven NASH (approximately 20% with established T2DM) who were randomly assigned to receive either rosiglitazone, rosiglitazone and metformin, or rosiglitazone and losartan daily for 48 weeks, combination therapy with rosiglitazone alone conferred no higher benefit on histopathological features (Torres et al., 2011). Losartan versus placebo for 96 weeks in patients with histological

evidence of NASH (about 60% with established T2DM) failed to recruit enough patients to determine if losartan had anti-fibrotic effects in the liver (McPherson et al., 2017). It is, therefore, possible to speculate that some ARBs, particularly losartan, may be beneficial in treating NASH/NAFLD and its complications, but additional and larger controlled clinical trials are needed to provide consistent data on this topic, particularly in those with T2DM.

Anti-Platelet Aggregation Agents

There is a scarcity of evidence from prospective trials on the impact of aspirin on fibrosis in NAFLD patients. Daily aspirin usage was linked with less severe histologic characteristics of NAFLD and NASH, as well as a decreased risk of progression to advanced fibrosis over time in a recent observational analysis of individuals with biopsy-proven NAFLD (Simon et al., 2019). Some investigations have found that platelet-derived GPIb may have a role in the development of NASH independent of von Willebrand factor (vWF), p-selectin, and Mac-1 (also known as integrin M2, or CD11b/CD18). These processes may provide a fresh potential target for NASH (Malehmir et al., 2019).

Vitamin E

According to animal NASH models, vitamin E is a strong antioxidant that may be useful in the treatment of NAFLD. Vitamin E has been found to enhance lipid and glucose metabolism by activating the Nrf2/CES1 signalling pathway (He et al., 2019), as well as to decrease oxidative stress by downregulating iNOS and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Uchida et al., 2018). Over 96 weeks, the PIVENS trial, which enrolled adults with NASH and randomly assigned them to receive vitamin E or placebo, found significant improvements in serum liver enzymes as well as some histological features of NASH (steatosis, inflammation, ballooning) in the vitamin E group (Sanyal et al., 2010). At present, there is no sufficient evidence to recommend the use of vitamin E in NAFLD patients with T2DM. Nonetheless, the AASLD and NICE practice guidelines for NAFLD treatment support the use of vitamin E in non-diabetic patients with NASH, especially in those with secondary/tertiary care settings. However, more evidence might be required to support the usage of this agent in the long run (Chalasani et al., 2018; Glen et al., 2016).

Potential new drugs for NAFL/NASH

Several more possible NAFLD agents have been explored in recent years. However, it is crucial to highlight that in the majority of the studies that have been conducted thus far, only a fraction of NASH patients had developed T2DM at the outset (Mantovani and Dalbeni, 2021).

In animal NASH models, synthetic ligands that activate the nuclear receptor farnesoid X receptor (FXR) reduces insulin resistance, control glucose and lipid metabolism, and have direct anti-inflammatory and anti-fibrotic actions (Friedman et al., 2018a; Kong et al., 2009). The paradigm for this class of drugs is obicholic acid (OCA), a synthetically modified counterpart of chenodeoxycholic acid (Friedman et al., 2018a). The FLINT trial, which included patients with non-cirrhotic NASH (almost half of whom had T2DM), discovered that OCA improved histological features of NASH, including fibrosis (Neuschwander-Tetri et al., 2015). Yonoussi et al. reported that in the 18-month interim analysis of the multicenter, randomized, placebo-controlled phase 3 trial REGENERATE, which evaluated the safety and efficacy of two doses of OCA relative to placebo in patients with biopsy-proven stage F2–F3 fibrosis, 23% of patients in the OCA high-dosage mg group improved fibrosis, compared to 12% in the placebo group. Obeticholic acid causes several adverse effects, including pruritus and increased LDL cholesterol levels. In detail, the effect of OCA on the lipid profile was found to be clinically significant, as NAFLD patients have an elevated risk of CVD, the leading cause of mortality in these patients. LDL cholesterol levels increased by approximately 20% from baseline in the REGENERATE study, however, it was postulated that the rise in LDL cholesterol levels was transitory and reduced by statin therapy. Moreover, OCA treatment at 12 weeks was linked with increases in small VLDL particles, big and small LDL particles, and a decrease in HDL particles in another research involving FLINT trial participants. Following medication cessation, such changes in lipoprotein concentrations returned to baseline (Siddiqui et al., 2020). The combination of OCA and atorvastatin is being investigated for safety in NASH patients in the randomized, placebo-controlled, double-blind "combination OCA and statins for monitoring of lipids (CONTROL)" phase 2 trial (NCT02633956) (Pockros et al., 2019). A pilot genome-wide association research utilizing FLINT participants, similar to pioglitazone, found that some genetic variations may be linked with histological improvement in NASH patients taking OCA. Among the studied genetic variations, the rs75508464 mutation near the chymotrypsin-like elastase 3B (CELA3B) gene appears to have a substantial influence on NASH resolution in OCA individuals (Gawrieh et al., 2019).

Non-bile acid farnesoid X activated receptor (FXR) agonists such as tropifexor, cilofexor, EDP-305, and nidufexor are being studied for their ability to not raise LDL cholesterol levels (or other lipoproteins) or induce pruritus (Friedman et al., 2018a).

Another significant route that might increase FXR activity is the release of the growth factor FGF-19 from the gut in response to bile acid-binding to FXR, which has been shown to have beneficial effects in animal NASH models (Friedman et al., 2018a). In a phase 2a trial (NCT02443116), the

FGF-19 analogue NGM282 significantly reduced hepatic fat content and liver enzymes in individuals with biopsy-proven NASH (Harrison et al., 2018b, p. 282).

Thyroid hormone receptor (THR)-β-selective agonists have been tried in animal NASH models to decrease lipotoxic burden in the liver. Importantly, it improves circulating lipids in healthy people (Friedman et al., 2018a). Resmetirom (MGL-3196), a liver-directed, orally active, selective thyroid hormone receptor-agonist, showed a relative reduction in hepatic fat when compared to placebo in a multicenter, randomized, double-blind, placebo-controlled, phase 2 trial of NAFLD patients with and without established T2DM at weeks 12 and 36. The resmetirom group experienced more transient mild diarrhoea and nausea than the placebo group (Harrison et al., 2019).

DGAT2 is one of two enzyme isoforms (DGAT1 and DGAT2) that catalyze the final stage of triglyceride synthesis by increasing the coupling of diacylglycerol to acyl-coenzyme A (Loomba et al., 2020; Stone et al., 2009). While DGAT1 is mainly found in the small intestine, DGAT2 is mostly found in the liver (Loomba et al., 2020). Antisense inhibition of DGAT2 in animal NASH models has been shown to reduce triglyceride production and hepatic triglyceride levels, as well as ameliorate hepatic steatosis and plasma lipoprotein profiles. IONIS-DGAT2Rx is a 20-O-methoxyethyl chimeric antisense oligonucleotide inhibitor that inhibits DGAT2 protein synthesis by mediating enzyme-mediated degradation of DGAT2 mRNA (Loomba et al., 2020; Stone et al., 2009). Loomba et al. reported that in a recent small randomized, placebo-controlled phase 2 trial of IONIS-DGAT2Rx that enrolled NAFLD patients with T2DM who were randomly assigned to receive IONIS-DGAT2Rx or placebo for a total of 13 weeks, the mean absolute reduction of liver fat content from baseline was approximately 5.2% in the IONIS-DGAT2Rx group compared to approximately 0.6% in the placebo group. However, a limited number of patients in the IONIS-DGAT2Rx group developed several serious adverse events, in contrast to the placebo group (Loomba et al., 2020). NASH development is linked to the innate and adaptive immune systems. Based on data from animal models, the C–C motif chemokine receptor 2 (CCR2)-CCR5 chemokine axis enhances the innate immune response into the liver and stimulates the activation of hepatic stellate cells, which are important collagen makers, leading to fibrosis (Friedman et al., 2018a; Krenkel et al., 2018). Cenicriviroc inhibited CCR2-CCR5 in a limited clinical study of NASH patients and decreased short-term fibrosis progression (Friedman et al., 2018b). A phase 3 study (NCT03028740) is now underway (Friedman et al., 2018a).

Some experimental investigations have concentrated on the particular suppression of the fibrosis process in the liver. An inhibiting antibody to lysyl oxidase-2 (LOXL-2), an enzyme that chemically crosslinks fibrillary collagen, is one possible mechanism. In this respect, LOXL-2 inhibition has

been shown in animal NASH models to improve macrophage-mediated collagen breakdown (Rotman and Sanyal, 2017). However, it was reported that a monoclonal antibody against LOXL-2, simtuzumab, was ineffective in decreasing hepatic collagen content and hepatic venous pressure gradient in two phase 2b trials of patients with bridging fibrosis caused by NASH who were randomly assigned to groups given weekly subcutaneous injections of simtuzumab or placebo for 240 weeks (Harrison et al., 2018a).

Caspases are intracellular proteases that cleave cytoskeletal proteins to cause apoptotic cell death. NASH patients have enhanced apoptosis, and serum levels of cleaved keratin-18 (CK-18) are linked to liver fibrosis (Barreyro et al., 2015; Friedman et al., 2018a). Emricasan is a pan-caspase inhibitor that has previously been found to reduce caspase-3/7 activity, CK-18, and increase blood ALT levels in NAFLD patients (Friedman et al., 2018a). However, a double-blind, placebo-controlled study that enrolled biopsy-proven NASH patients with or without established T2DM and randomly assigned them to emricasan or placebo for 72 weeks found that emricasan treatment did not improve liver histology and, more importantly, may have worsened fibrosis and ballooning in a subset of patients (Harrison et al., 2020a).

The apoptosis signal-regulating kinase 1 (ASK1) protein is important in hepatocyte damage, inflammation, and fibrosis. Experimental data from animal NASH models suggests that selonsertib's specific inhibition of ASK1 may have a significant antifibrotic impact in NASH (Friedman et al., 2018a). However, two recent randomized, double-blind, placebo-controlled phase 3 studies of selonsertib in patients with NASH and bridging fibrosis (F3; STELLAR-3) or compensated cirrhosis (F4; STELLAR-4) failed to show a decrease in liver fibrosis with selonsertib therapy (Harrison et al., 2020b). Nonetheless, it is commonly accepted that it is critical to carefully stratify NAFLD patients when conducting future studies.

A new Bayesian network meta-analysis incorporating direct and indirect treatment comparisons assessed the comparative efficacy of several pharmacological treatments for the treatment of NASH. There were nine randomized, controlled studies with biopsy-proven NASH comparing vitamin E, glitazones, pentoxifylline, or OCA against placebo or each other. According to this study, pentoxifylline and OCA reduced fibrosis whereas vitamin E, glitazones, and OCA improved ballooning degeneration in NASH patients (Singh et al., 2015). However, these findings do not give clear recommendations for NAFLD medication therapy.

The gut microbiota plays an important role in the onset and progression of NAFLD. Probiotics, prebiotics, and bovine colostrum carrying antibodies against endotoxin are now being studied for NASH (Koopman et al., 2019; Suk and Kim, 2019). Scorletti et al. reported that in a double-blind

phase 2 trial of UK patients with NAFLD, with or without established T2DM, who were randomly assigned to receive synbiotic agents (i.e., fructooligosaccharides plus Bifidobacterium animalis subspecies lactis BB-12) or placebo for 14 months, the administration of a symbiotic combination (probiotic plus prebiotic) altered the faecal microbiome (Scorletti et al., 2020).

1.2.7. Preclinical models in drug discovery

Basic and preclinical studies continue to be critical for improving our understanding of NAFLD pathogenesis and developing pharmacotherapies. These biological systems used to day span in complexity and size from monolayer cell culture to sophisticated three-dimensional (3D) organoids and model organisms.

1.2.7.1. *In Vitro* Cell Culture Models

Hepatic Cell Sources

Cell lines are frequently utilized in drug development and research. They have a greater replicative capacity than primary cells and a stable phenotype, allowing them to be employed for an extended length of time. They are also reasonably priced. Primary cells, on the other hand, proliferate directly from tissues, have a shorter life and growth capability than cell lines, but they retain the morphological and functional features of their origin for a limited time.

Human Hepatic Cell Lines

Human cell lines are immortalized cells derived from resected tumour tissue (e.g., HepG2, HepaRG, or HuH7 hepatoma cell lines) or from genetically modified primary liver cells (e.g., SV40 Large T, hTERT). The main benefits of immortalized cell lines are their limitless growth capacity and stable phenotypic throughout time (Ramboer et al., 2014). As a result, adopting these models makes standardized methods and reproducible investigations possible.

HepG2 cells have been frequently utilized for in vitro NAFLD modelling. Valproate therapy increased lipid accumulation by increasing the expression of CD36, a fatty acid transporter, and DGAT2. FFA-exposed cells demonstrated a dose-dependent increase in lipid accumulation as well as increased TNFa production. Additionally, Bisphenol A treatment increased lipid accumulation, SREB1 expression, and other genes involved in de novo lipogenesis in a dose-dependent manner (Bai et al., 2017; Gómez-Lechón et al., 2007; Lin et al., 2017). Furthermore, a high-content imaging assay was designed to examine lipid accumulation, oxidative stress, mitochondrial membrane potential, and cell survival of 16 previously reported steatosis-inducing medications,

all of which tested positive for lipid accumulation (Tolosa et al., 2015). However, one significant disadvantage of employing HepG2 is the lack or poor expression of drug-metabolizing enzymes, which may be partially addressed by transfecting with vectors carrying appropriate enzymes (Gerets et al., 2012).

When grown to confluence, HuH7 cells display efficient CYP3A4 activity (Sivertsson et al., 2010, p. 4) and have been utilized to explore the mechanism of steatosis-ameliorating drugs as well as uncovering new processes in the pathogenesis of NAFLD (Chavez-Tapia et al., 2012; Takahara et al., 2017).

HepaRG cells have emerged as a popular cell line with drug-metabolizing enzyme and drug transporter expression patterns comparable to PHH. Differentiated HepaRG cell cultures, derived from a human hepatocellular carcinoma, generally comprise hepatocyte- and biliary-like cells and have been found to express several drug-metabolizing enzymes, including CYPs and phase II transporters. As a result, it has been employed in toxicological and drug metabolism research (Andersson et al., 2012; Lübberstedt et al., 2011). HepaRG cells exposed to FFAs demonstrated an increase in triglyceride levels and a decrease in CYP2E1 activity, resulting in a greater loss in cell viability when treated with acetaminophen (Michaut et al., 2016). Furthermore, a high-content examination of HepaRG cells treated to 16 previously recognized steatosis-inducing drugs identified them as positive for lipid accumulation in this test (Tolosa et al., 2016). Additionally, HepaRG cells have been utilized to investigate the mechanism of steatosis induction by Amiodarone and tetracycline following short-term and recurrent treatment (Anthérieu et al., 2011). By exposing HepaRG cells to cyproconazole, they have recently attempted to examine critical events from the bad outcome pathway of chemically induced liver steatosis. In this study, retinoic acid receptor alpha and pregnane X receptor activation was identified as molecular initiating events, together with lipid accumulation and mitochondrial failure. However, molecular alterations that differed from those described in the adverse outcome pathway were identified (Luckert et al., 2018).

However, because of their tumour phenotype or the immortalization process, hepatic cell lines are typically different in key metabolic activities, limiting the direct comparison to the human scenario, particularly when investigating NAFLD. Thus, 2D cell models are appropriate for studying several key events in NAFLD, but most studies have focused on steatosis and oxidative stress, as well as the underlying mechanisms, rather than tracking multiple parallel cellular events and how this networked interaction affects NAFLD risk.

Primary Human Hepatocytes

Primary human hepatocytes (PHHs) are cells isolated from resected liver tissue and cultured for a few days utilizing a two-step collagenase perfusion or magnetic cell separation method (Aoudjehane et al., 2020; Lee et al., 2013). PHHs are the closest model to match the phenotype of a hepatocyte in vivo due to direct separation from tissue; particularly, their plasma membrane maintains active uptake/secretion mechanisms and metabolism (M. et al., 2003). Because of their excellent functioning in comparison to the human organ in vivo, are regarded as the gold standard short-term human in vitro liver model (Müller and Sturla, 2019).

Nonetheless, PHHs exhibit phenotypic instability and their accessibility and culture time are restricted (Schyschka et al., 2013). Furthermore, human sample collection requires ethical approval, and non-pathological tissues are required and difficult to obtain in clinical practice. Cell viability is highly sensitive to tissue collection surgical and transit circumstances, and genetic diversity between donors might create bias, resulting in a lack of repeatability between studies and experiments (Richert et al., 2004). FFA-exposed PHHs increased lipid accumulation, expression of C/EBP homologous protein, a key protein leading to endoplasmic reticulum (ER) stress, induction of the profibronetic gene transforming growth factor-beta (TGFb), fibronectic activation of hepatic stellate cells via conditioned media from lipid-loaded PHH, and expression of the chemokine regulated upon activation (Wanninger et al., 2011; Wobser et al., 2009). As a result, PHHs appear to be particularly well-suited for drug metabolism investigations that do not need a lengthy culture period, and to a lesser extent for mimicking specific liver disorders such as NAFLD (M. et al., 2003).

Hepatocyte-Like Cells

Hepatocyte-like cells (HLCs) are cells that have been differentiated from human stem cells (hSCs), which include liver stem cells and embryonic pluripotent stem cells (Hu and Li, 2015). Human SCs can be differentiated into HLCs in vitro by exposing them to growth agents and nutrients at the same time (Touboul et al., 2010). Furthermore, methods for producing human-induced pluripotent stem cells (hiPSCs) from somatic cells such as fibroblastic cells are now available, representing an infinite reservoir of stem cells capable of secondary differentiation into HLCs (Hannan et al., 2013; Takahashi et al., 2007). HLCs have morphological and functional properties similar to primary hepatocytes, but without the constraint of culture time (Lu et al., 2015). Graffmann et al. created a NAFLD model using HLCs generated from iPSCs, and after exposure to FFA lipid buildup, overexpression of perilipin 2, and genes implicated in the peroxisome proliferator-activated and secreted receptor pathway were detected (Graffmann et al., 2016). Nonetheless, HLCs pose concerns about the ethical use of embryonic stem cells, inadequate hepatic differentiation, and

the lack of a defined differentiation process, which restricts the repeatability of investigations (Zeilinger et al., 2016).

2D Cell Culture Models

Monoculture

Steatosis may be produced in 2D cultures of hepatocytes from various sources, by adding free fatty acids (FFA) to the cell culture media, particularly oleic and/or palmitic acids (Aoudjehane et al., 2020; C. Chavez-Tapia et al., 2011; Wobser et al., 2009). FFA induction promotes triglyceride buildup in the cytoplasm of hepatocytes, which leads to endoplasmic reticulum (ER) stress, inflammation, and cell death, all of which are hallmarks of NASH (Lebeaupin et al., 2018). Steatosis may also be produced in cultured cells by medicines like Bisphenol A, which promotes lipid accumulation owing to SREBP1 overexpression, or Valproate, which increases fatty acid absorption and triglyceride synthesis (Bai et al., 2017; Bucher et al., 2017; Lin et al., 2017). Furthermore, triglyceride buildup can be accelerated in the presence of ER-stress inducers (Parafati et al., 2018). Hepatocyte 2D monoculture allows researchers to investigate all main metabolic pathways, including carbohydrates, lipids, and amino acids (Green et al., 2015; Ling et al., 2013). It is also appropriate for preclinical drug testing in order to assess drug effectiveness and cellular tolerance. However, 2D human monocellular models fall short of expectations owing to a lack of essential hepatocyte–non-parenchymal-cell (NPC) interactions, which are required for the start of inflammatory processes leading to fibrogenesis in NAFLD (C. Chavez-Tapia et al., 2011; Ling et al., 2013).

Co-culture

In vitro interactions between hepatocytes and NPCs are studied using 2D co-culture models. Different cell types coexist in the same environment, and steatosis may be produced in the same way as in monoculture by adding FFA to the culture medium. Co-culture of hepatocytes with hepatic stellate cells (HSCs), which are important effectors of fibrosis in NAFLD, is one model of distinct interest (Giraudi et al., 2015). Barbero-Becerra et al. investigated this interaction by developing a model of co-culture of HuH7 (human hepatocyte cell line) and LX-2 (human HSC line) cells. They found that FFA exposure only increased SMA expression in LX-2 cells when co-cultured with Huh7 and that HSC activation was independent of FFA accumulation but needed cell-to-cell contact with hepatocytes (Barbero-Becerra et al., 2015). Co-culture of primary hepatocytes with Kupffer or endothelial cells is a great tool for determining the pathways by which FFA induces inflammatory responses in vitro (Bale et al., 2015; Suurmond et al., 2019). Nonetheless, co-

culture is rather difficult to execute since, for example, growth media optimization is necessary to choose the medium that best maintains the various cell populations. Human cells are also scarce, complicating co-culture models even more. For all of these reasons, compared to monoculture and animal models, these models are uncommon in the literature (Soret et al., 2020).

3D Cell Culture Models

Collagen Gel Sandwich

In the collagen gel sandwich model, hepatocytes are cultured between two layers of collagen gel, allowing them to recreate the cellular polarity present in the liver. Sandwiched hepatocytes secrete albumin, transferrin, fibrinogen, bile acids, and urea for at least 6 weeks, in comparison with hepatocytes grown on a single layer of collagen gel halt secretion after 1 to 2 weeks. Adding a second layer of collagen to the monolayer of collagen gel culture has been proven to re-establish this secretion (Dunn et al., 1991). This restoration in the bipolarity of PHHs allows for longer life and better maintenance of hepatic metabolism (Berthiaume et al., 1996; Schyschka et al., 2013). A more complex 3D model, in which hepatocytes are co-cultured with liver sinusoidal endothelial cells (LSECs) inside collagen gels to imitate liver lobular architecture, has recently been presented. Hepatocytes and LSECs account for around 80% of the liver mass, with LSECs lining the sinusoidal walls and acting as a barrier between hepatocytes and blood. This co-culture model creates an environment in which hepatocytes and LSECs improve cell function and survival via cell-cell interactions and/or soluble substances (Bale et al., 2015). However, due to the thickness of the collagen in this model, cell-cell interactions can be concealed or inhibited (Janorkar et al., 2011). As a result, the sandwich structure has been utilized as a NAFLD model very infrequently.

Hepatic Spheroids and Organoids

Spheroids, also known as 3D cell aggregates, are the most widely used 3D culture model. Hepatic spheroids are hepatocyte aggregates composed mostly of hepatic progenitor cells but also of PHHs. Spheroid production is begun by spontaneous self-aggregation of hepatocytes and does not need the development of an extracellular matrix. Several methods based on non-adherent surfaces and gravitational adherence have been developed (van Grunsven, 2017). PHHs in spheroids can be differentiated for at least 3 to 4 weeks, and hepatic steatosis can be induced by supplementing the culture medium with pathophysiological levels of FFA, carbohydrates, and insulin (Kozyra et al., 2018; Pingitore et al., 2019). Cyclosporine A can induce hepatic steatosis in another PHH spheroids model (Bell et al., 2016). Hepatic spheroids can be formed in collaboration with NPCs such as stellate cells and endothelial cells to form more complex liver-like structures (Baze et al.,

2018; Hurrell et al., 2020; Pingitore et al., 2019). However, it is difficult to maintain uniform spheroid size in standard non-adhesive plates, and clusters of spheroids can form, limiting nutrient and oxygen absorption to the distal spheroids and ultimately leading to cell death (Underhill and Khetani, 2018).

Organoids are relatively novel tools used in research, and are defined as artificially grown aggregates of cells that resemble miniature organs, are new research tools. Liver organoids are created by isolating and growing stem and progenitor cells from hepatic stem cell niches or primary liver cells into small self-organizing 3D structures that mimic many of the functions of a native liver (Lancaster and Knoblich, 2014; Wu et al., 2019). To stimulate the different cell niches, successful organoid formation necessitates a careful orchestration of spatiotemporal signals from growth factors to supportive matrices. In detail, human pluripotent stem cells can be co-differentiated into epithelial and mesenchymal lineages to form spheroids to create human liver organoids (HLOs). These spheroids are then embedded in Matrigel and cultured with retinoic acid before being differentiated into hepatocytes using a specific maturation medium. The resulting HLOs are self-organized clusters of cells composed of liver epithelial cells (cholangiocytes, hepatocytes) and NPC (stellate cells, biliary stem cells, and Kupffer cells (Ouchi et al., 2019). When these HLOs were exposed to FFA, they developed steatosis and ballooning, while Kupffer cells released pro-inflammatory cytokines, and stellate cells produced collagen, all of which are important processes in NASH (Kostrzewski et al., 2017). Furthermore, elevated ROS levels and overexpression of lipid- and carbohydrate-related genes were observed (Ramli et al., 2020). Collin de l'Hortet et al. recently developed HLOs based on the control of silent information regulator 1 (SIRT1) expression, which is known to exert protective functions in hepatocytes and macrophages by modulating metabolic and inflammatory pathways, respectively. Downregulation of SIRT1 in these HLOs causes a rapid accumulation of lipid droplets in hepatocytes, which is accompanied by a pro-inflammatory response of neighbouring cells. Furthermore, the majority of the metabolic pathways seen in NAFLD patients' livers were upregulated in these HLOs (Ding et al., 2017; l'Hortet et al., 2019). As a result, HLOs appear to be promising tools for studying the pathophysiology of NAFLD. Organoids, as opposed to 2D cell culture, can more closely mimic natural physiological processes such as stem cell differentiation, cellular movement, and cell-cell interactions. Furthermore, organoids can undergo extensive expansion and culture while maintaining genomic stability, allowing for long-term storage and high-throughput screening. Organoids, when compared to animal models, require fewer animal experiments and are more easily accessible to live imaging techniques; in some cases, they can provide a more accurate model of human development and disease than animal models. Cell differentiation, on the other hand, is sometimes

incomplete, and cell organization is random, resulting in a lack of reproducibility. Methods and techniques still need to be refined in order to achieve complete differentiation and standardized organoid architecture (Kim et al., 2020).

Liver-On-A-Chip

The aim of liver-on-a-chip technology is to create microscale functional liver constructs on a chip using microstructures and microfluidic devices. A polymeric scaffold made up of hundreds of small channels mimics the microarchitecture of the liver (Banaeiyan et al., 2017). When hepatocytes are seeded into these channels, they form a long doughnut-shaped arrangement that closely resembles the hepatic lobule through which a microfluid containing various nutrients and oxygen can pass to simulate blood flow (Hassan et al., 2020). A more complex liver-on-a-chi presented the integration of NPCs in a vascular layer of endothelial cells and macrophages with stellate cells co-cultured with hepatocytes in a hepatic layer. This 3D culture is then embedded in a microfluidically perfused biochip, which provides adequate nutrition and replicates the morphological aspects of the human liver sinusoid. The perfusion chamber in this model creates fluidic shear stress and mimics the microenvironment of the native liver (Rennert et al., 2015).

The use of liver-on-a-chip technology to create in vitro NAFLD models has been extensively researched. Gori et al. cultured HepG2 cells in a microfluidically perfused device that mimics the endothelial–parenchymal interface of a liver sinusoid and allows for nutrient diffusion and waste product removal similar to that seen in liver microcirculation. When compared to static cultures, the microfluidic dynamic in this chip allows for gradual and lower intracellular lipid accumulation, higher hepatic cell viability, and minimal oxidative stress. It closely resembles the chronic conditions associated with fat accumulation in NAFLD patients (Gori et al., 2016). Similarly, Kostrzewski et al. cultured PHHs in a 3D perfused platform and demonstrated that fat accumulation in the cultured cells occurred gradually over time. Furthermore, the metabolic activities (e.g., cytochrome P activity) of PHHs were gradually reduced over the course of the culture, which is consistent with what has been observed in the livers of NAFLD patients (Kostrzewski et al., 2017). Lee et al. also created a "gut-liver-on-a-chip" to investigate the gut–liver axis in the context of NAFLD. FFAs were absorbed through a gut layer in this microfluidic chip, and the subsequent secretion of chylomicrons was observed, which coincided with fat accumulation in hepatocytes (Lee and Sung, 2018).

Liver-on-a-chip models vastly improve the ability to model NAFLD in vitro, including the interplay between the liver and other organs, which is critical for understanding metabolic disorders. Chip

technology is more reproducible than organoids due to standardized protocols and bioengineering fabrication. However, because of its complexity and high cost, it is not widely used in laboratories.

Human Precision-Cut Liver Slices Model

Human precision-cut liver slices (hPCLS) present a robust ex vivo model in which the multicellular histoarchitecture of the hepatic environment remains functional for at least 5 to 21 days in culture, though viability typically decreases afterwards (Kartasheva et al., 2018; Van de Bovenkamp et al., 2007). The presence of inflammation can distinguish between steatohepatitis from simple steatosis. Thus, hPCLS that retain liver-infiltrating immune cells like lymphocytes and macrophages allow researchers to study the changes observed during the various stages of NAFLD and test the therapeutic efficacy of new compounds (Hundertmark and Tacke, 2020; Palma et al., 2019). Treatment of hPCLS with FFA leads to induction of hepatic steatosis, resulting in the activation of inflammation and fibrogenesis in NPCs (Geoffrey et al., 2017; Palma et al., 2019). This model is also well suited for investigating whether intrahepatic lipid accumulation can be modulated via epigenetic manipulation (Palma et al., 2019).

Co-culture or 3D models that replicate the architecture of liver tissue are excellent tools for elucidating the cellular processes of fatty liver disease to research cell interactions in the course of the illness from simple steatosis to steatohepatitis. However, further study is required to standardize cultural circumstances and enhance the availability of next-generation technology.

NAFL/NASH induction strategies

Free Fatty Acids

Among the numerous variables involved in the pathogenesis of NAFLD, the function of free fatty acids (FFA) is the most direct, as demonstrated in vivo by the tight connection between adipocytes, which supply FFA, and other cell targets such as hepatocytes. FFAs are obtained from three sources: 1) dietary fatty acids (primarily from intestine-derived chylomicron remnants); 2) increased lipolysis of peripheral fat stored in white adipose tissue, which flows to the liver as plasma non-esterified fatty acids; and 3) fatty acids newly synthesized by the liver via de novo lipogenesis (Girard and Lafontan, 2008).

The most direct method for studying the effect of FFA on hepatocytes is to expose them to high concentrations of FFA, which results in the development of intracytoplasmatic lipid droplets associated with redox state changes. Intracellular ROS levels rose fast and peaked after 6 hours in FaO hepatic cells exposed to 0.1% triglycerides, suggesting that ROS generation is a possible early trigger for cell death and apoptosis. (lipo-apoptosis). The use of a powerful flavoprotein

inhibitor, such as nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and NADPH reductase, dampened the increase in ROS production, indicating that the excess in ROS production was flavoprotein-dependent and possibly originated from mitochondrial complex-1 or NADPH oxidase (Srivastava and Chan, 2008; Tirosh et al., 2009).

In addition to oxidative stress, FFA-related cytotoxicity occurs through caspase-dependent apoptosis, and JNK activates the mitochondrial apoptosis pathway via Bim-dependent Bax activation (Malhi et al., 2006). Surprisingly, data suggests that by utilizing unsaturated FFA, it is feasible to replicate the "two hits theory" that may occur in NASH. FFA sensitizes hepatocytes to apoptosis produced by relatively low concentrations of glycochenodeoxycholic acid, whereas non-steatotic cells are unaffected. This behaviour is linked to increased production of the proinflammatory cytokines IL-8 and IL-22, which occurs exclusively in steatotic cells (Joshi-Barve et al., 2007; Pusl et al., 2008). Transfecting H35 rat hepatoma cells with plasmid DNA expressing a fluorescent GFP reporter protein under the transcriptional control of a single transcription factor response element resulted in an intriguing model of hepatocyte-derived reporter cells. This model revealed that FFA sensitization might be mediated by a number of mechanisms, including an increase in ROS coupled with a drop in mitochondrial membrane potential, which leads to a decrease in NF-kB and heat shock response element (HSE) activation (Janorkar et al., 2009). The data acquired by this approach, however, is still contentious since the necrotic and inflammatory activities may be explained by culture medium properties (saturated / unsaturated FFA and the FFA-albumin ratio(Chavez-Tapia et al., 2012)) rather than intracellular processes.

The effects of FFA varies depending on the fatty acid moiety utilized, with stearic > palmitic > palmitoleic > oleic acid being the most efficient FFA to cause apoptosis. In accordance with this result, oleic and palmitic acid had distinct effects on cell survival, caspase activation, and DNA fragmentation in hepatocytes. This discovery highlights the significance of the saturated-to-unsaturated fatty acid ratio in experimental settings (Gentile and Pagliassotti, 2008; Pusl et al., 2008).

Amiodarone

Amiodarone is a large amphiphilic drug used for the treatment of atrial and ventricular arrhythmias. Long-term amiodarone therapy is hampered by a slew of extracardiac adverse effects, including pulmonary toxicity, skin hyperpigmentation, thyroid dysfunction, and liver failure caused by mitochondrial oxidation inhibition (Willebrords et al., 2015). When mice are given 150 mg/kg/day for 4 to 7 days, hepatic steatosis generally develops (McCarthy et al., 2004). Amiodarone may easily penetrate the outer mitochondrial membrane in its unprotonated state. It

diffuses across the inner mitochondrial membrane after protonation. Amiodarone loses its protonated state inside the more alkaline mitochondrial matrix, lowering the membrane potential (Fromenty et al., 1990). Amiodarone also inhibits microsomal triglyceride transfer protein, which is capable of transferring TGs and assembling them into extremely low-density lipoproteins and chylomicrons (Varbiro et al., 2003). Dronedarone, an amiodarone-like anti-arrhythmic medication, was presented as a less hepatotoxic alternative to amiodarone for the treatment of atrial fibrillation. Nonetheless, dronedarone has been shown to have at least the same ability to block the mitochondrial respiratory chain and -oxidation (Felser et al., 2013).

Tamoxifen

Tamoxifen is a selective estrogen receptor modulator that can act both agonistically and antagonistically, resulting in a variety of beneficial and harmful effects such as lower low-density lipoprotein (LDL) levels, increased bone mineral density, promoted cardioprotection, and an increased risk of endometrial cancer. It is considered the gold standard in the treatment of estrogen-related breast cancer in women. Tamoxifen, as an estrogen receptor antagonist in breast tissue, can inhibit estrogen signalling, resulting in a lower breast cancer mortality rate (Willebrords et al., 2015). Another well-known side effect of tamoxifen includes the occurrence of liver steatosis (Nishino et al., 2003), which was discovered in mice given 150 mg/kg tamoxifen for 24 hours or 40 mg/kg/day tamoxifen for two weeks (Gudbrandsen et al., 2006; Lee et al., 2010). Tamoxifen, like amiodarone, is an amphiphilic medication that is protonated in the intermembranous region and unprotonated in the matrix of mitochondria after crossing the inner membrane. The following proton release decouples oxidative phosphorylation. Tamoxifen, in fact, weakens the electron transport chain and slows the regeneration of oxidized cofactors such nicotinamide adenine dinucleotide and flavin adenine dinucleotide (Cardoso et al., 2001; Tuquet et al., 2000).

Valproic acid

Valproic acid (VPA), or its derivative sodium valproate, was found to have anticonvulsive action and is the most widely used anti-epileptic medication globally. VPA is used to treat bipolar disorders, clinical depression, absence seizures, tonic-clonic seizures, complex partial seizures, and juvenile myoclonic epilepsy in addition to anticonvulsant characteristics (Willebrords et al., 2015). VPA has been identified as a histone deacetylase inhibitor with therapeutic promise in the field of cancer (Phiel et al., 2001). Although VPA is thought to have a generally benign profile, adverse responses such as encephalopathy, hypersensitivity syndrome, and teratogenicity have been documented in VPA patients (Nanau and Neuman, 2013). VPA can enter mitochondria after being converted to valproyl-CoA in hepatocyte cytosol by coenzyme A (CoA). VPA inhibits

mitochondrial fatty acid oxidation since it undergoes oxidation to numerous products and so competes with endogenous lipids for enzymes in the oxidation pathway (Ponchaut et al., 1992). In rats given VPA dosages ranging from 100 mg/kg/day for two weeks to 1000 mg/kg/day for 24 hours, microvesicular steatosis develops (Lee et al., 2008). The initial step in VPA bioactivation is dehydrogenation by cytochrome P450 (CYP) 2C9 and CYP2A6 enzymes, which results in 4-ene-valproate. This activates a secondary mechanism of -oxidation inhibition. In vivo and in vitro investigations have shown that when 4-ene-valproate is transformed to 2,4-diene-valproyl-CoA, it is more steatogenic and cytotoxic than the parent drug (Kingsley et al., 1983), perhaps due to inhibition of oxidation enzymes (Sadeque et al., 1997). VPA serves as an anionic uncoupler in the mitochondrial matrix by translocating protons. This protonophoric impact can partially uncouple mitochondrial respiration and may lead to the opening of the mitochondrial permeability transition pore, which functions as an additional trigger for VPA-induced cell death (Lemasters et al., 1998). The suppression of carnitine palmitoyltransferase 1, which transfers FFA into the mitochondria, by VPA-CoA has recently been described as a new mechanism of VPA-induced hepatic steatosis. As a result, mitochondrial FFA absorption diminishes, leading to an increase in TG accumulation (Aires et al., 2010).

Tetracyclines

Tetracyclines, a broad-spectrum antibiotic family, are widely employed in the treatment of human and animal infections due to their action against gram-positive and gram-negative bacteria, as well as atypical organisms including chlamydiae and mycoplasmas. Tetracyclines were among the first medicines shown to cause microvesicular steatosis, with symptoms generally appearing 4 to 10 days after the first intravenous administration of large dosages (Thiim and Friedman, 2003). Microvesicular steatosis is induced in mice by dosages ranging from 100 to 200 mg/kg/day (Yin et al., 2006). Because tetracyclines are readily taken up by mitochondria in vitro and in vivo, the primary mechanism behind the development of hepatic steatosis is reduced TG evacuation via inhibition of mitochondrial triglyceride transfer protein. Different examples of severe hepatotoxicity during tetracycline or minocycline therapy have led to a reduction in the use of intravenous tetracyclines, as having the discovery of oral alternatives and the advent of resistant strains of bacteria (Anthérieu et al., 2011; Willebrords et al., 2015).

Non-steroidal anti-inflammatory drugs

Acetylsalicylic acid, an analgesic, antipyretic, and anti-inflammatory medication, is known to induce Reye's syndrome, an often fatal complication in children characterized by vomiting, high fever, encephalopathy, and liver failure (Willebrords et al., 2015). The latter is associated with

lipid droplet formation in hepatocytes and is primarily caused by salicylate, the major toxic metabolite of acetylsalicylic acid. Salicylate induces mitochondrial swelling, oxidative phosphorylation uncoupling, mitochondrial respiration impairment, and adenosine triphosphate (ATP) production inhibition (You, 1983).

Nonsteroidal anti-inflammatory drugs such as ibuprofen and pirprofen have also been linked to mitochondrial -oxidation and microvesicular steatosis. Both enantiomers of ibuprofen suppress -oxidation and oxidative phosphorylation, but only the R-enantiomer can sequester CoA and restrict fatty acid intake and oxidation stereoselectively (Browne et al., 1999). Pirprofen, on the other hand, inhibits the activity of mitochondrial triglyceride transfer protein (Lettéron et al., 2003).

Moreover, diclofenac and nimesulide can both inhibit oxidative phosphorylation and increase mitochondrial permeability transition pore opening (Berson et al., 2006). Acetaminophen inhibits oxidative phosphorylation by destroying mitochondrial DNA, opening the mitochondrial permeability transition pore, and destroying mitochondrial DNA (McGill et al., 2012).

Other compounds

Methotrexate is an anti-inflammatory medication that is used to treat rheumatoid arthritis, psoriasis, psoriatic arthritis, inflammatory bowel disease, and Crohn's disease. It works by inhibiting folic acid production, which is a crucial regulator in the synthesis of RNA and DNA. Methotrexate enters cells through the organic anion-transporting polypeptide 1B1 and inhibits the respiratory chain. Patients with risk factors, such as obesity and diabetes, are more susceptible to methotrexate's hepatotoxic effects and may develop steatohepatitis-like histological patterns (Kolli et al., 2014; Willebrords et al., 2015).

Irinotecan is an antineoplastic medication that inhibits topoisomerase 1, and it is a component of current chemotherapy regimens for the treatment of lung and colorectal cancer. The usage of irinotecan has been linked to the development of NASH (Vauthey et al., 2006).

In excessive dosages, amineptine and tianeptine, both tricyclic antidepressants, impair -oxidation and induce microvesicular steatosis in mice (Fromenty et al., 1989; Le Dinh et al., 1988). In addition to directly inhibiting FFA oxidation, both medications have a detrimental impact on mitochondrial triglyceride transfer protein function(Lettéron et al., 2003).

The antiretroviral nucleoside reverse-transcriptase inhibitors stavudine and zidovudine are essential medicines in the treatment of individuals infected with the human immunodeficiency virus. They can produce hepatic steatosis by depleting mitochondrial DNA, which is a process shared by didanosine and zalcitabine (Igoudjil et al., 2008; Walker et al., 2004).

Perhexiline is an anti-anginal drug used for prevention that inhibits oxidative phosphorylation and carnitine palmitoyltransferase 1. Perhexiline maleate users had mild to moderate fatty alterations in their liver, as well as steatohepatitis (Kennedy et al., 1996).

Cycloheximide, which inhibits tRNA-mRNA binding, causes hepatic steatosis in rats. Several investigations have shown that this is due to increased hepatic FFA absorption, an imbalance in FFA oxidation and esterification, and inhibition of very low-density lipoprotein secretion. Cycloheximide has recently been demonstrated to reduce hepatic lipid production owing to a reduction in apolipoprotein B, a lipoprotein responsible for the packing of extremely low-density lipoproteins (Murakami et al., 2011).

Other drugs interfering with mitochondrial functions include disulfiram (mitochondrial permeability transition pore opening and inhibition of mitochondrial respiratory chain reaction) (Balakirev and Zimmer, 2001), fialuridine (inhibition of mitochondrial DNA synthesis) (Horn et al., 1997), alpidem (mitochondrial permeability transition pore opening) (Berson et al., 2001), nilutamide (inhibition of mitochondrial respiratory chain reaction) (Berson et al., 1994), troglitazone (damaging of mitochondrial DNA and mitochondrial permeability transition pore opening) (Okuda et al., 2010), tacrine (impairment of oxidative phosphorylation and inhibition of mitochondrial DNA synthesis) (Melo et al., 2012), buprenorphine (inhibition of oxidative phosphorylation and mitochondrial respiratory chain reaction) (Tanaka et al., 1985), orotic acid (Feo and Garçon, 1973), clozapine (Zhang et al., 2007) and thioacetamide (Hammes et al., 2012).

1.2.7.2. *In vivo* models

Mouse Models

High-Fat Diet Feeding

A high-fat diet has been linked to the development of obesity and associated metabolic disorders such as NAFLD in several studies as mentioned before. As a result, high-fat diets (HFDs) rich in fat, ranging from 45% to 75% of total calories and made up of saturated fatty acids, poly-saturated fatty acids, and various combinations thereof, have been developed to induce obesity in mice. Mice on a 45% fat high-fat diet develop obesity, glucose intolerance, insulin resistance, and hepatic steatosis, all of which are key features of obesity and associated metabolic syndrome in humans. Importantly, depending on the genetic background of the mice strains, inconsistency in the development of NAFLD has been documented. In brief, despite its widespread usage, HFD is not the optimal model for studying NAFLD due to differences in dietary content (supply and type of fatty acids), mice strains, gender, and feeding length. For all of these reasons, various feeding

regimens have evolved in recent years, intending to reduce variability and cover the whole range of NAFLD (Hariri and Thibault, 2010; Wang and Liao, 2012).

Western-Style or Fast-Food Diet Feeding

The Western diet is a contemporary dietary pattern found in industrialized countries that is characterized by a high intake of fat and sugar. It's known as the "fast food" diet, and it's been linked to the development of obesity. To induce NAFLD in rats, a comparable dietary model based on fat, fructose, and sometimes cholesterol has been established. When compared to HFD, this fast-food-diet-induced obesity appears to be a more robust model for human obesity, although the lack of a consistent diet interferes with repeatability (M. Charlton et al., 2011).

Methionine-α and Choline-Deficient Diet (MCD) and Derivative Feeding

Methionine-α and choline-deficient diet is a traditional NASH dietary model with high sucrose and fat (40% and 10% correspondingly) but deficient in the minerals methionine and choline, which are important components in both animal and human nutrition. Choline is a precursor to phosphatidylcholine, which is required for the formation of very-low-density lipoproteins (VLDL), while methionine is required for the production of glutathione, a key anti-oxidant protein (Itagaki et al., 2013). As a result of increased fatty acid absorption and reduced VLDL secretion, mice given an MCD diet develop hepatic steatosis quickly. Furthermore, oxidative stress and an increase in cytokines and adipokines occur, which leads to liver damage in this dietary model. The MCD diet is simple to obtain and apply, and it causes a more severe type of steatohepatitis than other dietary models, but it has significant drawbacks (Greene et al., 2014).

The MCD diet is the basis for the Choline-deficient, Ethionine-supplemented (CDE) diet. Ethionine is a non-proteinogenic amino acid produced from methionine that induces steatohepatitis in a relatively short amount of time (Passman et al., 2015). Because ethionine has hepatocarcinogenic characteristics CDE feeding is of special interest in the investigation of steatohepatitis-related hepatocellular carcinoma (HCC). This nutritional approach, however, is associated with weight loss and a high rate of death (Gogoi-Tiwari et al., 2017). Some studies alternate the use of a CDE diet with a standard chow diet to reduce mortality. This alternate mechanism reduces morbidity while maintaining steatosis, inflammation, and hepatocarcinogenesis (Passman et al., 2015).

The CDAA diet is a choline-deficient diet in which proteins are substituted by an equimolar combination of L-amino acids. CDAA, like the MCD diet, inhibits fatty acid oxidation in hepatocytes and promotes lipid production, oxidative stress, and inflammation, leading to liver fibrosis. Mice given a CDAA diet lose considerably less weight than mice fed MCD or CDE diets, but they do not

develop hepatic insulin resistance, gain weight, or changes in peripheral insulin sensitivity (Ibrahim et al., 2016). Despite this, insulin resistance can develop as a result of CDAA use. It might be determined by the dietary components and the time of the meal. As a result of the limited repeatability of the different sources of CDAA, this diet should not be utilized to investigate the disease's metabolic profile (Soret et al., 2020).

STAM Model

The STAM model links a single injection of streptozotocin (STZ) as the first hit with an HFD diet as the second hit. STZ is an antibiotic generated by *Streptomyces achromogens* that works as a DNA alkylating agent and was originally utilized to cause type 1 diabetes owing to significant pancreatic islet inflammation and destruction. This model causes steatohepatitis at 8 weeks, liver fibrosis at 12 weeks, and HCC (Fujii et al., 2013). Despite the pharmacological intervention and the lack of obesity in this paradigm, the hepatic lipidomic profile of STAM mice was shown to be remarkably comparable to that of humans with NASH (Saito et al., 2015)[116].

ob/ob Mice

To better understand NAFLD, genetically engineered mice with leptin insufficiency have been created. Ob/ob mice have a homozygous point mutation in the leptin gene. They are hyperphagic and physically sedentary, and they acquire severe obesity, hyperlipidemia, hyperglycemia, hyperinsulinemia, and insulin resistance (Bingxuan et al., 2014). Although hyperphagia contributes to obesity, leptin insufficiency does not play a significant role in NAFLD. Fat buildup in the liver causes steatosis and hepato-lipotoxicity in ob/ob mice but seldom progresses to steatohepatitis and fibrosis. As a result, a second stimulus, such as hepatotoxic drugs or the MCD diet, is required to initiate fibrosis, and steatohepatitis can be investigated in these animals using high-caloric diets (Farrell et al., 2019).

db/db Mice

Unlike ob/ob mice, db/db mice acquire leptin resistance as a result of a spontaneous mutation in the gene encoding the leptin receptor. Hepatic steatosis develops in obese, insulin-resistant, hyperglycemic, hyperphagic, and hyperinsulinemic db/db mice. However, db/db mice, like ob/ob animals, do not display the entire spectrum of human NASH histology, and additional triggers are required to cause steatohepatitis and fibrosis. As a result, results from monogenic models like db/db and ob/ob mice may differ from those from the human population, where obesity is regarded as a complicated condition (H. H. Hansen et al., 2017).

Mc4r-Deficient Mice

The binding of leptin to pro-opiomelanocortin (POMC) neurons results in the release of alpha-melanocyte-stimulating hormone (α-MSH), a hormone that mediates the anorectic signal following binding to the hypothalamic Melanocortin-4 receptor (MC4R). MC4R deficiency is responsible for 6% of monogenic obesity in humans (Loos et al., 2008). As a result, genetically engineered mice with Mc4r deficiency have been created to better understand NAFLD. Under a typical chow diet, MC4R-deficient mice had an early development of obesity, hyperphagia, hyperinsulinemia, and hyperglycemia. Furthermore, when given an HFD, MC4R-deficient animals develop steatohepatitis and fibrosis, and the prevalence of HCC increases over time. As a result, MC4R-deficient mice appear to be a good model for studying the impact of medicines on the development of steatohepatitis when given an HFD (Itoh et al., 2011).

Srebp1c-Overexpressing Mice

Sterol regulatory element-binding proteins (SREBPs) are a kind of transcription factor that promotes the expression of genes involved in the production of FFA and cholesterol. Overexpression of SREBP1c in hepatocytes causes lipid buildup and ER stress. Furthermore, these animals exhibit higher serum FFA and triglycerides, which correlate with higher visceral adipose tissue, showing that pathological liver dysfunctions contribute to visceral adipogenicity (Shimomura et al., 1999). However, hepatic SREBP1c overexpression is insufficient to cause inflammation and fibrosis, and a fast-food diet is required to cause steatohepatitis and fibrosis in these animals (Soret et al., 2020).

FATZO Mouse Model

The FATZO mouse model, or MS-NASH model, was created by crossing two strains of mice with a high proclivity for obesity when given a high-fat diet (C57BL/6J and AKR/J mice). Obesity, metabolic syndrome, and insulin resistance susceptibility resulted from the crossing of these two strains, as well as subsequent generations' selective inbreeding. FATZO mice given a fast-food diet supplemented with 5% fructose develop NASH symptoms such as hepatic steatosis, lobular inflammation, ballooning, and fibrosis. When compared to FATZO animals on a regular chow diet, the FATZO mice developed hypercholesterolemia and progressive elevations of ALT and AST. Unlike the monogenic leptin-deficient ob/ob and db/db mouse models, the FATZO mouse model has a polygenic inheritance of obesity and diabetes propensity with an intact leptin pathway, making it more translatable to human illness. Selective inbreeding, on the other hand, can result in a considerable decrease in genetic diversity, which may create bias in preclinical drug testing (Sun et al., 2019).

Rat Models

Most dietary animal models of NAFLD have been widely utilized in rats, including DIO such as HFD and Western diet, but also non-obesogenic diets such as MCD and CDAA. Rats are more prone to diet-induced NAFLD than mice, and they frequently proceed spontaneously to steatohepatitis and fibrosis. Zucker fatty (fa/fa) rats, like db/db mice, have a mutant leptin receptor that reduces their affinity for leptin. The same variations found in mice apply to the rat model of NAFLD, and dietary compositions (supply and type of fatty acids), strains, gender, and feeding time must all be thoroughly established before to studies (Ibrahim et al., 2016).

Non-Rodent Models

NAFLD has been investigated in many mini-pig species. Mini-pigs acquire obesity and metabolic syndrome comparable to humans due to their longer lifespan than rodents, allowing for long-term research. As a result, medication toxicity studies in mini-pigs appear to be more controllable and predictive of human toxicity than rodents. DIO, on the other hand, does not produce hepatic steatosis in mini-pigs, most likely due to the lack of liver de novo lipogenesis. As a result, alternative non-obesogenic feedings must be employed; for example, steatohepatitis may be produced in minipigs given a CDAA diet within 8 weeks (Pedersen et al., 2020). Although mini-pig physiology is more similar to that of humans than rodents, the liver metabolic pathways are more different, which may restrict the interpretation of the findings. Furthermore, the cost-effectiveness ratio, as well as ethical and legal issues, constrain the use of mini-pigs. Primates, namely rhesus monkeys, have been suggested for NAFLD study because of their genetic closeness to humans. However, because of ethical issues and the high cost of investigations, they are rarely used (B. C. Hansen et al., 2017).

Translation to clinical research

Treatment efficacy is highly reliant on the selection of appropriate preclinical models, which aid in simulating certain elements of the illness and therefore evaluating medication efficacy and safety (Rinella et al., 2019).

Although the complexity of in vitro models that better replicate cell interactions in a native liver have risen considerably in recent years, these cell culture models remain unsatisfactory and require more technological advancements and cost reductions. Important clinical features of the disease, such as the recruitment of circulating immune cells or the influence of extrahepatic signals, such as those from the gut, are difficult to represent in those models, and medication effects on other organs (e.g., adipose tissue) cannot be assessed (Hundertmark and Tacke, 2020).

As a result, in vivo models, particularly mouse models, are still frequently utilized to discover metabolic pathways leading to NASH and to study novel treatment targets. Although transferring data from animal models to people is difficult, they remain the greatest way to research liver disease processes and the development of new medicines in vivo. This interspecies disparity, which is currently a research issue in NAFLD, might be explained by differences in microbiome composition, which appears to play a significant role in NAFLD pathogenesis (Ratziu and Friedman, 2020).

Recently published clinical studies failed to translate encouraging rodent research findings to human illness, which is likely owing to fundamental differences in disease starting pathways across species, as well as significant heterogeneity in human NAFLD patients (Alonso et al., 2017). Comparing gene expression patterns revealed significant transcriptome variations between mice and men throughout the development of NAFLD, which might lead to discrepancies in medication metabolism (Teufel et al., 2016). The progression of illness differs between rats and humans, especially in terms of fibrosis progression. It has been demonstrated that NASH development in mice is strongly affected by strain, implying that comparable divergent pathways exist in humans (Farrell et al., 2014). Other variables, such as age, sex, hormonal status, or microbiome, have also been demonstrated to drive illness heterogeneity in people and hence create a lack of medication effectiveness, indicating that effective therapy may require more customized techniques (Ratziu and Friedman, 2020).

Preclinical studies are also frequently intended to reduce extrinsic factors such as gender, genetic background, nutrition, or age that may restrict translatability. Furthermore, only a few models incorporate comorbidities such as insulin resistance or obesity, which contribute to the complicated aetiology of human NAFLD. Likewise, poor proof in preclinical data prior to proceeding to clinical trials may be the reason for reduced medication efficiency in patients (Ratziu and Friedman, 2020).

Thus, integrating NAFLD in vitro and in vivo models might be a method for accumulating enough preclinical data to explore therapeutic clinical development (Ratziu and Friedman, 2020).

1.2.8. Omic approaches and NAFL/NASH biomarkers

1.2.8.1. Genetic

Genome-wide association studies have been useful in identifying particular genetic alterations in NAFLD pathogenesis. These investigations also enabled the discovery of steatosis biomarkers across genomes. A mutation on chromosome 22 in the patatin-like phospholipase domain-containing family member A3 (PNPLA3), also known as adiponutrin, is significantly linked to elevated hepatic fat levels and hepatic inflammation in steatosis patients. This mutation has been linked to steatosis, portal inflammation, lobular inflammation, Mallory-Denk bodies, and fibrosis in NAFLD patients, as well as HCC (Anstee and Day, 2015).

NAFLD has also been related to a single nucleotide polymorphism in transmembrane 6 superfamily member 2 (TM6SF2). The rs58542926 (E167K) variation, a non-synonymous mutation in TM6SF2 appears to be strongly linked with higher liver fat content. The rs58542926 polymorphism has a substantial impact in regulating lipid characteristics, although it differs between genotypes (Carulli et al., 2019).

Toll-like receptor 4 (TLR4) is well-known to have a crucial role in the pathophysiology of NAFLD. Interestingly, allelic variations of TLR4 (Asp299Gly and Thr399IleTLR4) in humans may prevent NAFLD, since the frequency of individuals with the heterozygous mutation in NAFLD patients is substantially lower than in the control group (Kiziltas et al., 2014, p. 4). Furthermore, sterol regulatory element-binding factor 1c (SREB1c) polymorphism is associated with an increased risk of developing NAFLD, as well as more severe liver histology and abnormalities in glucose and lipoprotein metabolism (Musso et al., 2013). A common variation in the glucokinase regulatory (GKPR) gene and the MTP-493G> T polymorphism was linked to an increased risk of NAFLD, as well as more severe liver fibrosis and higher blood TG levels (Petta et al., 2014). Furthermore, apolipoprotein C3 (APOC3) genotypes with thymine-cytosine, cytosine-cytosine, and thymine-thymine polymorphism are more vulnerable to NAFLD. The cytosine-cytosine genotype is more prone to IR than the thymine-thymine genotype, and it is associated with a considerably greater risk of hypertension, hypertriglyceridemia, and low levels of high-density lipoprotein (M.-R. Li et al., 2014, p. 3).

1.2.8.2. Epigenetic

DNA methylation has been shown to control genes involved in lipid and glucose metabolism, fibrosis formation, and liver tissue remodelling. DBA/2J mice given a lipogenic methyl-deficient diet have less significant DNA demethylation in the liver than C57BL/6J counterparts. This is linked to cytosine methylation loss at repetitive sequences such as major and minor satellites, long

interspersed nuclear elements, short interspersed nuclear elements, and intracisternal A-particle elements (Pogribny et al., 2009). Genes encoding chromatin-remodelling enzymes, including jumonji C- trimethylation, were found to be significantly changed in the livers of steatotic hAPOE2 animals. This is accompanied by altered expression of PPAR and hepatic lipid catabolism genes. The degree of methylation of the mitochondrial nicotinamide adenine dinucleotide dehydrogenase 6 (NADH6) gene was shown to be elevated in NASH patients, and the degree of methylation was connected to the severity of NAFLD. This is associated with reduced mitochondrial NADH6 synthesis and increased DNA methyltransferase I expression (Delik et al., 2020). The DNA methylation status of the PPARγ coactivator 1 gene correlates with plasma fasting insulin levels and IR in NAFLD patients, but the mitochondrial transcription factor A gene does not. Circulating microRNAs (miRs) have sparked considerable interest as potential NAFLD diagnostic indicators in recent years (Sookoian et al., 2010).

MiR-122, a miR species that is abundantly expressed in the liver, has been demonstrated to be a key regulator in cholesterol metabolism, HCC, and hepatitis C infection. MiR-122 expression is downregulated in NASH patients, which is supported by increased levels of certain miR-122 targets, such as sterol regulatory element-binding protein 1c, fatty acid synthase, and 3-hydroxy-3-methyl-glutaryl-CoA reductase (Cheung et al., 2008). Serum levels of miR-122, miR-21, miR-34a, and miR-451, all of which affect liver cholesterol and fatty acid homeostasis, were shown to be elevated in NAFLD patients, with miR-122 levels being linked to liver steatosis (Yamada et al., 2013). MiR-122 knock-out mice accumulate TGs due to overexpression of enzymes involved in TG production and storage in the liver. Inflammation, fibrosis, and HCC are all present in these animals. Furthermore, a diverse set of miRs is changed in NAFLD animal models. In this regard, miR-29 (a potential therapeutic target in NAFLD in mice), miR-34a (an apoptosis regulator), miR-155 (a tumour suppressor regulator), and miR-200b (targets transcriptional repressors of E-cadherin) are all positively affected when C57BL/6J and DBA/2J mice are fed a methyl-deficient diet (Baffy, 2015). In steatotic Sprague Dawley rats, miR-122, miR-451, and miR-27 are downregulated, whereas miR-200a, miR-200b, and miR-429 are upregulated (Alisi et al., 2011). Downregulation of enhancer of zeste homolog 2 (EZH2), a protein that regulates the epigenetic silencing of particular genes and/or miRs by trimethylating lysine27 on histone H3, is inversely associated with fat accumulation in HFD-fed Sprague Dawley rats (Vella et al., 2013)

1.2.8.3. Transcriptomic

Genes involved in fatty acid metabolism, such as CYP4A14, and cell proliferation and differentiation, such as fibroblast growth factor 21 (FGF21) and CYP4A10, are upregulated in VPA-treated ICR mice, while genes encoding glucose-6-phosphatase (G6Pase) and protein phosphatase

2 (PP2), a protein involved in cell growth, have been found to be downregulated (Lee et al., 2007). Similarly, tamoxifen treatment causes hepatic transcriptional upregulation of androgen receptor (AR), nuclear receptor subfamily 2 group F member 1 (NR2F1), hepatocyte nuclear factor 4 (HNF4), and retinoic acid receptor-related orphan receptor 1 (ROR1), all of which are involved in lipid and glucose metabolism (Lee et al., 2010). Furthermore, tetracycline causes overexpression of several phospholipid metabolism genes, including choline kinase, elongation of very-long-chain fatty acids, insulin-induced gene 2, and carbohydrate metabolism genes, including protein phosphatase 1, regulatory subunit 3C, and prostaglandin D2 synthase (Yin et al., 2006). Transcriptomics analysis of liver tissue from APOE-deficient mice fed a Western-type diet enriched in linoleic acid reveals upregulation of lymphocyte antigen 6D (Ly-6D), genes associated with adipocyte differentiation, such as fat-specific protein 27 (FSP27), genes associated with fatty acid metabolism, such as stearoyl-CoA desaturase-1 (SCD1) and CD36, and a concomitant downregulation of genes involved in bile duct formation. Decorin, a proteoglycan generated by monocytes and macrophages in areas of inflammation, has been shown to be elevated in obese human adipose tissue. This implies that decorin has a role in the development of inflammation in NASH. This is supported by the finding that decorin production increases in the livers of ob/ob, db/db, and C57BL/6J mice given an HFD. Other increased genes include endothelial membrane protein 1, a peripheral myelin protein thought to influence cell-matrix and cell-cell interactions, and IB kinase-interacting protein, which connects the transcription factor NF-kB to the endoplasmic reticulum apoptotic signaling pathway. Reduced expression of the former decreases cytokine expression, indicating an anti-IR action (Hennig et al., 2014). Protein tyrosine phosphatases play critical roles in insulin action and hepatic glucose metabolism. Although they are more prone to hepatic steatosis and have greater PPAR gene expression, protein tyrosine phosphatase 6 knock-out mice fed an HFD are protected from IR (Xu et al., 2014, p. 6). Human liver steatosis patients' liver tissue exhibits increased gene expression of evolutionarily conserved signalling intermediate in the Toll pathway (i.e. an adapter protein of the Toll-like and interleukin-1 receptor signalling pathways involved in the assembly of mitochondrial nicotinamide adenine dinucleotide), a Toll-interacting protein, and a single immunoglobulin interleukin-1-related molecule. This overexpression can be due to Kupffer cells or hepatocytes activating and releasing interleukins that act on hepatic stellate cells (Chiappini et al., 2006). mRNA levels of keratin type I cytoskeletal 23, a protein related to metabolism, and aldo-keto reductase family 1 member B10, a protein involved in cytoarchitecture control, are upregulated in steatohepatitis compared to steatosis and normal liver and have thus been proposed as potential biomarkers for steatohepatitis as well as HCC progression (Starmann et al., 2012).

1.2.8.4. Proteomic

Proteomics methods can be utilized to examine protein alterations on a wide scale, in cases of in vitro and in vivo liver steatosis models. Using these methods, it was discovered that HFD fed male C57BL/6J mice fed a high-fructose diet produce an abundance of proteins, including fatty acid-binding protein (FABP), carbamoyl-phosphate synthase (CPS), apolipoprotein A1 (APOA1), protein disulfide isomerase (PDI), anti-oxidation proteins such as peroxiredoxin 2 (PRDX2) and heat shock protein 70 (HSP70), fructose-1,6-biphosphatase (FBP), and glycerol kinase (GK), increased protein synthesis of cytokeratin 8 (CK8) and 18 (CK18), vimentin, and apolipoprotein E (APOE), but reduced glutathione peroxidase (GPx) (Park et al., 2011). Furthermore, in apolipoprotein E-null animals, major urinary protein 2 (MUP2 is overexpressed. This may be a compensating, defensive reaction generated in the liver. This, in turn, might affect plasma lipid homeostasis, because another component of major urine protein 2, major urinary protein 1, increases mitochondrial biogenesis and reduces lipid accumulation in the liver of db/db mice. Due to an increase in reactive oxygen species, mitochondrial antioxidant expression is enhanced in apolipoprotein E-null mice, particularly peroxiredoxin-4, thioredoxin-dependent peroxide reductase, and glutathione peroxidase 1 (Suski et al., 2011). Female C57BL/6N mice given an HFD have enhanced methylenetetrahydrofolate dehydrogenase 1 protein expression, whereas translational levels of glucose transporter 1, methylthioadenosine phosphorylase, and methionine adenosyltransferase 1 are reduced (Thomas et al., 2012). Similarly, db/db mice have a number of overexpressed proteins, such as annexin 5 (apoptotic marker), cadherin 2 (a protein involved in insulin signalling), transporter 2, 24-dehydrocholesterol reductase (protein related to inflammation and possibly to insulin signalling), and guanosine triphosphate-binding protein SAR1 gene homolog B, while others, such as epidermal growth factor, are underexpressed (Kim et al., 2013). Heat shock proteins are substantially downregulated in the livers of rats given a high-fat diet. Human patients have also shown steatosis-related protein alterations. Thus, FABP1 is overexpressed in steatosis patients' livers but is downregulated at various phases of NASH development (Charlton et al., 2009, p. 1). Furthermore, protein profiling of lipid droplets in human steatotic liver tissues showed a hitherto unknown lipid droplet-associated protein that is increased during the fat buildup. Its protein expression has been confirmed in db/db mice as well as HFD-fed mice (Su et al., 2014).

1.2.8.5. Metabolomic

Using metabolomics approaches, it was shown that PPAR knock-out mice had a decrease in glucose and glycogen content, as well as an increase in linoleic acid, oleic acid, and di-homo-y-linolenic acid in the liver (Atherton et al., 2009). In a similar study, an increase in TGs, free and esterified

cholesterol, oleic acid, leucine, valine, lysine, and methionine was observed in the liver of low-density lipoprotein knock-out mice fed a cholesterol-rich diet, while a decrease in arachidonic acid, eicosapentaenoic acid, docosahexaenoic acid, and carnitine was observed (Vinaixa et al., 2010). AflatoxinB1 causes hepatic steatosis in rats, which is linked with higher levels of glucose, amino acids, choline, phosphocholine, and glycerophosphocholine, as well as reduced lipid levels in the blood. AflatoxinB1 simultaneously increases lipids, tyrosine, histidine, phenylalanine, leucine, isoleucine, valine, choline, inosine, adenosine, and uridine while decreasing glycogen and glucose in the liver (Zhang et al., 2011, p. 1). The liver of paraoxonase-1-deficient animals given an HFD includes high amounts of methionine sulfoxide, taurine, fructose, glucose, and 1,3-dihydroxyacetone, but low levels of glycine, glutamate, cysteine, hypotaurine, methionine, homocysteine, glutathione, 3-phosphoglycerate, phosphoenolpyruvate, lactate, citrate, cis-aconiate, succinylcarnitine, fumarate, malate and phosphate (García-Heredia et al., 2013). The presence of abundant e oleic acid, linoleic acid, non-esterified fatty acids, tauro—muricholate, taurocholate, and 12-hydroxyeicosatetraenoic acid in the serum of male C57BL/6NCr mice fed an MCD diet, as well as low production of stearoyl lysophosphatidylcholine, oleoyllysophosphatidylcholine, and palmitoyllysophosphatidylcholine have been described, all which points to alterations in phospholipid and bile acid metabolism (Tanaka et al., 2012). Similarly, taurochendeoxycholate and taurocholate are increased in the serum of tetracycline-treated male C57BL/6NCr mice, but glycochenodeoxycholate and glycocholate are downregulated. These findings suggest that bile acid homeostasis disruption is an early event in drug-induced liver steatosis and that these metabolites may serve as sensitive and early indicators for the diagnosis of hepatic steatosis (Yamazaki et al., 2013). Four metabolites in the liver of mice fed an HFD are likewise changed in liver samples from individuals with steatosis and NASH, notably glucose, glutamate/glutamine, lactate, and taurine, implying a function as clinically relevant read-outs (Li et al., 2011). Upregulated metabolites in human liver steatosis and NASH patients include glutamyl dipeptides, glutamyl valine, glutamyl leucine, glutamyl phenylalanine, glutamyl tyrosine, free carnitine, butyrylcarnitine, glutamate, lysine, tyrosine, and isoleucine, while downregulated metabolites include cysteine-glutathione. Furthermore, human NASH livers had higher amounts of taurine, taurocholic acid, and taurodeoxycholic acid, with lower levels of cholic acid and glycodeoxycholic acid (Lake et al., 2013). Furthermore, increased synthesis of glycerophosphocholine, glycerylphosphorylethanolamine, taurine, and glycine conjugates was discovered in steatosis patients' liver tissue, indicating abnormalities in lipid and bile acid balance as well as mitochondrial dysfunction. Additionally, these individuals had increased levels of

hypoxanthine, creatinine, glutamate, glutamine, glutathione, and ATP, indicating changes in energy metabolism and amino acid metabolism (Li et al., 2011).

1.3. Drug repositioning

Drug repositioning (also known as drug repurposing or drug re-profiling) has gain considerable attention since it has the potential for discovering new uses for existing drugs. Lately, it has attracted considerable attention due to COVID-19 pandemic,that spurred the urgency for developing new drugs fast to fight the pandemic. The process of de novo drug discovery and authorization is generally known to be costly and can take 10–15 years. This lengthy discovery process opens the door to drug repositioning as an alternative strategy for reducing drug development time. Repurposing is the use of pharmaceuticals that have been approved by regulatory authorities for a new application, and it has the potential to significantly reduce the development cycle (Parvathaneni et al., 2019).

Identifying and developing a medication involves substantial expenditure, owing to the varied physicochemical characteristics of the chemical entities and the difficulties of scaling up manufacturing. This constraint also allows pharmaceutical firms or academic institutions to swiftly and effectively use already-approved medicines for a novel indication that is not yet available to patients suffering from that condition. Investigational compounds that fail to demonstrate effectiveness for a predefined indication usually give a suitable starting point for repurposing. They can be rediscovered for a new indication(s), eventually becoming viable treatments, which is especially beneficial in situations of rare diseases (Delavan et al., 2018). Some autoimmune diseases, bacterial infections, and uncommon malignancies, for example, are not hereditary, making treatment more challenging because they are idiopathic. When compared to the time-consuming traditional research and development methods, drug repurposing offers a less expensive and faster way to bring effective treatments to patients. Furthermore, this method aids in overcoming the rising costs of drug research, cutting out-of-pocket expenditures for patients and, eventually, lowering the real cost of therapy (Parvathaneni et al., 2019).

Safety and effectiveness data for a novel experimental compound are not yet available, resulting in higher attrition throughout the drug discovery process and the most failures regarding safety or efficacy. In contrast, all safety, preclinical, and effectiveness data for a repurposed chemical are readily available, allowing the investigator to make an educated judgment at each step of drug development. Prior knowledge of safety, effectiveness and the proper administration route greatly decreases development costs and shortens development time, resulting in less work necessary to effectively bring a repositioned medication to market (Padhy and Gupta, 2011).

One of the successful repurposing attempts is sildenafil, a phosphodiesterase type 5 (PDE5) inhibitor. Sildenafil was originally designed to treat hypertension, but it was subsequently discovered to offer substantial advantages in the treatment of erectile dysfunction and was authorized by the FDA for this purpose. It was then repurposed for the treatment of pulmonary hypertension, an uncommon disease (Hatzimouratidis, 2006). With the introduction of new technology and the availability of computational tools, drug discovery is a considerably more cost-effective strategy when starting with an already-approved medication. In recent years, this technique has accounted for 30% of all newly authorized medicines by the FDA (Parvathaneni et al., 2019). Because these medicines have qualities that are appealing to potential purchasers, drug repositioning has a high potential for out-licensing. Although appealing, the identification of a new disease target must not jeopardize the drug's marketing prospects for its initial indication (Sleigh and Barton, 2010). Rare diseases, which have a high unmet medical need due to the lack of conventional treatments and worsening clinical outcomes, might be a key area of interest for medication repurposing (Delavan et al., 2018).

Even though drug repurposing has recently gained popularity, there are fewer applications than predicted due to numerous obstacles in efficient implementation. Because there are no hard and fast regulatory rules for repurposing drug candidates, emerging start-ups face a difficult challenge in providing appropriate information to regulatory agencies. Furthermore, the exclusivity afforded by patents and the Orphan Drug Act may be ostensibly relevant when using a repurposed drug for a new purpose. However, such exclusivities may not preclude a clinician from using the medication off-label. A few medicines, such as thalidomide and rapamycin, have acquired extra exclusivity as a result of an unexpected regulatory shift, allowing patients to access critical therapies at a cheap cost. However, due to the lack of a defined exclusivity path, using repurposed medicines remains a significant challenge (Parvathaneni et al., 2019; Pushpakom et al., 2019).

A repurposing candidate, in general, carries a potential temporal risk, especially if it has previously failed for an intended application. In this situation, it is advisable to design a branching development program in which the lead chemical or medicine is studied for many indications concurrently. This method reduces the temporal risk and the possibility of intellectual property infringement, which would otherwise demand significant reinvestment in re-profiling the same molecule (Novac, 2013). Drug repurposing necessitates an in-depth understanding of the biological and molecular pathways that a drug might influence, as well as its interactions with endogenous biomolecules. This hypothesis risk may be greatly reduced with a comprehensive understanding of the medication and its affected pathways, leading to effective drug repurposing (Agrawal, 2015).

Pharmaceutical firms place a high value on cost-effective and lucrative discovery areas. However, no guarantee repurposing a drug for rare and neglected diseases would result in significant commercial benefits. As a result, it is more possible for an industry to focus on a more focused and well-established research directive. Another issue that pharmaceutical firms face is a lack of financial incentives and research funding. For example, in the case of uncommon malignancies such as pediatric tumours, there are limited incentives available for pharmaceutical firms to participate in research on medicines with no guarantee of return on investment. Due to investment hesitancy on the side of pharmaceutical firms and medication developers, this issue impedes the usual drug development process (Pantziarka et al., 2018).

Other possible roadblocks may arise while performing therapeutic trials with a repurposed candidate. Proof-of-concept and preclinical research may not be sufficient to show scientifically verified efficacy, and a large financial commitment may be necessary to begin the process with Phase I clinical trials (Padhy and Gupta, 2011). There may also be worries about restricted patient enrolment for big clinical studies necessary for some rare diseases due to patient scarcity. Furthermore, product safety in the elderly and other specific patient populations with comorbidities must be proven. If a drug's or dosage form's intellectual property rights have expired, discovering a new indication will generally result in a low return on investment, as well as significant legal difficulties for the creator. Before generic medications hit the market, the developer may only have a very limited window of opportunity to recoup development expenses, much alone earn a return on investment. Given all of these obstacles, medication repurposing necessitates innovative tactics and tenacity on the side of pharmaceutical corporations. These roadblocks can deter the development of a novel medication for a new indication (Croset, 2014).

1.3.1. Drug Repositioning Approaches

Novel indications for a therapeutic candidate can be discovered by chance or through hypothesis-driven techniques. Hypothesis-driven drug repurposing techniques might comprise experimental and computational approaches that have great potential to improve knowledge of disease pathogenesis processes and pathways (Padhy and Gupta, 2011).

Before moving the candidate medicine farther down the research pipeline, a drug repurposing approach typically consists of three steps:

1. Identifying a potential compound for a specific indication (hypothesis generation)
2. Mechanistic evaluation of drug impact in preclinical models

3. Assessment of effectiveness in phase II clinical trials (assuming there is sufficient safety data from phase I studies).

Step 1 - identifying the correct medication for an indication of interest with a high level of confidence — is the most important of these three phases, and it is here where current techniques to hypothesis generation might be most beneficial. These systematic techniques are further split into computational approaches and experimental approaches, both of which are increasingly utilized in tandem. These two major categories include drug repurposing based on clinical evidence (Pushpakom et al., 2019).

Binding assays and phenotypic screening techniques are two experimental repurposing approaches that may be used to discover binding interactions of ligands to assay components and lead compounds from vast chemical libraries, respectively (Pushpakom et al., 2019). Target-based, knowledge-based, signature-based, pathway- or network-based, and target-mechanism-based techniques are the most common types of computational approaches. These techniques have been shown to be cost-effective and valuable in the discovery of new therapeutic compounds. Computational techniques, in particular, improve the drug discovery process by efficiently utilizing cheminformatics, bioinformatics, network biology, and systems biology. These techniques, in particular, use existing targets, medicines, disease biomarkers, or pathways to develop innovative methodologies and expedite the preparation of critical clinical trials (Parvathaneni et al., 2019).

1.3.1.1. Experimental Drug Repositioning (eDR)

Binding assays

Targets for various substances can be identified using techniques like proteomics and mass spectrometry. The cellular thermostability assay (CETSA) method, for example, predicts the thermal stability of target proteins by binding molecules with the highest cellular affinity. Cellular targets for the tyrosine kinase inhibitor (TKI) crizotinib have recently been validated, and quinone reductase 2 has been identified as acetaminophen-off-target at the cellular level (Miettinen and Björklund, 2014).

The need to address the promiscuity of protein kinase inhibitors has long been acknowledged, and protein kinases are expected to be the primary therapeutic targets of the twenty-first century. This has spurred attempts to produce better probe chemicals for preclinical research that can influence clinical drug development and repurposing via an evidence-based pharmacological audit trail in cells. It is also worth noting that the mistakes made in various kinase drug discovery approaches have a lot to contribute, and early-stage unbiased affinity approaches are especially

useful for understanding the likely effects of compounds in cells, which include paradoxical kinase activation by inhibitors, underpinning mechanistic off-target tumour initiation in patients (Blagg and Workman, 2017). Brehmer and colleagues, for example, incubated HeLa cell lysate extracts with a matrix containing covalently attached gefitinib to understand the complexity of compound effects; mass spectrometry of the resulting elutes identified more than 20 different protein kinases as putative gefitinib targets (Brehmer et al., 2005). Efforts have also been made to assess kinase inhibitors using affinity matrices including 'kinobeads,' which capture proteins before analytical quantification (Duncan et al., 2012), which frequently reveals intriguing unexpected off-targets for well-known medicines (Klaeger et al., 2016). Many of the molecular on and off-targets for important clinical agents have now been identified, including the first-to-market BCR–ABL inhibitor imatinib (Wisniewski et al., 2002), which has been successfully repurposed to treat KIT-driven gastrointestinal stromal tumours (Blanke et al., 2008), the newer BCR–ABL inhibitors nilotinib and dasatinib, and the very promiscuous kinase inhibitor ponatinib.

Chemical genetics can also help to clarify the connection between binding and effectiveness in the biological environment. These results can be quickly transferred into new therapeutic domains or used to address drug-resistance outcomes of extended exposure, which are almost unavoidable phenotypic responses to kinase inhibitor therapy in cancer. Many of these studies are the result of industry-driven high-throughput direct binding or catalytic assays, in which small-molecule–kinase binding is investigated across the kinome using a variety of in vitro and increasingly organism-based assays to generate heat maps of biologically important interactions (Pushpakom et al., 2019). Karaman et al. employed an in vitro competitive binding test to analyze 38 kinase inhibitors against a panel of 317 different human protein kinases; their analysis revealed a total of 3,175 binding interactions. Some kinase inhibitors, like sorafenib and dasatinib, exhibited greater affinity to secondary kinase targets than to their recognized main target, possibly informing (or invalidating) their usage in patient groups (Karaman et al., 2008). Non-kinase targets of small compounds initially meant to inhibit protein kinases are increasingly discovered in the kinase field, leading to repurposing prospects in cancer, as Zika virus modulators, and as possible antibiotic-resistant bacteria (Munoz, 2017).

Phenotypic strategies

Drug candidates are frequently discovered by accident using phenotypic drug screening approaches. Changes *in vitro* or *in vivo* models or even clinical observations might lead to the discovery of new therapeutic compounds (Parvathaneni et al., 2019). For example, it may entail screening a chemical library against cell lines to assess cellular response, then identifying

compounds that modify the disease phenotype, followed by identification of disease state and mechanism of action (Lage et al., 2018). The evaluation of a series of drugs in a variety of different models with the goal of determining effectiveness in one or more of the evaluated models demonstrates the critical conditions required for effective drug repurposing (Reaume, 2011).

To discover and validate candidates for repositioning, *in vitro* phenotypic screening requires examination of known drugs or compounds initially in disease-relevant phenotypic tests. To swiftly screen huge chemical libraries, robotic screening platforms and very sensitive detection methods are utilized. The identification of novel functionalities for authorized drugs can save time and money in drug research and development, while also lowering the chance of failure in early clinical trials. Direct knowledge about a prospective new disease setting may be acquired by using in vitro tests to detect repositioning action. Furthermore, numerous compounds with different modes of action can be evaluated to see if they have a therapeutic impact across the entire concentration range (Wilkinson and Pritchard, 2015).

New advances include the investigation of *in silico* methods for drug repurposing of known drugs and new molecules using structure-based methods and other approaches and have demonstrated a promising future in facilitating drug repositioning. Recent research has also shown that the use of induced pluripotent stem cell technology can be used to produce patient-derived cells for high-throughput *in vitro* screening of current medicines for effects on patient-specific cellular phenotypes (Nishimura and Hara, 2018; Parvathaneni et al., 2019).

In vivo screening of approves drugs for disease-related phenotypic effects has been performed using genome editing technologies such as the CRISPR/Cas-9 system in combination with small *in vivo* preclinical research (Nishimura and Hara, 2018). Because in vivo phenotypic screening approaches focus on high-quality treatment candidates or compounds rather than chemical libraries, these models can estimate efficacy as well as overall tolerance and safety (Ciallella and Reaume, 2017).

Jacquemet et al. used phenotypic screening to find FDA-approved calcium channel blockers that limit filopodia development in cancer cells. By treating cancer cells expressing MYO10-GEP with a library of chemicals, they discovered the relevance of L-type calcium channels in controlling calcium entrance and filopodia stability. They discovered that L-type calcium channel blockers including amlodipine besylate, felodipine, manidipine dichloride, and cilnidipine may prevent filopodia production and cancer cell invasion (Jacquemet et al., 2016). Zilbermintz et al. have described a method for investigating host-oriented treatments that involve the screening of an FDA-approved medication library against the harmful effects of numerous infections. They

discovered that amodiaquine, an antimalarial medication, is effective in protecting host cells against various infections by killing anthrax and Ebola toxins (Zilbermintz et al., 2015).

1.3.1.2. Computational Drug Repositioning (cDR)

Drug-centric

Drug-centric repurposing approaches revolve around predicting new indications for previously approved drug molecules. Most of the molecules involved in this approach follow a common theme of potentially interacting with multiple targets. Although polypharmacological agents are known to produce unwanted side effects, their actions can be exploited because they present potentially new indications for a particular drug. The discovery of a drug for a new indication can be determined through the study of the drug-receptor interactions of its "off-target" hits (Reddy and Zhang, 2013).

When searching for a new target for a previously known drug, assessing drug–target binding and interaction is helpful in discovering other structurally similar compounds that might possess binding ability for the same target. Polypharmacological approaches examine how a single drug acts on multiple targets of a unique disease pathway or multiple targets related to multiple disease pathogenesis. The polypharmacological approach also helps in revealing the unknown off-targets for existing drugs. However, this approach needs to combine all the data derived from methods such as computational modelling, in-vitro–in-vivo pharmacological testing and clinical studies (Reddy and Zhang, 2013).

Target-centric

Target-based screening is the study of a drug candidate with an isolated biological target (i.e., protein, receptor) to distinguish a biological response. In this approach, new indications are determined by linking a drug to a specific disease based on its protein targets. As discussed earlier, a new indication for a particular drug can be determined based on the primary target and also off-target proteins. If the new indication is treated by interacting with the same target protein as previously determined, the approach is known as target repositioning. Approximately 80% of drug repositioning projects have occurred based on this approach (Sawada et al., 2015). When the approved drug interacts with a secondary target and can treat a new indication, this kind of approach is known as off-target repositioning (Parvathaneni et al., 2019).

Although effective, blinded (unintentional, fortuitous discoveries) and target-based approaches are not used to explore new drug-target interactions. Knowledge-based approaches consolidate known information about a drug to anticipate previously unexplored mechanisms including the presence of unidentified drug targets for old drugs, undiscovered drug-drug similarities and new biomarkers. By integrating a considerable amount of information into drug repurposing, knowledge-based methods upgrade the prediction certainty (Parvathaneni et al., 2019). A combination of biological, chemical and clinical knowledge enables the most encouraging repurposing outcome and can pave the way toward determining a new target for an already-approved drug along with a deep vision into its mechanism of action. Knowledge-based approaches can be broken down into three categories: bioinformatics, cheminformatics and text analytics (Loging et al., 2011).

The importance of bioinformatics and data mining in repurposing is discussed. Traditional drug discovery processes make it challenging to identify multiple uses of a single drug. Conversely, this can be accomplished by drug repurposing through in-depth scientific analysis and/or simple serendipity. Given this perception and availability of large datasets, it is important that methods are in place for investigators to adequately make use of such data. More specifically, utilizing proper experimental design and the addition of different biological, chemical and clinical datasets can result in identifying novel and unexplored relationships or targets for a certain drug. These conclusions can result in a profound understanding of disease biology, target or compound selection, as well as drug toxicity (Loging et al., 2011).

In biological data mining (bioinformatics), a drug discovery corporation can either access data achieved internally or obtain publicly sourced information; and implement data mining approaches to discover new inter-relationships and potentially new intellectual property in a particular field of interest. An organization that identifies its inherent biological data for repurposing studies will often investigate against multiple disease states. By contrast, generalist organizations do not focus their endeavours toward a specific disease; instead, they constitute a panel of disease-relevant models, and then screen compounds to be repurposed universally. Specialists lean on their ability and specialization within a particular disease area to screen large numbers of compounds against a targeted set of diseases (Loging et al., 2011). There are several different types of protein interaction databases available for data mining. Bioinformatics-based approaches have also successfully been used to discover new relationships between biomedical entities such as genes, biological pathways and diseases in a drug repurposing approach (Harpaz et al., 2012). Using

transcriptional data for drug repurposing is another pathway where compounds consisting of opposite transcriptional signatures toward a disease are identified. Despite the underwhelming performance of this method, newer transcription-based methods such as CuGuCtD are providing further understanding and ability to identify whether a compound has the potential to upregulate the genes in the same way as compounds already treating that particular disease (Himmelstein et al., 2017).

In chemical data mining (cheminformatics), demand for drug repurposing can also come after achieving successful Phase I clinical trial outcomes, and subsequent recruitment of an appropriate patient population for Phase II/III trials. Determination of pertinent biomarkers provides validation of the desired mode of action hypothesis. If the drug is a validated hit to the target, a subsequent failure could be due to a lack of understanding of the target protein's role in the pathogenesis and progression of the disease.

To comprehend how to effectively repurpose these safe drug entities to appropriate indications, two vital concepts are required for comprehensive scrutiny. First, any given small-molecule drug candidate can interact with numerous proteins. Second, complex diseases are frequently the result of sophisticated crucial intra- and inter-cellular molecular interactions, which are repeatedly standardized and ordered. Information about drug-protein interactions is frequently inadequate and much of the computational data assessment for repurposing comes from an integration of distinct data sources. More specifically, this arises across the boundaries of traditional regulations of clinical medicine, chemistry, biology and toxicology (Loging et al., 2011).

Genome-wide association studies

Genotyping technology along with the completion of the Human Genome Project have lowered genotyping costs and led to an increase of genome-wide association studies (GWAS). GWAS aim to provide insights into the biology of a studied disease by identifying genetic variants associated with it. Further analysis of the obtained data can also lead to the identification of new targets shared between different diseases, hence enhance drug repositioning approaches. Sanseau et al. refined the catalogue of published GWAS traits from the US National Human Genome Research Institute (NHGRI) and reported that genes that were associated with a disease trait were more likely to code for proteins that can serve as drug targets than the rest of the genome, with the GWAS gene set enriched by 2.7-fold in targets being pursued by the pharmaceutical industry. They also found 92 individual genes with a GWAS trait that was different from the original drug indication, suggesting that it is possible to evaluate drugs that target the products of these 92 genes for a new disease indication (Sanseau et al., 2012). Another recent study by Grover and

colleagues used a bioinformatics approach to match gene targets identified for coronary artery disease with drug information obtained by integration of three different drug–target databases (DrugBank, Therapeutic Target Database and PharmGKB) to identify potential repositioning opportunities (Grover et al., 2015).

However, there are challenges in the use of GWAS information for drug repositioning, and its utility at present is unclear. GWAS signals in gene-rich loci with strong linkage disequilibrium may make identification of causal gene and/or gene variants difficult. Another issue is the lack of information on the direction of effect of the gene variant; functional studies will need to be conducted to ascertain this before deciding whether an activator or a suppressor is required to control the disease (Sanseau et al., 2012). GWAS data do not provide detailed pathophysiological information, and hence, rational use of GWAS data is advocated before predicting repurposing targets. It should also be noted that the current understanding of the human genome is not final and there may be many more new genes discovered (Wang and Zhang, 2013).

Retrospective clinical analysis: use of electronic health records

Sildenafil is the greatest example of retrospective clinical analysis leading to the repurposing of a candidate compound (Hatzimouratidis, 2006). Aspirin cardiovascular disease and in colorectal cancer (Dehmer et al., 2016), raloxifene in breast cancer, and propranolol in osteoporosis (Cavalla and Singal, 2012) are further examples of repurposing potential resulting from retrospective clinical and/or pharmacological analysis. The aforementioned examples, however, did not develop as a consequence of a systematic examination of clinical data, and a systematic approach to clinical data analysis is increasingly being recommended for discovering drug repurposing potential (Jensen et al., 2012).

Retrospective clinical data may be collected from a variety of sources, including electronic health records (EHRs), post-marketing surveillance data, and clinical trial data. EHRs include massive amounts of organized and unstructured data on patient outcomes. Diagnostic and pathophysiological data, such as laboratory test results and drug prescribing data, are more structured; however, EHRs also contain significant amounts of unstructured information, such as clinical descriptions of patient symptoms and signs (important in defining disease phenotype) and imaging data. This plethora of data in EHRs might be utilized to detect signs for drug repurposing whereas the massive volume of EHR data gives great statistical power (Hurle et al., 2013; Paik et al., 2015). For example, Paik et al. extracted clinical signatures from over 13 years of EHRs from a single hospital, including approximately 10 million laboratory tests from half a million patients, as well as diverse genomics signatures, to identify over 17,000 known drug-disease associations;

this approach resulted in the identification of terbutaline sulfate, an anti-asthmatic, as a promising candidate for further research (ALS) (Paik et al., 2015).

A lot of sources of patient, disease and drug data that could be utilized as sources for drug repurposing analyses exist thus far (United Kingdom's Clinical Practice Research Datalink, Medicines and Healthcare Products Regulatory Agency's Yellow Card scheme, European database of suspected adverse drug reaction reports managed by the EMA, the FDA Adverse Event Reporting System, World Health Organization's global database for adverse drug reactions). However, significant problems remain in obtaining and exploiting EHR data, including ethical and legal barriers that limit data access and difficulty retrieving the unstructured information available in these systems. Adding greater research capabilities to EHR databases might increase their value for a variety of downstream prospects such as drug repurposing. Natural language processing and machine learning approaches may also be beneficial.

Other significant large data sources include post-marketing surveillance data and clinical trial data, however, access may be restricted for commercial or confidentiality reasons. However, there is a growing recognition that allowing access to such a plethora of information might assist in future drug development research. The EMA began offering direct public access to clinical trial data provided by pharmaceutical firms in October 2016 and has released reports on six different medicines to date (European Medicines Agency Clinical Data) (Pushpakom et al., 2019).

Molecular Docking

Molecular docking is a computational approach that uses structure to predict binding site complementarity between a ligand (drug) and a target. If a receptor target implicated in a specific disease is known in advance, various medicines might be tested against that specific target (conventional docking: one target and multiple ligands). Drug libraries, on the other hand, may be tested against a variety of target receptors (inverse docking: multiple targets and one ligand) to find new interactions that can be used for repurposing (Kitchen et al., 2004). Dakshanamurthy et al. used high-throughput computational docking to compute molecular fit on more than 3000 FDA-approved medicines spanning approximately 2000 human protein crystal structures. Mebendazole, an anti-parasitic medication, was shown to have the structural capacity to block vascular endothelial growth factor receptor 2 (VEGFR2), a modulator of angiogenesis; this was also validated experimentally (Dakshanamurthy et al., 2012).

However, the utilization of molecular docking for drug repurposing has numerous concerns. First, 3D structures for some protein targets of interest may not be accessible, especially because

pharmacological targets are frequently membrane proteins, such as G protein-coupled receptors (GPCRs), despite recent significant improvements in GPCR crystallography (Cooke et al., 2015). Second, there is a scarcity of well-curated macromolecular target databases that give precise structural information, but this is improving (Huang et al., 2018). Finally, the efficacy of docking methods to forecast binding affinities has been questioned, and while it is improving, there can be discrepancies across software packages, and some prediction constraints (for example, mode of binding and entropic effects) persist (Pagadala et al., 2017).

Pathway or network mapping

Pathway-based or network-based methods have been frequently utilized to discover compounds or therapeutic targets with repurposing potential (Smith et al., 2012). As previously stated, while some of the prospective targets discovered by GWAS or other techniques may be immediately accessible as drug targets, these genes are not always good druggable targets. In such cases, a pathway-based method may offer information on genes that are either upstream or downstream of the GWAS-associated target, which may be used for repurposing (Greene and Voight, 2016). To aid the discovery of repurposing candidates, network analysis involves the construction of drug or disease networks based on gene expression patterns, disease pathology, protein interactions, or GWAS data, and network analysis techniques are often used in signature matching studies (Iorio et al., 2013b). For example, Greene et al. used an approach called network-wide association study (NetWAS) to discover disease–gene correlations considerably more precisely than GWAS alone. They integrated genetic variant information derived from GWAS with tissue-specific functional interaction networks. When they applied this method to hypertension and compared the resulting data to drug–target data from DrugBank, they discovered that antihypertensive medication targets were enriched to a larger extent among the top genes using NetWAS than with GWAS (Greene et al., 2015). In another study, pathway analysis of gene expression data sets from studies using a diverse variety of respiratory viruses in human host infection models revealed 67 similar biological pathways that may be relevant in respiratory viral infections. When these pathways were compared to the DrugBank database, many compounds with potential anti-host-viral effects were discovered. Pranlukast, a leukotriene receptor 1 antagonist used to treat asthma, and amrinone, a phosphodiesterase inhibitor used to treat congestive heart failure, was among them. Because of their potential capacity to modify the immune response, it has been proposed that both of these medicines might be beneficial in treating viral infections (Smith et al., 2012).

Signature matching is based on comparing a drug's distinctive features, or "signature," to those of another drug, disease, or clinical phenotype. A drug's signature might be produced from one of three forms of data: transcriptomic, proteomic, metabolomic, chemical structures, or adverse event patterns (Keiser et al., 2009).

Matching transcriptomic signatures may be utilized to make drug-disease comparisons (Dudley et al., 2011) and drug-drug comparisons (Iorio et al., 2013a) by calculating drug-disease similarity.

In the first case, the transcriptomic signature of a specific drug is generated by comparing the gene expression profile of biological material before and after treatment with the drug. The resulting differential gene expression signature, which serves as the drug's molecular signature, is then compared to a disease-associated expression profile produced similarly by differential expression analysis of disease versus healthy states.

The degree of negative correlation between the gene expression signature of the drug and that of the disease, hence the genes up-regulated in the disease that are down-regulated with the drug and vice versa, would therefore allow inference of whether the drug may potentially affect the disease (Sirota et al., 2011). This computational approach is based on the signature reversion principle (SRP). SRP states that if a drug can reverse the expression pattern of a given set of genes that are a hallmark for a specific disease phenotype, then that drug may be able to reverse the disease phenotype itself. Although simple, this method has been demonstrated to be promising for metabolic disorders (Wagner et al., 2015) and to be effective in finding prospective drug repositioning possibilities across a wide variety of therapeutic areas, including medicines that might be repositioned as chemo-sensitizers (Hsieh et al., 2016; Malcomson et al., 2016; Mirza et al., 2017; Wei et al., 2006, p. 1).

Drug-drug similarity methods seek to uncover shared mechanisms of action among otherwise divergent drugs, meaning drugs that belong to different classes or with different structures. This principle is known as guilt by association (Chiang and Butte, 2009) and it can help identify alternate targets for current drugs, as well as possible off-target effects that might be studied for therapeutic applications (Keiser et al., 2009). A shared transcriptomic signature between two drugs could therefore suggest that they also share a therapeutic application, independent of their chemical structures' similarity or dissimilarity (Iorio et al., 2010). This concept has been demonstrated to be successful even when comparing transcriptional signatures that are reflective

of a secondary mechanism of action, such as those shared by a group of pharmacologically different mild correctors of a particular disease phenotype (Iorio et al., 2015).

Both drug-disease and drug-drug similarity approaches require the matching of transcriptomic signatures and hence rely significantly on publically available gene expression data. The Broad Institute created the Connectivity Map (cMap) in 2006, which comprises gene expression profiles produced by dosing more than 1000 chemicals in a variety of cell lines (Lamb, 2006). cMap data may be viewed as a proxy phenotypic screen for a large number of drugs and has been effectively utilized to forecast medication repurposing for a variety of diseases. The third version of the cMap data repository (cMap 3.0) is currently accessible at the Library of Integrated Network-based Cellular Signatures at the National Institutes of Health (NIH) (LINCS). It includes transcriptional signatures created by hundreds of human cell lines after being treated with tens of thousands of compounds. This massive resource may be used along with other public repositories of transcriptomic data, such as the Gene Expression Omnibus (GEO) and Array Express, which include raw gene expression data from numerous diseases in humans and animal models. Manual curation or dedicated computational tools (Wang et al., 2016) can then be used to interrogate these disease signatures in association with the cMap data to uncover novel drug-disease connections and potential drug repositioning opportunities (Pacini et al., 2013).

The second approach of signature matching employed in drug repurposing is based on chemical structures and their link to biological activity; comparing the chemical signature of one drug with that of another to determine whether their chemical similarities might indicate common biological activity. The method entails choosing a set of chemical features for each drug and then constructing the corresponding networks based on the common chemical features (Oprea et al., 2007). This is demonstrated by the statistics-based cheminformatics approach utilized by Keiser et al. to forecast novel targets for more than 800 FDA-approved small molecules and more than 2000 drugs. They were able to uncover 23 novel drug–target relationships by using a similarity ensemble method (SEA) to analyze the 2D structural similarity of each molecule to each target's ligand set (Keiser et al., 2009).

Chemical similarity approaches have drawbacks: errors in chemical structures, as well as physiological effects that exist outside of the structural relationship (for example, a metabolite of the original drug with a modified structure could be the active molecule), may limit their use in drug repurposing (Dudley et al., 2011).

Lastly, each drug has a rather distinct adverse effect profile that may be utilized to predict its phenotype. The matching of a drug’s adverse effect signature is based on the concept that two

drugs that induce the same adverse effect may be operating on the same target or protein or in the same pathway (Dudley et al., 2011). The possibility also exists that the adverse effect phenotype of a certain medication resembles that of a disease, and this would imply that the drug and the disease share pathways and physiology. The adverse effect similarity method was utilized by Peer Bork et al. to uncover unique drug-target interactions for more than 700 approved drugs. They collected relevant adverse effect profiles from drug package inserts, weighted them based on frequency, then rated these drugs based on adverse effect similarities using the Unified Medical Language System (UMLS) ontology for medical symptoms. This method not only verified previously known drug-drug pairings that had the same protein target but also discovered novel common targets for over 700 drug pairs (Campillos et al., 2008). Yang and Agrawal employed a different approach to link adverse drug effects to disease. They were able to anticipate repurposing indications for 145 disorders by combining adverse effect information from drug labels with drug-disease correlations gathered from the PharmGKB database (Yang and Agarwal, 2011).

Even though this is a reasonable method for discovering repurposing opportunities, the difficulties in mining adverse effect information from drug package inserts, as well as the lack of well-defined adverse effect profiles and causality evaluations for a number of drugs, may restrict its application. Artificial intelligence systems capable of text mining and natural language processing, on the other hand, provide possible future prospects to overcome these constraints (Dudley et al., 2011).

Validation of computational drug repositioning models

Computational drug repositioning studies are ideal for identifying new applications for current medicines and optimizing the pre-clinical process of creating new pharmaceuticals by saving time and money as compared to the traditional de novo drug discovery and development strategy. Researchers validate/evaluate their data before suggesting a list of drug repositioning candidates.

Nonetheless, validation or evaluation models may differ from the proposed computational models in certain conditions, or specific validation models may be inaccurate and unreliable. Consequently, understanding and selecting appropriate validation models is critical for the success of the suggested computational models. Additionally, due to many considerations such as high price, high degree of toxicity, and limited bioavailability, as well as certain drugs having been abandoned or not favoured by physicians or biologists, selecting the proper group of drug repositioning candidates for validation is critical.

Practically speaking, validation/evaluation models differ from one research to the next and can, to some extent, be influenced by the nature of the targeted objectives. These models are divided

into eight categories: (1) *in vitro* experiments, (2) *in vivo* experiments, (3) electronic health records, (4) leave-one-out and cross-validation, (5) benchmarking against previous models, (6) case studies, (7) literature cross-referencing, and (8) consultation with domain experts.

Despite numerous well-known drawbacks, in vitro and in vivo experimental validation models have been widely used to evaluate medication repositioning prospects. In vitro and in vivo validation models are used to conduct research in a controlled environment. Furthermore, in a recent study, literature-based validation models have been frequently employed. Furthermore, in machine learning-based research, K-fold cross-validation is commonly used to train models to prevent over-optimistic estimation of model performance, which may also be addressed using a fresh testing dataset independent of the training set, if such data is available (Jarada et al., 2020).

1.3.2. Barriers to drug repositioning

Although there have been significant successes with drug repurposing, repurposing does not always succeed. Some failures in late-stage development are unavoidable, as with the *de novo* drug development, but these failures should be less likely to be related to toxicity because the candidates' safety profiles have already been defined in clinical trials. However, there are additional causes for failure in the repurposing sector, including the inability to even pursue a promising candidate beyond initial trials, that are connected to drug repurposing-specific constraints, such as patent issues, regulatory considerations, and organizational impediments.

Drug repurposing is hampered by a variety of legal and intellectual property hurdles. Difficulties connected with patenting a new repurposed indication and enforcing patent rights are major barriers to encouraging medication repurposing because they have a significant influence on the potential profit expected from the repurposed product (Ashburn and Thor, 2004; Breckenridge and Jacob, 2019). In most major pharmaceutical markets, a new repurposed medical application of a known medicinal molecule can be protected, providing the new medical use is novel and innovative. Many of the possible repurposing applications, however, are already known in the scientific literature or clinical practice. Even if they have not been clinically proven to function, previous scientific knowledge of the repurposed usage may limit the potential to acquire patent protection unless the patentee can effectively separate their patent claims from information that is already in the public domain. To acquire granted patents for a new repurposed medical use, the patentee must additionally submit evidence in the patent application proving that the medicine is a credible therapy for the new indication in question (Breckenridge and Jacob, 2019).

Off-patent medicines can acquire a new method-of-use (MOU) patent for a new repurposed usage of an old generic medication if that use is new and inventive and can be supported by suitable data to render the new use credible. However, if the new repurposed indication makes use of the generic drug's existing formulations and dosage forms, enforcement may become a serious issue because the generic medicine may be readily available from other manufacturers and prescribed by doctors for non-patented uses. The generic manufacturer can lawfully label their product exclusively for non-patented indications (‘skinny labelling'), and it will be difficult to argue that they are infringing on the new MOU patent if they do not encourage usage in the patented indication in any other manner. In this case, it may be difficult to restrict off-label use for the newly patented repurposed indication, lowering the product's potential profitability [99]. However, off-label usage may be restricted if the new repurposed indication necessitates a unique formulation and/or dosage regimen that cannot be easily obtained with the drug's existing generic equivalents. Given the difficulties outlined above, it may be necessary to evaluate how and to what degree intellectual property may be protected for a repurposed result from the outset of the project (Breckenridge and Jacob, 2019).

Regulatory issues are important factors in the development of repurposed medicines. The commercial exclusivity given in the EU/EEA for repurposed pharmaceuticals with a designated orphan indication is 10 years of protection from market competition with comparable medicines with similar indications, plus an extra 2 years if they comply with an approved Paediatric Investigation Plan (PIP). All orphan drug applications must go through the centralized process. Ten years of data exclusivity are allowed for repurposed medicines that do not have orphan status. Applications for new indications of well-established drugs may be given one year of data exclusivity if filed. Data exclusivity provisions, on the other hand, do not apply to changes to current marketing authorizations. In the United States, the FDA grants 3 years of data exclusivity for a novel application of a previously marketed medicine; nevertheless, 3 years is insufficient time to recoup the money a firm has invested in repurposing a specific drug. Furthermore, off-label usage of a repurposed generic medication may devalue the drug even further (Breckenridge and Jacob, 2019).

Pharmaceutical corporations are recognizing the value of medication repurposing outside of their main medical areas of emphasis and are forming partnerships with smaller biotech businesses and academic institutions. However, repurposing in the pharmaceutical industry can face some organizational challenges, especially if the repurposed indication is not within the organization's core disease area or the compound has been discontinued in development, and thus there is no longer a "live" project within the R&D division to provide dedicated support for the new indication.

This would imply a dearth of employees to work on a prospective medication repurposing project, as well as a lack of funds and resources to move the concept forward inside the company (Ashburn and Thor, 2004; Breckenridge and Jacob, 2019).

1.3.3. Drug Repositioning for NAFLD

Thus far, *in silico* and *in vitro* approaches to DR for NAFLD and NASH are promising (Kashyap et al., 2019).

On the eDR front, Luo et al. (2018) utilized *in vitro* models and a lengthy high-content screening pipeline to screen the compound library LOPAC®1280 (The Library of Pharmacologically Active Compounds, MilliporeSigma, MA, USA), out of which only 5 were found to demonstrate some repositioning potential (Luo et al., 2018). In summary, this work used LOPAC® and high-content screening to identify chemicals that dramatically reduced intracellular lipid droplets (LD) after treatment with a high-fat medium (HFM). Among the 1280 compounds tested, 239 were shown to reduce LD by more than 50%, with 17 retaining cell viability. Nine were chosen for testing in normal primary hepatocytes, with five exhibiting dose-dependent efficacy. Using whole-genome transcriptome network analysis, the underlying regulatory network was constructed. According to gene enrichment and pathway analysis, CREB1 was proposed as a potential therapeutic target for liver disease.

Alternatively, Sookoian et al. (2019) employed a purely cDR strategy. They suggested a drug candidate selection technique based on the incorporation of disease pathogenesis molecular pathways into network analysis tools that employ OMICs data as well as data from other sources, such as text mining from the medical literature. In detail, they publicly available tools and data to map NAFLD and chemical interaction networks, and proposed 149 target genes and compounds interacting with these genes (Sookoian and Pirola, 2019). The lack of an eRD phase imposes the need for additional trials to assess the anti-steatogenic effect, before concluding on the actual repositioning potential.

To this end, what remains ambiguous is how effectively cDR can be combined with eDR, leading to repurposed compounds that can be effectively translated into therapeutic interventions. To the best of our knowledge, no robust framework combining cDR and eDR, paired with efficacy testing, for NAFL/NASH exists so far.

1.4. Aim

This project introduces an integrated eDR and cDR framework aiming to identify compounds that interfere with mechanisms of liver steatosis towards the amelioration of NAFL. The implemented approach was based on the null hypothesis that if a compound can reverse a pathway that is significantly altered by a disease, it can also reverse the disease phenotype, hence the clinical outcome (Signature Reversion Principle) (Iorio et al., 2015). The first part of the platform consists of the *in* silico selection of repurposing candidates (cDR) based both on gene expression data from clinical human samples and cell-based *in vitro* data. The selected compounds were subsequently validated with *in vitro* assays (eDR), minimizing the overall screening time and increasing hit rate, whilst broadening the pool of compounds. In detail, gene expression data from NAFL/NASH patients were obtained from GEO (NCBI) (Edgar et al., 2002) and drug repositories. Second, by combining signature matching and pathway mapping, networks significantly deregulated in NAFL/NASH were identified. These were then compared to networks of approved drugs and investigational compounds, and of compounds used on designed *in vitro* steatosis models, to reveal compounds that could interfere with NAFL's pathogenesis. Finally, the discovered compounds' efficacy was validated *in vitro* via high-content phenotype screening and proteomic assays.

Out of approximately 20'000 approved and investigational compounds, cDR screening pinpointed 46 candidates and 21 were moved onto the *in vitro* testing. Out of these, 6 were found to significantly improve the steatotic phenotype included Resveratrol (already been in clinical trials for NAFLD) (Berman et al., 2017; Tejada et al., 2021) and Sirolimus (already proven effective *in vitro* and *in vivo*) (J. Li et al., 2014; Wang, 2010). In addition, proteomic experiments revealed similarities in the compounds' mode of action, thus introducing the possibility for novel therapeutic interventions.

2 | METHODS

2. METHODS

2.1. Workflow of the Drug Repositioning Platform

A network-based computational approach was employed to propose compounds for treating NAFLD. Briefly, gene expression networks from NAFLD patients and pathways of known steatogenic compounds were matched to drug-induced networks. Through this matching, the platform identified compounds that interfere with NAFLD's mechanisms, as shown in Figure 1.

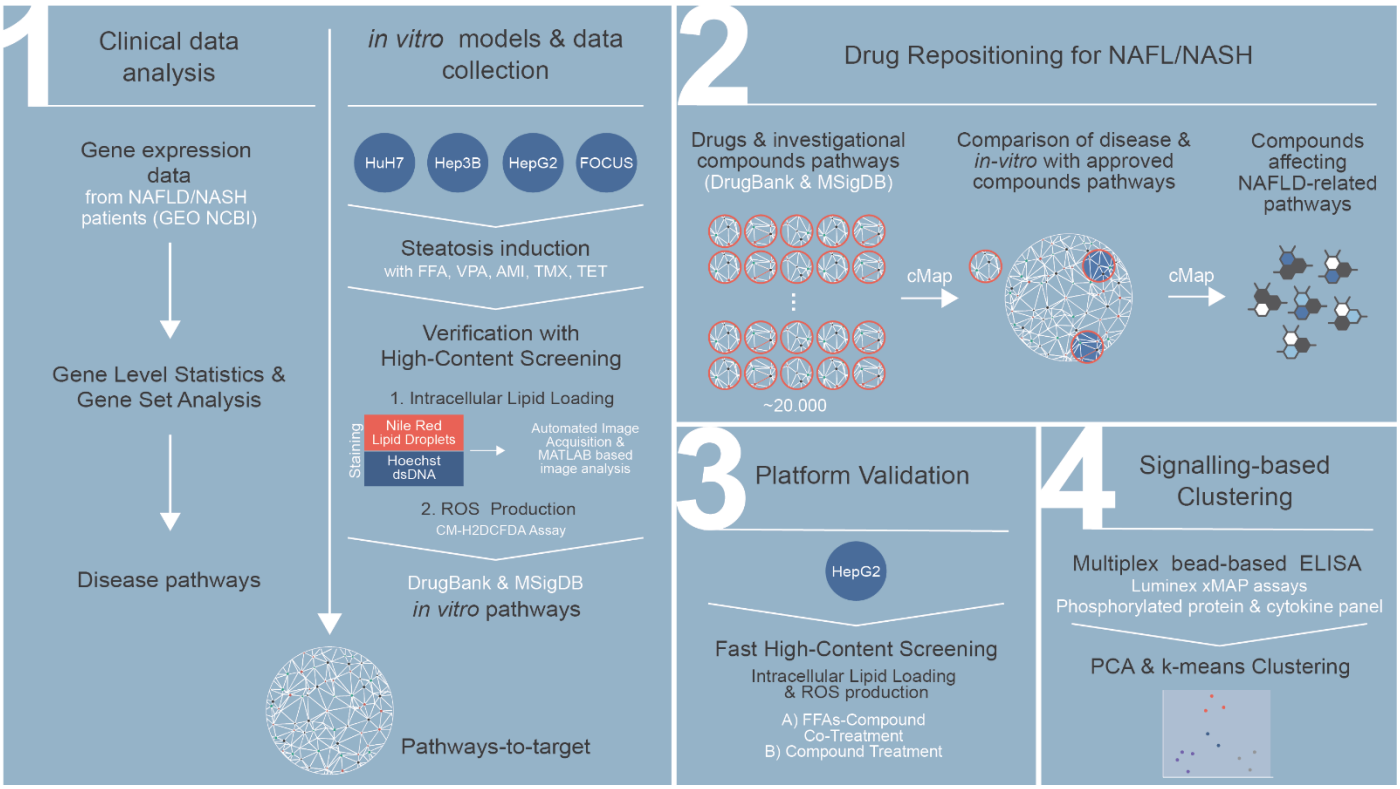


Figure 1 Schematic representation of the drug reposition platform and the experimental pipeline. *In vitro induction and verification of steatosis and identification of pathways affected, along with identification of disease-related pathways through analysis of clinical data were the first step of the DR platform (1). Disease pathways were compared against known therapeutic compounds pathways to identify those affecting NAFLD/NASH-related pathways (2). The results of the computational analysis were validated using the developed in vitro steatosis models, testing for the compounds' ability to ameliorate the steatotic phenotype (3). The promising compounds were then submitted to proteomic analysis and statistical clustering to compare their mode of action in the signalling level (4).*

2.2. Isolation of Primary Human Hepatocytes

Primary human hepatocytes were isolated by double collagenase perfusion of histologically normal liver fragments using standard operating procedures (Biopredic International, Paris, France) from adult donors undergoing liver resection. All samples were acquired via approved ethics protocols and according to hospital's policies, and informed consents were obtained from all subjects (2nd Department of Surgery "Aretaieio" Hospital, 1st Department of Propaedeutic Surgery).

2.3. Experimental models details

Four hepatic cell lines, HUH7, HepG2, Hep3B and FOCUS, were cultured in Dulbecco's Modified Eagle's High Glucose Medium (DMEM) (Biosera, Nuaille, France) supplemented with 10% v/v Fetal Bovine Serum (FBS) (Biosera, Nuaille, France) and 1% v/v Penicillin-Streptomycin solution (Penicillin: 10'000 units/mL; Streptomycin: 10'000 ug/mL) (Biosera, Nuaille, France), at a 37°C, 5% CO_2 humidified incubator. For subculturing, cells were detached by treatment with 0.25% trypsin/0.02%EDTA at 37 ◦C. Cultures were used at 80% confluency. For drug treatment, compounds were diluted in serum-free medium without phenol red (Thermo Fisher Scientific, MA, USA) at either 0.1% v/v DMSO or 1% v/v etOH.

Primary human hepatocytes were thawed and seeded in black flat-bottom 96-well plates coated with Rat Tail Collagen I (BD Biosciences, MA, USA) at a seeding concentration of 50,000 cells per well in Williams E cell culture medium (PAN Biotech, USA) supplemented with 2% v/v Fetal Bovine Serum (FBS) (Biosera, Nuaille, France), 1% v/v Penicillin-Streptomycin solution (Penicillin: 10'000 units/mL; Streptomycin: 10'000 ug/mL) (Biosera, Nuaille, France), 1% Insulin-Transferin-Selenium Solution (MerckMillipore, USA) and 10uM Dexamethasone (MerckMillipore, USA). Cells were overlayed with GelTrex (Thermo Fisher Scientific, MA, USA) 12-24h after seeding.

2.4. In vitro steatosis induction

Before treatment, the cells were seeded onto black flat-bottomed 96-well plates for 24h at the corresponding densities: HUH7: 15'000 cells/well, HepG2: 20'000 cells/well, Hep3B: 15'000 cells/well, FOCUS: 10'000 cells/well in serum-free medium.

Cells were treated for 24h in serum-free medium with known steatogenic compounds. Namely, free fatty acids (FFAs; oleic acid:palmitic acid) (Cayman Chemical, MI, USA), Valproic acid sodium salt (VPA) (Cayman Chemical, MI, USA), Amiodarone hydrochloride (AMI) (Cayman Chemical, MI, USA), Tamoxifen citrate (TMX) (Cayman Chemical, MI, USA) and Tetracycline hydrochloride (TET) (Cayman Chemical, MI, USA) were evaluated as known steatogens. Oleic acid (OA), palmitic acid

(PA), and VPA were diluted in 100% ethanol (etOH) at 1% v/v final (% in cell culture medium) concentration. TMX, AMI and TET were diluted in DMSO at 0.1% v/v final concentration. Exogenous FFAs (molar ratio OA:PA=2:1) were conjugated with Bovine Serum Albumin (BSA) (MilliporeSigma, MA, USA) at a molar ratio of FFAs:BSA=4:1 (Figure 2).

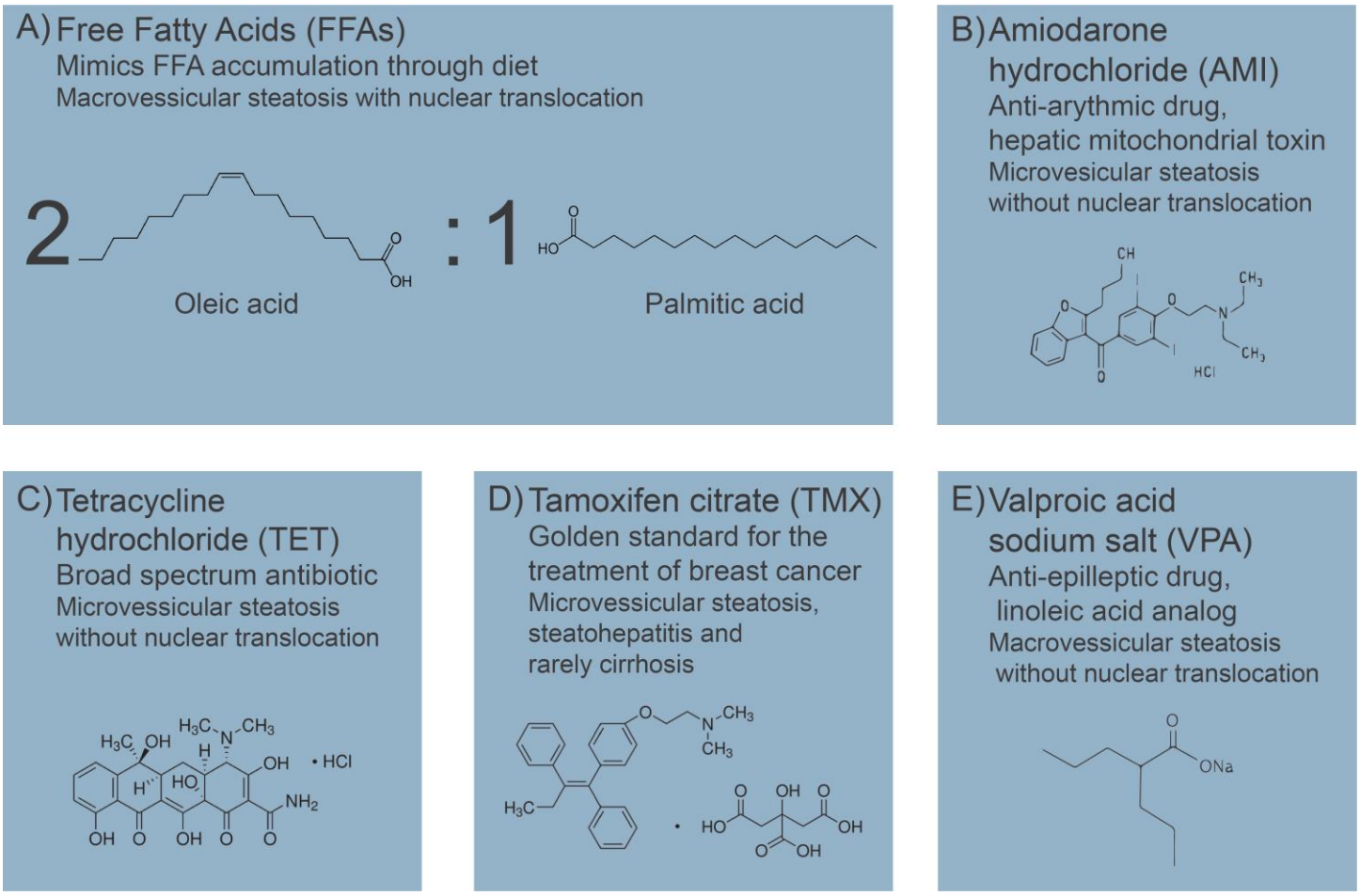


Figure 2 Drugs used to induce steatosis in vitro. *Cells were treated for 24h in serum-free medium with known steatogenic compounds. Oleic acid (OA), palmitic acid (PA), and VPA were diluted in 100% ethanol (etOH) at 1% v/v final (% in cell culture medium) concentration. TMX, AMI and TET were diluted in DMSO at 0.1% v/v final concentration. Exogenous FFAs (molar ratio OA:PA=2:1) were conjugated with Bovine Serum Albumin (BSA)) at a molar ratio of FFAs:BSA=4:1.*

2.5. Verification of lipid droplet accumulation via High-Content Screening (HCS)

Lipid droplets were fluorescently stained with Nile Red (Thermo Fisher Scientific, MA, USA) and cell nuclei were counterstained with Hoechst 33342 (Thermo Fisher Scientific, MA, USA). The culture medium was first aspirated and the cells were rinsed three times with Phosphate-buffered Saline (PBS) buffer (Biosera, Nuaille, France). Nile Red and Hoechst 33342 were diluted in Phenol-Red-free culture medium at 4ug/mL and 5ug/mL final concentrations respectively. Thirty (30) uL

of the imaging medium were added into each well and plates were then incubated for 45min at 37ºC. Images were acquired automatically using JuLI™ Stage Real-Time CHR (NanoEnTek, Seoul, Korea) with a 20x objective lens of a high-sensitivity monochrome CCD camera (Sony sensor 2/3") at room temperature.

2.6. Image analysis

Lipid accumulation was computationally quantified with image analysis, given the images obtained with HCS. Five images were acquired per well and all experiments were performed in technical and experimental replicates, hence a minimum of 50 images were analysed per treatment. An image analysis pipeline was created in MATLAB (v.2018a, Mathworks, USA). In short, multi-channel images were divided into the corresponding channels and converted into a binary format through a number of filters. Object sharpening and background elimination led to the identification and labelling of cell boundaries, nuclei and lipid droplets. For the output, the number of nuclei and the number of lipid droplets and droplet area were extracted.

The first step of the algorithm is the preprocessing of the images, where a Laplacian filter with a value of -8 in the centre of a 3×3 window with all the other elements defined by value 1 is applied. This filtering is used to sharpen the edges of objects, however, "salt and pepper" noise is added as a consequence of the differential operator. To remove this noise, a Median filter in the 3×3 neighbour area of each pixel is applied. In the processing step, a foreground and a background marker of each image are necessary to separate the objects of interests that are bounded. A Sobel edge mask is used to compute the gradient magnitude of the image, which presents a local maxima at the objects' borders, and minima inside the objects of interest. With the purpose of getting a smoother area in the inner part of the objects, an opening by reconstruction is applied, followed by closing by reconstruction, both with a disc element of 3 pixels radius. By computing the regional maxima of this image a sufficient foreground marker is obtained. The next step is the thresholding of the smoother image that marks the background with Otsu's algorithm. However, the Background marker is too close to the edges of the objects destined for segmentation, and to overcome this obstacle a thinner marker is used by computing the Skeleton by Influence Zones of the foreground, which is the watershed transform of the distance transform of the thresholded image. Finally, the watershed-based segmentation is computed and the objects are labelled, enabling the quantification of the necessary metrics (quantity, area, intensity).

2.7. Quantification of oxidative stress

Reactive oxygen species (ROS) were quantified with the CM-H_2DCFDA fluorescent substrate (Thermo Fisher Scientific, MA, USA). Cells, treated with 100uM of H_2O_2 for 30min, were used as a positive control. CM-H_2DCFDA was diluted in Phenol-Red-free and sodium-pyruvate-free culture medium at 10uM final concentration (Thermo Fisher Scientific, MA, USA). Thirty (30) uL of the staining solution were added per well. The plates were then incubated for 45min at 37°C. After incubation, fluorescence was measured at Ex495nm/Em520nm and normalized per ug of protein. The total protein concentration was deduced using BCA™ assay (Thermo Fisher Scientific, MA, USA) for each sample. CM-H_2DCFDA fluorescence and BCA absorbance were quantified with the Varioskan™ LUX multi-mode microplate reader (Thermo Fisher Scientific, MA, USA).

2.8. Resazurin reduction cell viability assay

Cell viability was quantified with Resazurin (MilliporeSigma, MA, USA). The Resazurin solution was added to the cell culture medium at a final concentration of 10ug/mL. The plates were then incubated for 2 hours at 37°C. Fluorescence was measured at Ex560nm/Em590nm, in Relative Fluorescent Units (RFU), with the Varioskan™ LUX multi-mode microplate reader (Thermo Fisher Scientific, MA, USA). The sample's viability was approximated as a percentage of treated over untreated cells. The experimental data were then fitted onto a 4-parameter logistic regression model in GraphPad Prism 9.0. IC_{10} values were extrapolated.

2.9. Identification of "clinical data pathways"

Datasets of microarray gene expression profiling, derived from biopsied NAFLD/NASH patients and healthy individuals, were obtained from the GEO (NCBI) database (Edgar et al., 2002). To assure robustness, only datasets with a sufficient number of samples and differentially expressed genes (DEGs) were selected, based on their degree of differential expression (Table 1). The degree of differential expression was calculated for each sample as the logarithm of the fold-change (FC) of expression values in the diseased condition (test) over the healthy condition (control). A student's t-test was used for statistical evaluation.

For every gene in each sample, the degree of differential expression was calculated by the logarithm of the fold change (FC) of expression values in the study condition compared to the control condition:

$$\log_2 FC(g) = \log_2 \frac{E(g)_{test}}{E(g)_{control}}$$

Thus, the neutral point of the quantity is defined by zero, whereas positive ratios correspond to overexpression and negative ratios correspond to under-expression of the gene, e.g. $\log_2 FC(g) = 1$ indicates an expression level two times higher than the control sample, while $\log_2 FC(g) = -1$ indicates a level two times lower.

However, to avoid using a value that appears to be significant only due to random fluctuation, a statistical evaluation of the data is needed. It is therefore necessary to compare a pair of distributions for each gene and not a pair of values. In this case, the statistical comparison was performed using Student's t-test. For graphically representing these data, $\log_2 FC$ and p-value for each gene were plotted in volcano plots. Each point on the plot corresponds to a gene, while the y-axis represents the negative decimal log of $p - value$ and the x-axis represents $\log_2 FC$. The higher the gene on the vertical axis, the more statistically significant its differential expression, and the farther from zero on the horizontal axis, the greater its intensity. $|\log_2 FC| > 1$ and $p - value \leq 0.05$. are usually used as the limits for the differential expression of a gene.

Next, each of the selected datasets was subjected to gene-level statistics (GLS), followed by pathway analysis (Gene Set Analysis; GSA). For the pathway analysis of clinical and compounds' data BIOCARTA (Nishimura, 2004), Protein Interaction Database (PID) (Schaefer et al., 2009), KEGG (Kanehisa and Goto, 2000; Mootha et al., 2003) and REACTOME (Fabregat et al., 2018; Jassal et al., 2020) databases were used as a knowledgebase.

GLS calculated the degree of differential expression for each gene via R Bioconductor's LIMMA (Linear Models for Micro-array and RNA-seq Data) package (Ritchie et al., 2015).

For every gene a linear model was generated, in order to quantify its differential expression level:

$$y_g = \beta_{g0} + \beta_{g1} * x_{g1} + \cdots + \beta_{gn} * x_{gn} + \varepsilon_g, g \in 1 \ldots G \quad (\mathbf{1})$$

Where:

y_g: Expression level of gene g

β_{g0} : Expression level of gene g in control samples

$\beta_{g1}, \ldots, \beta_{gn}$: The difference of expressions level of gene g on samples 1-n compared to control samples

$x_{g1}, \ldots, x_{gn}$: Variables with values 1 or 0, depending on the comparison made

ε_g: the computational error of gene expression

When eliminating the error value ε_g, the value of y_g is estimated by the value $\widehat{y_g}$, and the other parameters are transformed to $\widehat{\beta_{g0}}, \ldots, \widehat{\beta_{gn}}$. Thus the null hypothesis H_0 that there is no difference between the expression of the gene in the control sample and the individual sample is formed, i.e. $\widehat{\beta_{gn}} = 0$. From linear algebra this vector is calculated by:

$$\widehat{\beta_{gn}} = (X^T X)^{-1} X^T Y$$

Where,

X : the table of variables $x_{g0}, \ldots, x_{gn}$, where the rows correspond to the different genes and the columns to the different samples

Y : the vector of gene expression values

The standard error is calculated by: $se(\widehat{\beta_{gn}}) = \sqrt{s_g^2 (X^T X)^{-1}}$

Where s_g^2 represents the variance of the gene g, and the significance test from which the *t*-statistic for each gene is calculated takes the form: $t - test = \frac{\widehat{\beta_{gn}}}{se(\widehat{\beta_{gn}})}$

The above methodology poses a significant drawback, when the standard error is significantly low it generates a multitude of false positive (FP) results. This problem is addressed using hierarchical models, which describe the coefficients of variation $\widehat{\beta_{gn}}$ and the variation (s_g^2) as a function of the genes.

For each dataset, samples were divided into three clusters according to their health status (Healthy, NAFL or NASH). For every gene in a dataset, a linear model was generated in order to quantify the degree of differential expression. However, equation (1) only uses the variables x_{g1} and x_{g2}, as only three sets of samples are used instead of individual samples. The comparison is then made between healthy and NASH group, as well as between healthy and NAFL.

To eliminate false positives, hierarchical models were used to describe the coefficients of variation and to express variation as a function of the genes. The "Healthy" cluster was compared to both "NAFL" and "NASH". Finally, through the empirical Bayes methods, the B-value hyperparameter and a moderated t-statistic were calculated.

GSA (or pathway analysis) identifies significantly affected pathways. The analysis was performed with R Bioconductor's PIANO (Platform for Integrative Analysis of Omics Data) package under a functional class-scoring method (FCS) (Mathur et al., 2018; Väremo et al., 2013). Genesets,

provided by MSigDB, and GLS output underwent pathway analysis. For that, nine different statistical methods were utilized to identify the prevalent expression trend within a pathway.

- *"Fisher's combined probability test"*: The basic form of Fisher's tests the independence of multiple variables for $n \times n$ relevance tables by combining p-values from different statistical tests, based on the log-transformed gene-level p-values.
- *"Stouffer's method"*: Stouffer's method combines p-values from different tests using the inverse normal cumulative distribution function and generates Z-scores.
- *"Reporter features"*: The Reporter Features algorithm maps gene analysis to gene networks by identifying groups of adjacent genes that are differentially expressed. This algorithm allows for background distribution correction before calculating the gene set's significance.
- *"Tail strength":* Tail strength (TS) calculates gene set statistics by ranking the genes according to their level of significance and respectively lending the weight, meaning that it is more susceptible to tail deviations.
- *"PAGE"*: This method calculates a Z-score using fold change values for each set of genes and calculates the statistical significance using a normal distribution. (Parametric Analysis of Gene Set Enrichment; page)
- *"maxmean"*: This method calculates the average of the positive and negative expression values for each gene in each set of genes and selects the set of genes with the highest absolute value.
- *"sum", "mean", "median":* These methods calculate the sum, the mean and the median for each set of genes. The former uses the size *t*, while the other two uses the *fold change*.

Pathways were classified and ranked into five groups based on the gene expression trend and p-value within a pathway, namely "distinct up", "mixed up", "non-directional", "mixed down" and "distinct down" according to their given p-value.

- *Distinct up*: The genes that are overexpressed and the under-expressed genes are mutated. Overall there is a higher overexpression in the path.
- *Mixed up*: There is a high component of overexpressed genes and the component of under-expressed genes is not taken into account.
- *Non-directional*: The absolute value of the differential expression of the genes is used, as there is a high differential expression of genes, without regard to its direction.

- *Mixed down*: There is a high component of under-expressed genes and the component of overexpressed genes is not taken into account.
- *Distinct down*: The genes that are overexpressed and the under-expressed genes are mutated. Overall there is a higher under-expression in the path.

Those with low *p*-values were considered to be the most significant in terms of differential expression. Taken together, they formed a group of "clinical data pathways".

GEO Series Accession	Contributors	Number of control samples	Number of diseased samples	Pathological phenotype of NAFL
GSE48452	Ahrens M et al. (Ahrens et al., 2013)	14	59	Healthy obese, NASH, Steatosis
GSE63067	Frades I et al. (Frades et al., 2015)	7	11	NASH, Steatosis
GSE89632	Arendt et al. (Arendt et al., 2015)	24	39	NASH, Steatosis

Table 1 *Datasets of microarray gene expression profiling from biopsy-proven NAFLD/NASH patients and healthy individuals obtained from GEO (NCBI)*

2.10. Identification of "steatogenic compounds' pathways"

The compounds' used to induce steatosis *in vitro* were interrogated against the DrugBank (Wishart et al., 2006) and MSigDB (Liberzon et al., 2015) databases to identify the targets and pathways they affect, forming the "steatogenic compounds' pathways" group.

2.11. Identification of "DR compounds' pathways"

The known steatogens were input to the Connectivity Map (cMap) database (Broad Institute) (Lamb, 2006; Subramanian et al., 2017). cMap compares differences in gene expression levels, termed "signatures", between a disease, genetic perturbation or treatment with a small molecule ("signature questions"), to all perturbational signatures available. The similarity comparisons are evaluated through the Kolmogorov-Smirnov (K-S) test (Kim and Volsky, 2005), a non-parametric method that determines whether the two-sample distributions differ.]. Each gene in the "signature question" bears a signal indicating whether it is overexpressed or under-expressed. When the "signature question" is compared to the drugs in the data base, it is examined whether the overexpressed genes are at the top of the list of each drug and the under-expressed genes at the

bottom of the list, or vice versa. In the first case the drugs present a similar gene signature, while in the second case a reverse gene signature. The above procedure calculates a value called Enrichment Score; ES and takes values in the interval [-1,1]. Positive values correspond to similar gene signatures, while negative ones correspond to opposite. For each "signature question" asked, a table of bioactive substances is produced.

For each of the known steatogens, posed as the input "signature question", a table of bioactive compounds was produced. Only compounds with significantly (p-value<0.05) similar (ES>0) or opposite (ES<0) mode of action were selected. Out of those, known hepatotoxic and steatogenic compounds were reviewed on the ToxDB (Hardt et al., 2016) and LiverTox (Hoofnagle et al., 2013) databases to be excluded from further analysis. Each of the selected compounds was then introduced to the DrugBank and MSigDB databases to identify the pathways it respectively affects. Those pathways, taken together, formed a group of "DR compounds' pathways".

2.12. Validation of the *in silico* predictions with High-Content Screening (HCS)

An HCS pipeline was devised to verify the *in vitro* effects of the proposed DR compounds. HepG2 cells were seeded in black, flat-bottomed, 384-well plates at 5'000/well density and treated with FFAs at 200uM. Control samples were treated only with the candidate DR compounds at 10uM, while the rest were treated with a co-treatment of FFAs and DR compounds to evaluate the anti-steatogenic potential. All treatments were applied for 24h before HCS. Intracellular lipid content and ROS production were quantified according to the assays described in §2.1.2, §2.1.3 and §2.1.4.

2.13. Protein isolation

Total protein isolation protocols were followed for phospho-proteomic measurements. The cells were seeded on flat-bottomed, 96-well plates at their aforementioned densities. After 24h, they were co-treated with FFAs at 200uM and the DR compounds at 10uM for another 24h before lysis. The lysis buffer (ProtAtOnce Ltd, Athens, Greece) was supplemented with a protease/phosphatase inhibitor mix (ProtAtOnce Ltd, Athens, Greece) at 100x v/v and with Phenylmethanesulfonyl fluoride (PMSF) (MilliporeSigma, MA, USA) at 50x v/v. The samples were maintained at -80^{o}C. Before collection, thawed samples were first sonicated and then centrifuged at 2400rpm for 30min.

2.14. Multiplex ELISA – xMAP assays

All lysates were adjusted to a total protein concentration of 250ug/mL. xMap assays were performed on a Luminex FlexMAP 3D platform (Luminex, Austin TX, USA). The customized 17-plex phosphoprotein panel (ProtAtOnce Ltd, Athens, Greece) included: mothers against

decapentaplegic homolog-3 (SMAD3), transcription factor AP-1 (JUN), insulin receptor substrate-1 (IRS1), tyrosine-protein phosphatase non-receptor type-11 (PTN11 or SHP2), proline-rich AKT1 substrate-1 (AKTS1), mitogen-activated protein kinase-3 (ERK1 or MK03), RAC-alpha serine/threonine-protein kinase (AKT1), glycogen synthase kinase-3 alpha (GSK3A), heat shock protein beta-1 (HSP27 or HSPβ1), signal transducer and activator of transcription-3 (STAT3), mitogen-activated protein kinase (p38 or MAPK), transcription factor p65 (NFKβ), dual specificity mitogen-activated protein kinase kinase-1 (MEK1 or MP2K1), focal adhesion kinase-1(FAK1), cyclic AMP-responsive element-binding protein-1 (CREB1), nuclear factor erythroid 2-related factor-2 (NRF2 or NFE2L2), and 40S ribosomal protein S6 (RS6). For cytokine release measurements a 20-plex antibody assay was developed (ProtAtOnce Ltd, Athens Greece): interleukin 13 (IL13), growth-regulated alpha protein (GROA), interleukin 1a (IL1a), interleukin 8 (IL8), interleukin 12 (IL12), interleukin 6 (IL6), C-C motif chemokine 3 (CCL3), C-X-C motif chemokine 10 (CXCL10), tumour necrosis factor (TNFA), resistin (RETN), ciliary neurotrophic factor (CNTF), C-X-C motif chemokine 11 (CXCL11), transcriptional regulator NRG1 (NRG1), fibroblast growth factor (FGF), metalloproteinase inhibitor 1 (TIMP1), transforming growth factor beta-1 proprotein (TGFB1), C-C motif chemokine 5 (CCL5), C-C motif chemokine 2 (CCL2), matrix metalloproteinase-9 (MMP9) and interleukin 1b (IL1b). Additional details and the relationship of these proteins with NAFL/NASH are presented in Table 2.

#	Protein	Alliases	UNIPROT name	Pathways related with NAFL/NASH
1	**ERK**	MAPK3, MKO3	Mitogen-activated protein kinase 3	**KEGG_PATHWAYS:** MAPK signaling pathway, cAMP signaling pathway, Chemokine signaling pathway, FoxO signaling pathway, mTOR signaling pathway, Alcoholism, TNF signaling pathway, GnRH signaling pathway, Hepatocellular carcinoma, Insulin signaling pathway, AGE-RAGE signaling pathway in diabetic complications **REACTOME_PATHWAYS:** MAPK3 (ERK1) activation, MAPK1 (ERK2) activation, MAPK1/MAPK3 signaling, RAF-independent MAPK1/3 activation, MAP2K and MAPK activation, Activated NTRK3 signals through PI3K, RAF/MAP kinase cascade, Signalling to ERKs, FCERI mediated MAPK activation, MET activates PTPN11, MAP kinase activation, MET activates RAS signaling, MAPK family signaling cascades, Oncogene Induced Senescence, MAP3K8 (TPL2)-dependent MAPK1/3 activation

2	**SMAD3**	MAD, LDS3	Mothers Against Decapentapleg ic Homolog 3	**KEGG_PATHWAYS:** TGF-beta signaling pathway, FoxO signaling pathway, Hippo signaling pathway, Hepatitis B, Wnt signaling pathway, Endocytosis, Cellular senescence, Hepatocellular carcinoma, Diabetic cardiomyopathy, AGE-RAGE signaling pathway in diabetic complications **REACTOME_PATHWAYS:**, Downregulation of TGF-beta receptor signaling, TGF-beta receptor signaling activates SMADs, Signaling by TGFB family members, Signaling by TGF-beta Receptor Complex, FOXO-mediated transcription of oxidative stress, metabolic and neuronal genes
3	**P38 - MAPK**	MAPK14	P38 Mitogen Activated Protein Kinase	**KEGG_PATHWAYS:** Non-alcoholic fatty liver disease, Hepatitis B., MAPK signaling pathway, Autophagy, VEGF signaling pathway, GnRH signaling pathway, TNF signaling pathway, FoxO signaling pathway, Lipid and atherosclerosis, Cellular senescence, Toll-like receptor signaling pathway, NOD-like receptor signaling pathway Toll-like receptor signaling pathway, IL-17 signaling pathway, AGE-RAGE signaling pathway in diabetic complications, **REACTOME_PATHWAYS:** PKA-mediated phosphorylation of CREB, Platelet sensitization by LDL, JNK (c-Jun kinases) phosphorylation and activation mediated by activated human TAK1, Oxidative Stress Induced Senescence, TAK1 activates NFkB by phosphorylation and activation of IKKs complex, MAP kinase activation, Drug-mediated inhibition of ERBB2 signaling, Mitochondrial biogenesis, PTEN Regulation, CREB phosphorylation, Interleukin-1 family signaling, Interleukin-7 signaling, Signaling by Leptin, Activation of PPARGC1A (PGC-1alpha) by phosphorylation
4	**AKT**	AKT1	RAC-alpha serine/threoni ne-protein kinase	**KEGG_PATHWAYS:** Non-alcoholic fatty liver disease, PI3K-Akt signaling pathway, Insulin signaling pathway, Insulin resistance, Autophagy ,ErbB signaling pathway, VEGF signaling pathway, MAPK signaling pathway, mTOR signaling pathway, AMPK signaling pathway, Toll-like receptor signaling pathway, JAK-STAT signaling pathway, TNF signaling pathway, FoxO signaling pathway, cAMP signaling pathway, cGMP-PKG signaling pathway, HIF-1 signaling

				pathway, Phospholipase D signaling pathway, Hepatocellular carcinoma, Chemokine signaling pathway, JAK-STAT signaling pathway, Lipid and atherosclerosis, Hepatitis B, Hepatitis C, AGE-RAGE signaling pathway in diabetic complications, Adipocytokine signaling pathway, Glucagon signaling pathway, Regulation of lipolysis in adipocytes, Carbohydrate digestion and absorption **REACTOME_PATHWAYS:** PI3K/AKT activation, PIP3 activates AKT signaling, AKT phosphorylates targets in the cytosol, AKT phosphorylates targets in the nucleus, Negative regulation of MAPK pathway, Signaling by EGFR, KT-mediated inactivation of FOXO1A, CD28 dependent PI3K/Akt signaling, Negative regulation of the PI3K/AKT network, MET activates PI3K/AKT signaling, PI5P, PP2A and IER3 Regulate PI3K/AKT Signaling, Activation of AKT2, Signaling by Receptor Tyrosine Kinases, Activated NTRK3 signals through PI3K, FOXO-mediated transcription of cell death genes, Negative regulation of NOTCH4 signaling, PTEN Regulation, Regulation of TP53 Degradation, TP53 Regulates Metabolic Genes, PTEN Loss of Function in Cancer, Signaling by ERBB2, Downregulation of ERBB2:ERBB3 signaling, Signaling by ERBB2, PI3K events in ERBB4 signaling, FOXO-mediated transcription of oxidative stress, metabolic and neuronal genes, IRS-related events triggered by IGF1R, Signaling by Type 1 Insulin-like Growth Factor 1 Receptor (IGF1R), IGF1R signaling cascade, Signaling by Leptin
5	**HSP27**	HSPB1	Heat shock protein beta-1	**KEGG_PATHWAYS:** MAPK signaling pathway, VEGF signaling pathway **REACTOME_PATHWAYS:** MAPK6/MAPK4 signaling, activated TAK1 mediates p38 MAPK activation, Signaling by VEGF, AUF1 (hnRNP D0) binds and destabilizes mRNA
6	**STAT3**	-	Signal transducer and activator of transcription 3	**KEGG_PATHWAYS:** JAK-STAT signaling pathway, EGFR tyrosine kinase inhibitor resistance, FoxO signaling pathway, HIF-1 signaling pathway, Adipocytokine signaling pathway, Hepatitis B, Hepatitis C, Lipid and atherosclerosis, Chemokine signaling pathway, AGE-RAGE signaling pathway in diabetic complications, Insulin resistance

				REACTOME_PATHWAYS: Signalling to STAT3, Signaling by NOTCH2, Signaling by PTK6, Interleukin signaling, Signaling by Leptin
7	**JUN**	c-JUN AP-1	Activator Protein 1	**KEGG_PATHWAYS:** Non-alcoholic fatty liver disease , MAPK signaling pathway, FoxO signaling pathway, Wnt signaling pathway, TNF signaling pathway, C-type lectin receptor signaling pathway, Lipid and atherosclerosis, cAMP signaling pathway, Toll-like receptor signaling pathway, ErbB signaling pathway, Hepatitis B, AGE-RAGE signaling pathway in diabetic complications **REACTOME_PATHWAYS**: MAP kinase activation, PTEN Regulation JNK (c-Jun kinases) phosphorylation and activation mediated by activated human TAK1, MAPK targets/ Nuclear events mediated by MAP kinases, activated TAK1 mediates p38 MAPK activation, Interleukin-1 family signaling, Interleukin-3, Interleukin-5 and GM-CSF signaling, Macroautophagy , Oxidative Stress Induced Senescence , TNFR1-induced NFkappaB signaling pathway, Activation of the AP-1 family of transcription factors
9	**NRF2**	NFE2L2	Nuclear factor erythroid 2-related factor-2	**KEGG_PATHWAYS:** Hepatocellular carcinoma, Lipid and atherosclerosi **REACTOME_PATHWAYS**: ROS sensing by NFE2L2
9	**GSK3**	GSK3A/B	Glycogen synthase kinase-3 alpha/beta	**KEGG_PATHWAYS:** Non-alcoholic fatty liver disease, PI3K-Akt signaling pathway**,** Wnt signaling pathway, mTOR signaling pathway, Chemokine signaling pathway, Hedgehog signaling pathway, Hippo signaling pathway, Insulin resistance
10	**CREB1**	-	Cyclic AMP-responsive element-binding protein 1	**KEGG_PATHWAYS:** PI3K-Akt signaling pathway, cAMP signaling pathway, cGMP-PKG signaling pathway, AMPK signaling pathway, TNF signaling pathway, Glucagon signaling pathway, Alcoholism **REACTOME_PATHWAYS**: Signaling by NOTCH2, Transcriptional Regulation by MECP2, CREB1 phosphorylation through the activation of Adenylate Cyclase, Transcriptional activation of mitochondrial biogenesis

11	**FAK1**	PTK2, FADK	Protein Tyrosine Kinase 2	**KEGG_PATHWAYS:** VEGF signaling pathway, Chemokine signaling pathway, PI3K-Akt signaling pathway, ErbB signaling pathway, GnRH signaling pathway , Phospholipase D signaling pathway **REACTOME_PATHWAYS**: Signaling by Non-Receptor Tyrosine Kinases, Signaling by VEGF
12	**AKT1S1**	PRAS40	Proline-rich AKT1 substrate 1	**KEGG_PATHWAYS:** mTOR signaling pathway, AMPK signaling pathway, Autophagy **REACTOME_PATHWAYS**: AKT1S1 (PRAS40) binds mTORC1, AKT phosphorylates AKT1S1 (PRAS40)
13	**IRS1**	HIRS-1	Insulin receptor substrate-1	**KEGG_PATHWAYS:** Non-alcoholic fatty liver disease, Type II diabetes mellitus, Insulin resistance, Adipocytokine signaling pathway, mTOR signaling pathway, PI3K-Akt signaling pathway, cGMP-PKG signaling pathway, FoxO signaling pathway, Autophagy, AMPK signaling pathway, Insulin signaling pathway, Regulation of lipolysis in adipocytes, **REACTOME_PATHWAYS:** IRS activation, IGF1R signaling cascade, IRS-related events triggered by IGF1R, Activated NTRK3 signals through PI3K, Signaling by Leptin
14	**RS6**	S6K1, P70 S6K-Alpha, P70S6K	Ribosomal Protein S6 Kinase B1	**KEGG_PATHWAYS:** Insulin signaling pathway, PI3K-Akt signaling pathway, ErbB signaling pathway, HIF-1 signaling pathway, TGF-beta signaling pathway,
15	**PTN11**	PTPN11, SHP-2, CFC, NS1	Protein Tyrosine Phosphatase Non-Receptor Type 11	**KEGG_PATHWAYS:** MAPK signaling pathway, JAK-STAT signaling pathway, Natural killer cell mediated cytotoxicity, Phospholipase D signaling pathway, Adipocytokine signaling pathway, Insulin resistance **REACTOME_PATHWAYS**: Interleukin-6 family signaling
16	**NFKB**	-	Nuclear Factor Kappa B	**KEGG_PATHWAYS:** Non-alcoholic fatty liver disease, NF-kappa B signaling pathway, PI3K-Akt signaling pathway, MAPK signaling pathway, PI3K-Akt signaling pathway, NOD-like receptor signaling pathway, cAMP signaling pathway, TNF signaling pathway, Chemokine signaling pathway, Toll-like receptor signaling pathway, HIF-1 signaling pathway, Hepatitis B, Hepatitis C, Lipid and atherosclerosis, Adipocytokine signaling pathway, Insulin resistance, AGE-RAGE signaling pathway in diabetic complications

				REACTOME_PATHWAYS: MAP3K8 (TPL2)-dependent MAPK1/3 activation, Interleukin-1 signaling, Toll Like Receptor 4 (TLR4) Cascade, Interleukin signaling, Signaling by NOTCH, Evasion of Oxidative Stress Induced Senescence Due to Defective p16INK4A binding to CDK4 and CDK6, Diseases associated with the TLR signaling cascade
17	**MEK**	MEK1, MAP2K1	Mitogen-Activated Protein Kinase Kinase 1	**KEGG_PATHWAYS:** MAPK signaling pathway, Chemokine signaling pathway, FoxO signaling pathway, Osteoclast differentiation, Toll-like receptor signaling pathway, Autophagy, TNF signaling pathway, GnRH signaling pathway, Insulin signaling pathway **REACTOME_PATHWAYS**: MAPK1 (ERK2) activation, MAP2K and MAPK activation, Negative regulation of MAPK pathway, RAF/MAP kinase cascade, RAF-independent MAPK1/3 activation
22	**IL13**	-	Interleukin 13	**KEGG_PATHWAYS:** JAK-STAT signaling pathway **REACTOME_PATHWAYS**: Interleukin-4 and Interleukin-13 signaling, STAT6-mediated induction of chemokines
23	**IL8**	CXCL8	Interleukin 8	**KEGG_PATHWAYS:** Non-alcoholic fatty liver disease, NF-kappa B signaling pathway, Chemokine signaling pathway, Phospholipase D signaling pathway, Cellular senescence, Toll-like receptor signaling pathway, NOD-like receptor signaling pathway, Hepatitis B, AGE-RAGE signaling pathway in diabetic complications **REACTOME_PATHWAYS**: Interleukin-10 signaling, Interleukin-17 signaling
24	**CCL3**	MIP1A	C-C Motif Chemokine Ligand 3	**KEGG_PATHWAYS:** Chemokine signaling pathway, Toll-like receptor signaling pathway
25	**IL1a**	-	Interleukin 1A	**KEGG_PATHWAYS:** Non-alcoholic fatty liver disease, Cellular senescence, MAPK signaling pathway, AGE-RAGE signaling pathway in diabetic complications, Type I diabetes mellitus **REACTOME_PATHWAYS**: Interleukin-1 family signaling, Interleukin-1 processing, Senescence-Associated Secretory Phenotype (SASP)

26	**CXCL10**	IFI10	C-X-C Motif Chemokine Ligand 10	**KEGG_PATHWAYS:** TNF signaling pathway, Chemokine signaling pathway, Toll-like receptor signaling pathway, Hepatitis C **REACTOME_PATHWAYS**: Interleukin-12 signaling
27	**GROA**	CXCL1	C-X-C Motif Chemokine Ligand 1	**KEGG_PATHWAYS:** Chemokine signaling pathway, TNF signaling pathway, IL-17 signaling pathway, Toll-like receptor signaling pathway, NF-kappa B signaling pathway, NOD-like receptor signaling pathway **REACTOME_PATHWAYS**: MAP3K8 (TPL2)-dependent MAPK1/3 activation
28	**CCL2**	MCP1	C-C Motif Chemokine Ligand 2	**KEGG_PATHWAYS:** Chemokine signaling pathway, TNF signaling pathway, NOD-like receptor signaling pathway, AGE-RAGE signaling pathway in diabetic complications **REACTOME_PATHWAYS**: ATF4 activates genes in response to endoplasmic reticulum stress, STAT6-mediated induction of chemokines, NF-kappa B signaling pathway
29	**TNFA**	TNFSF2	Tumor Necrosis Factor	**KEGG_PATHWAYS:** Type II diabetes mellitus, Type I diabetes mellitus, Insulin resistance, TNF signaling pathway, NF-kappa B signaling pathway, NOD-like receptor signaling pathway, IL-17 signaling pathway, Adipocytokine signaling pathway **REACTOME_PATHWAYS**: TNFR2 non-canonical NF-kB pathway,
30	**RETN**	RSTN, ADSF	Resistin	**REACTOME_PATHWAYS**: FOXO-mediated transcription of oxidative stress, metabolic and neuronal genes
31	**CNTF**	HCNTF	Ciliary Neurotrophic Factor	**KEGG_PATHWAYS:** JAK-STAT signaling pathway, NF-kappa B signaling pathway **REACTOME_PATHWAYS**: IL-6-type cytokine receptor ligand interactions, Interleukin-6 family signaling
32	**IL1B**	-	Interleukin 1B	**KEGG_PATHWAYS:** NOD-like receptor signaling pathway, NF-kappa B signaling pathway, IL-17 signaling pathway, TNF signaling pathway, MAPK signaling pathway, Toll-like receptor signaling pathway **REACTOME_PATHWAYS**: Inflammasomes, Interleukin-1 family signaling, Interleukin-1 processing
33	**IL6**	-	Interleukin 6	**KEGG_PATHWAYS:** Non-alcoholic fatty liver disease, Insulin resistance, Hepatitis B, JAK-STAT signaling pathway, EGFR

				tyrosine kinase inhibitor resistance, HIF-1 signaling pathway, PI3K-Akt signaling pathway, IL-17 signaling pathway, TNF signaling pathway, FoxO signaling pathway, Cellular senescence, Toll-like receptor signaling pathway, NOD-like receptor signaling pathway, AGE-RAGE signaling pathway in diabetic complications **REACTOME_PATHWAYS**: Interleukin-10 signaling, Senescence-Associated Secretory Phenotype (SASP), Interleukin-12 signaling, Interleukin-6 family signaling, Interleukin-17 signaling, Toll Like Receptor 4 (TLR4) Cascade, MAP3K8 (TPL2)-dependent MAPK1/3 activation, Interleukin-10 signaling
34	**IL12**	IL12B. IL12P40	Interleukin 12, Subunit P40	**KEGG_PATHWAYS:** JAK-STAT signaling pathway, Toll-like receptor signaling pathway, Type I diabetes mellitus **REACTOME_PATHWAYS**: Interleukin-12 signaling, Interleukin-23 signaling, IL-6-type cytokine receptor ligand interactions, Interleukin-10 signaling
35	**CXCL11**	IP9	C-X-C Motif Chemokine Ligand 11	**KEGG_PATHWAYS:** Chemokine signaling pathway, Toll-like receptor signaling pathway
36	**NRG1**	NDF, HRG	Neuregulin 1	**KEGG_PATHWAYS:** ErbB signaling pathway **REACTOME_PATHWAYS**: Signaling by ERBB2, Signaling by ERBB4
37	**FGF**	-	Fibroblast Growth Factor	**KEGG_PATHWAYS:** MAPK signaling pathway, PI3K-Akt signaling pathway, Endocytosis, Hippo signaling pathway **REACTOME_PATHWAYS**: Spry regulation of FGF signalling, Signaling by FGFR family, Downstream signaling of activated FGFR family, Signaling by FGFR1 in disease, Negative regulation of FGFR signalling, FGFR ligand binding and activation
38	**TIMP1**	EPO, TIMP	TIMP Metallopeptidase Inhibitor 1	**KEGG_PATHWAYS:** HIF-1 signaling pathway
39	**TGFB1**	TGFbeta, TGFB	Transforming Growth Factor Beta 1	**KEGG_PATHWAYS:** Non-alcoholic fatty liver disease, Cellular senescence, TGF-beta signaling pathway, AGE-RAGE signaling pathway in diabetic complications, MAPK signaling

				pathway, FoxO signaling pathway, Hippo signaling pathway, Endocytosis, Hepatitis B **REACTOME_PATHWAYS**: Signaling by TGFB family members, Signaling by BMP
40	**CCL5**	RANTES	C-C Motif Chemokine Ligand 5	**KEGG_PATHWAYS:** TNF signaling pathway, Chemokine signaling pathway, Toll-like receptor signaling pathway, NOD-like receptor signaling pathway
41	**MMP9**	-	Matrix Metallopeptidase 9	**KEGG_PATHWAYS:** Hepatitis B, TNF signaling pathway **REACTOME_PATHWAYS**: MAPK6/MAPK4 signaling

Table 2 *Proteins targeted for proteoic measuremens and their relationshi with NAFL/NASH mechanisms.*

2.15. Quantification and statistical analysis

Statistical analysis of the intracellular lipid accumulation, ROS production and viability assessment was performed using R-programming language. Ordinary one-way ANOVA test with Tukey's multiple comparisons was performed to compare cell treatments to the respective controls. Biologically relevant comparisons were made between all samples and either the compounds' diluent (*in vitro* steatosis models, novel steatogenic compounds) or FFAs 200uM + DMSO 0.1% (novel anti-steatogenic compounds). Data are presented as mean ± SEM of at least three independent experiments. Comparisons with a $p\text{-value} \leq 0.05$ were considered statistically significant. Calculation of IC_{10} values of the steatogenic compounds was made via 4-parameters logistic regression fitting of the percentage of viability. Heatmaps and volcano plots were created using the R-programming language. Principal component analysis and k-means clustering were also performed using the R-programming language.

3 | RESULTS

RESULTS

3.1. Known steatogens induce steatosis *in vitro*

Four hepatic cell lines (HepG2, HuH7, Hep3B, FOCUS) were treated with a mixture of free fatty acids (FFAs; oleic acid:palmitic acid) and the known steatogenic compounds: Valproic acid sodium salt (VPA) (Rodrigues et al., 2016; Szalowska et al., 2014; Verrotti et al., 2009), Amiodarone hydrochloride (AMI) (Anthérieu et al., 2011; Szalowska et al., 2014), Tamoxifen citrate (TMX) (Cole et al., 2010; Yang et al., 2016; Zhao et al., 2014) and Tetracycline hydrochloride (TET) (Anthérieu et al., 2011; Choi et al., 2015; Szalowska et al., 2014). Concentrations lower than IC_{10} were used, so to avoid cytotoxic effects. The IC_{10} concentrations were extrapolated from cell-line- and compound-specific dose-viability curves (Table 3).

		Free Fatty Acids (FFA)	**Valproic acid (VPA)**	**Amiodarone (AMI)**	**Tamoxifen (TMX)**	**Tetracycline (TET)**
IC^{10}	**pHH**	255.21±1.73μM	516.0±1.13μM	18.0±2.24μM	7.53±1.1.49μM	150.2±5.05μM
R^2		0.9217	0.9906	0.9670	0.9683	0.9290
IC^{10}	**HepG2**	182.8±1.33μM	535.5±1.51μM	28.30±1.23μM	6.54±1.1.11μM	91.55±1.03μM
R^2		0.9563	0.9415	0.9772	0.9285	0.9698
IC^{10}	**HuH7**	216.1±1.32μM	151.8±1.63μM	34.87±1.08μM	20.43±1.09μM	53.93±1.18μM
R^2		0.9553	0.9613	0.9723	0.9285	0.9698
IC^{10}	**Hep3B**	101.8±1.35μM	299.6±5.03μM	19.55±1.08μM	6.04±1.02μM	76.05±1.08μM
R^2		0.9550	0.9584	0.9815	0.9909	0.9627
IC^{10}	**FOCUS**	128.3±1.38μM	139.3±1.11μM	47.19±1.40μM	14.77±1.09μM	35.3±1.91μM
R^2		0.9360	0.9906	0.9670	0.9683	0.9290

Table 3 IC_{10} and R^2 of dose-response fitting upon treatment of hepatic cells with compounds known to induce steatosis in vitro. *Cell viability was calculated as the percentage of Relative Fluorescent Units (RFU) of treated cells to untreated cells (control). Data were fitted to a 4 parameters logistic regression model.*

Intracellular lipid loading was quantified as the intensity of lipid droplets per cell (intensity of nucleus), after Nile Red and Hoechst33342 staining, via MATLAB-based image analysis. Reactive oxygen species (ROS) production was expressed in Relative Fluorescent Units (RFU) per ug of protein. In both, the fold-change (FC) of treated-over-control samples was calculated and the compound's diluent was used as control. The results for the pHH cells are shown in Figure 3 and the HepG2, HuH7, Hep3B and FOCUS cells in Figures 4-7, respectively. Briefly, all treatments led

to a significant increase in both intracellular lipid droplet formation and ROS production across all the cell lines, except for TMX on HuH7 cells, thus recapitulating the steatotic phenotype. The steatogens' targets and pathways affected were identified through DrugBank and MSigDB databases forming the "steatogenic compounds' pathways".

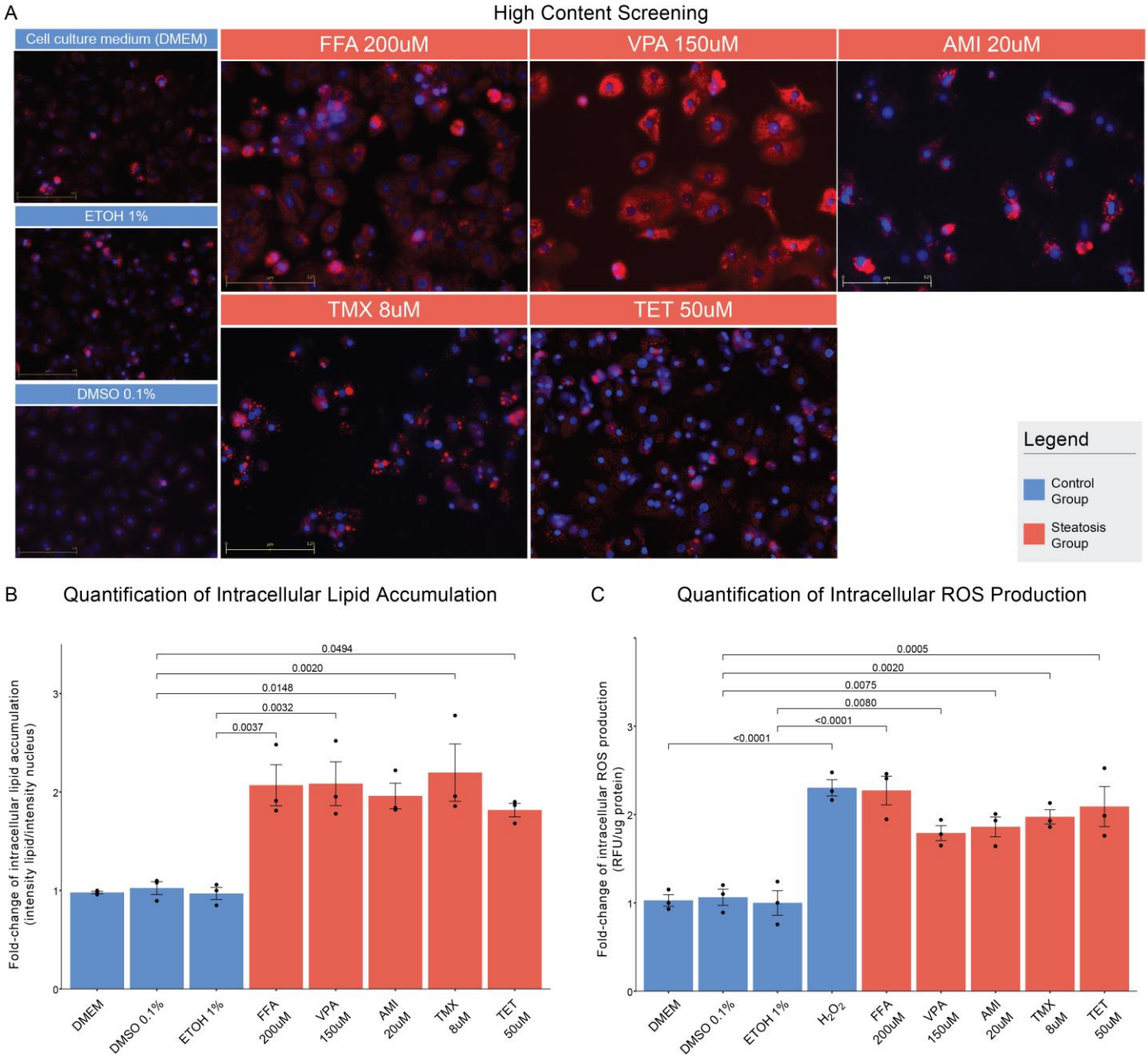


Figure 3 Formation of intracellular lipid droplets and increase of ROS production in primary human hepatocytes (pHH), after treatment with FFA, VPA, AMI, TMX and TET*. A) Intracellular lipid accumulation observed via HCS-based fluorescent microscopy with Nile Red staining. Hoechst33342 was used for staining the nuclei. Images were acquired under 20x optical magnification. B) Quantification of lipid accumulation via MATLAB-based image analysis. Bars represent the FC of lipid droplet intensity per cell in*

treated cells over respective controls. C) FC of intracellular ROS production compared to controls. H_2O_2 was used as a positive control. (B, C) Data expressed as mean±SEM of n=3 independent experiments, and the p-value is denoted by brackets.

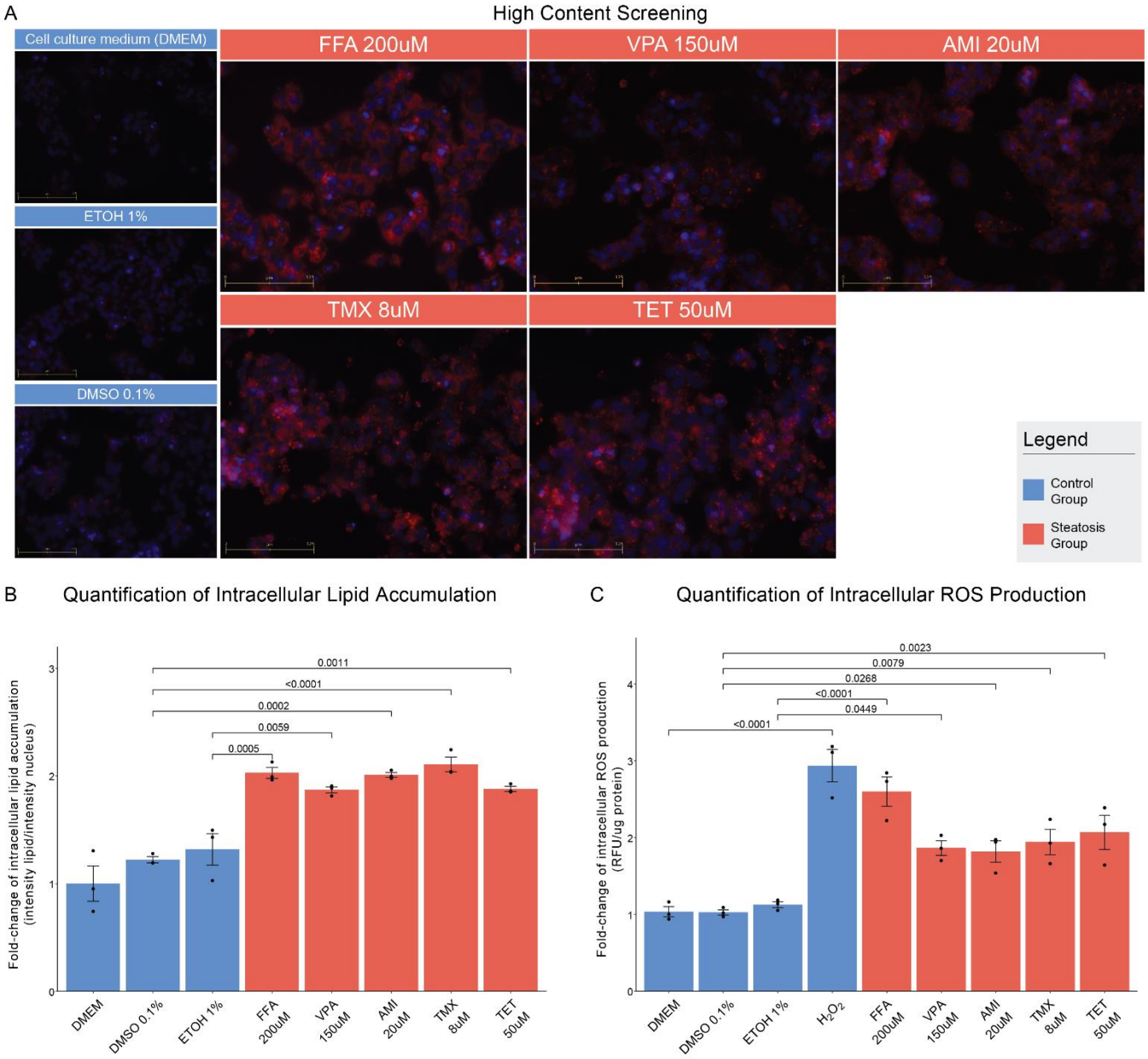


Figure 4 Formation of intracellular lipid droplets and increase of ROS production in HepG2 cells, after treatment with FFA, VPA, AMI, TMX and TET. *A) Intracellular lipid accumulation observed via HCS-based fluorescent microscopy with Nile Red staining. Hoechst33342 was used for staining the nuclei. Images were acquired under 20x optical magnification. B) Quantification of lipid accumulation via MATLAB-based image analysis. Bars represent the FC of lipid droplet intensity per cell in treated cells over respective controls. C) FC of intracellular ROS production compared to controls. H_2O_2 was used as a positive control.*

(B, C) Data expressed as mean±SEM of n=3 independent experiments, and the p-value is denoted by brackets.

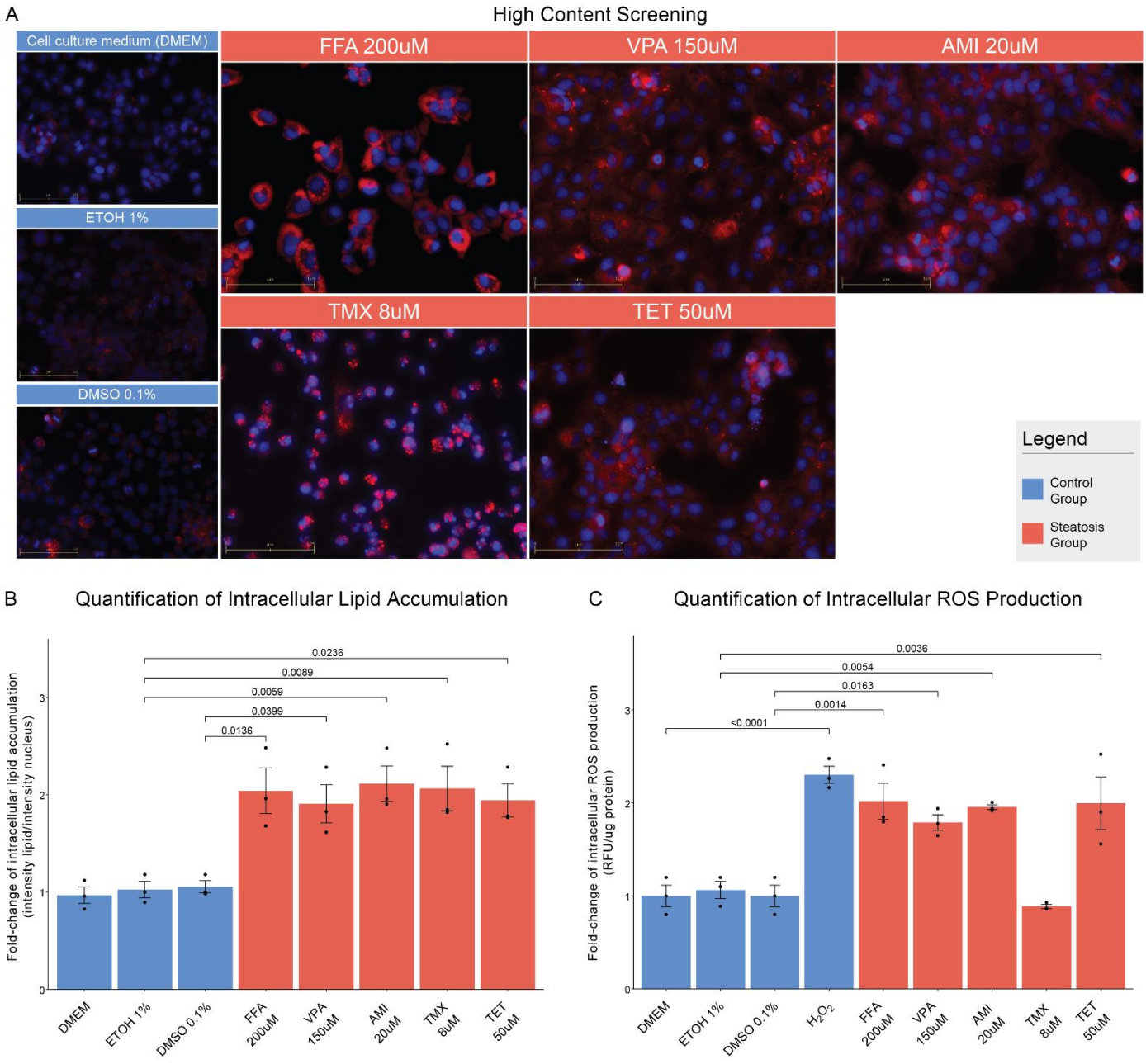


Figure 5 Formation of intracellular lipid droplets and increase of ROS production in HuH7 cells, after treatment with FFA, VPA, AMI, TMX and TET. *A) Intracellular lipid accumulation observed via HCS-based fluorescent microscopy with Nile Red staining. Hoechst33342 was used for staining the nuclei. Images were acquired under 20x optical magnification. B) Quantification of lipid accumulation via MATLAB-based image analysis. Bars represent the FC of lipid droplet intensity per cell in treated cells to the respective controls. C) FC of intracellular ROS production compared to controls. H_2O_2 was used as a positive control. (B, C) Data expressed as mean±SEM of n=3 independent experiments, and (*) denotes p-value ≤ 0.05*

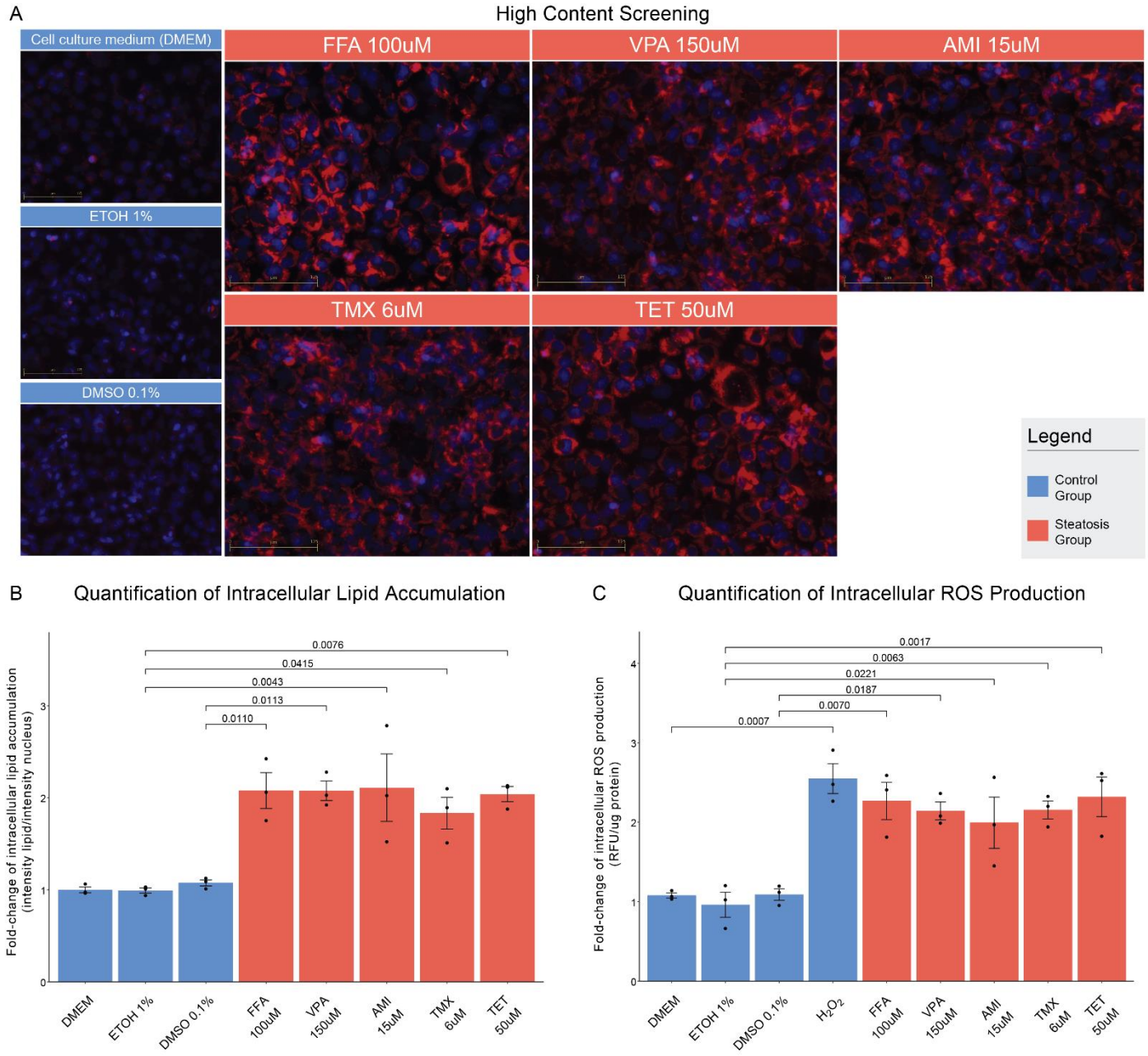


Figure 6 Formation of intracellular lipid droplets and increase of ROS production in Hep3B cells, after treatment with FFA, VPA, AMI, TMX and TET. *A) Intracellular lipid accumulation observed via HCS-based fluorescent microscopy with Nile Red staining. Hoechst33342 was used for staining the nuclei. Images were acquired under 20x optical magnification. B) Quantification of lipid accumulation via MATLAB-based image analysis. Bars represent the FC of lipid droplet intensity per cell in treated cells to the respective controls. C) FC of intracellular ROS production compared to controls. H_2O_2 was used as a positive control. (B, C) Data expressed as mean±SEM of n=3 independent experiments, and (*) denotes p-value ≤ 0.05*

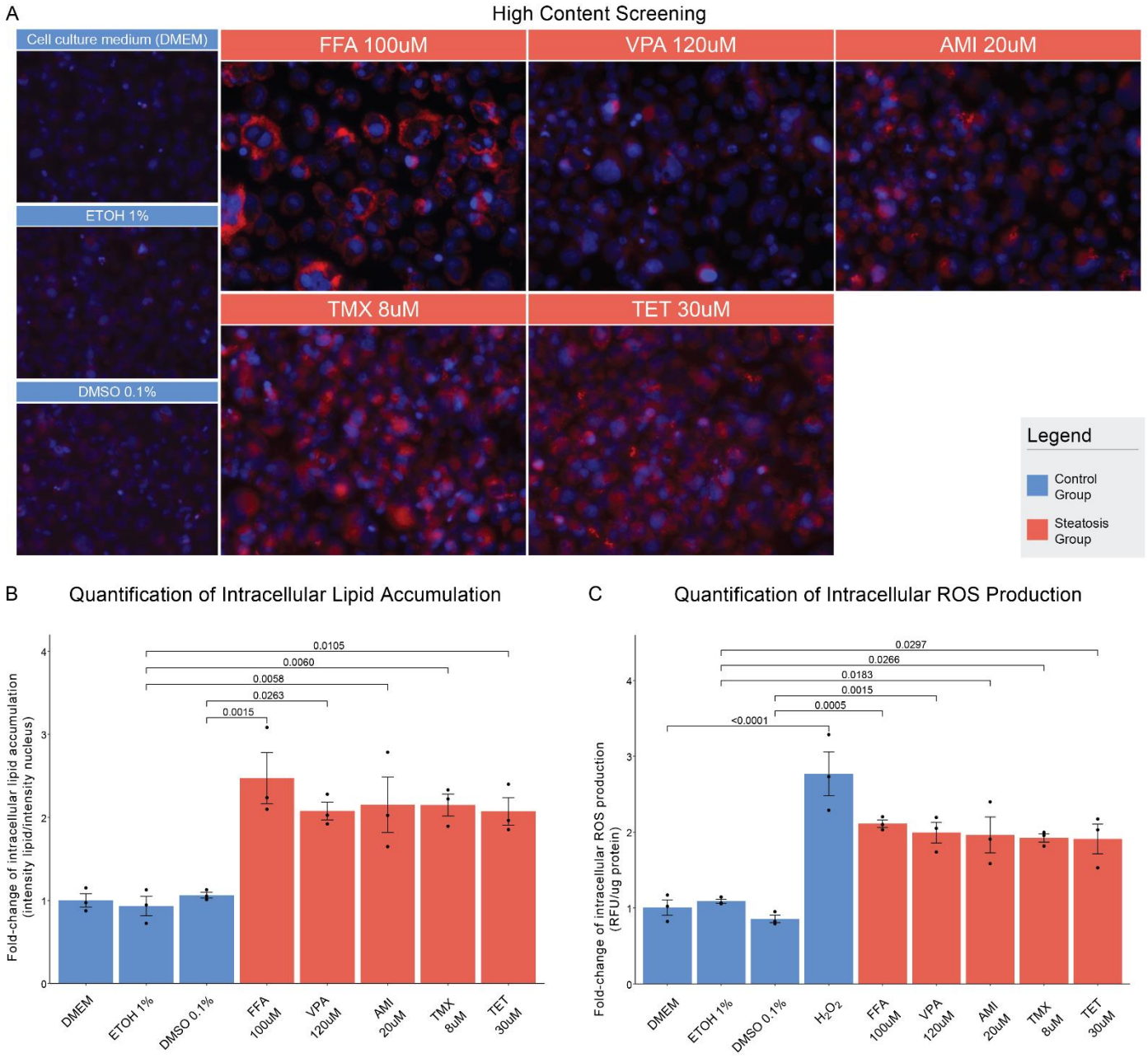


Figure 7 Formation of intracellular lipid droplets and increase of ROS production in FOCUS cells, after treatment with FFA, VPA, AMI, TMX and TET. *A) Intracellular lipid accumulation observed via HCS-based fluorescent microscopy with Nile Red staining. Hoechst33342 was used for staining the nuclei. Images were acquired under 20x optical magnification. B) Quantification of lipid accumulation via MATLAB-based image analysis. Bars represent the FC of lipid droplet intensity per cell in treated cells to the respective controls. C) FC of intracellular ROS production compared to controls. H_2O_2 was used as a positive control. (B, C) Data expressed as mean±SEM of n=3 independent experiments, and (*) denotes p-value ≤ 0.05.*

3.2. Significantly deregulated pathways in NAFL and NASH via GLS and GSA analysis on clinical data

For each dataset selected, gene-level statistics (GLS) via the limma package (Ritchie et al., 2015) revealed the statistically significant differentially expressed genes (DEGs) in the "NAFL" and "NASH" states against the "Healthy" control state. The results were input for the gene set analysis PIANO package (Väremo et al., 2013). The analysis produced significantly affected pathways in "NAFL" and "NASH" and divided them into five classes, according to their expression trend (Figure 8).

As mentioned before, pathways were classified and ranked based on the gene expression trend and p-value within a pathway, according to their given p-value.

- *Distinct up*: The genes that are overexpressed and the under-expressed genes are mutated. Overall there is a higher overexpression in the path.
- *Mixed up*: There is a high component of overexpressed genes and the component of under-expressed genes is not taken into account.
- *Non-directional*: The absolute value of the differential expression of the genes is used, as there is a high differential expression of genes, without regard to its direction.
- *Mixed down*: There is a high component of under-expressed genes and the component of overexpressed genes is not taken into account.
- *Distinct down*: The genes that are overexpressed and the under-expressed genes are mutated. Overall there is a higher under-expression in the path.
-

The significantly affected pathways formed the group of "clinical data pathways".

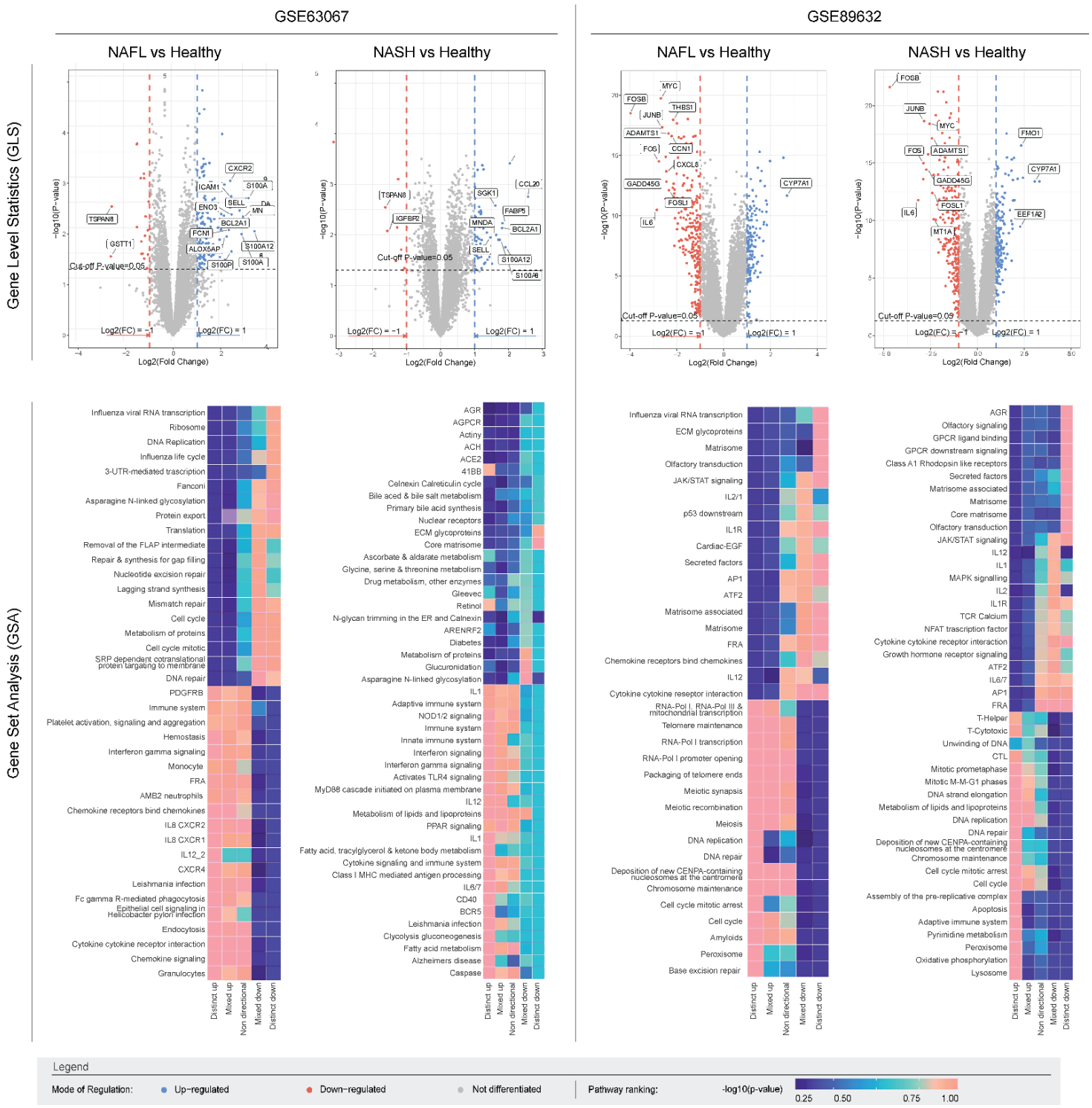


Figure 8 Gene levels statistics (GLS) and gene set analysis (GSA) from microarray gene expression data sets denoting the 15 most statistically significant differentially expressed genes and the differentially altered pathways. *The degree of differential gene expression was calculated for each sample as the logarithm of the fold-change (FC) of expression values in the diseased stages (test) over the healthy state (control). A student's t-test was used for statistical evaluation. For graphically representing these data,* $log_2 FC$ *and p-value for each gene was plotted in volcano plots. Each point on the plot corresponds*

to a gene, while the y-axis represents the negative decimal log of $p - value$ and the x-axis represents $log_2 FC$. The greater the difference of the gene on the vertical axis compared to the control group, the more statistically significant the differential expression, and the farther from zero on the horizontal axis, the greater the intensity. $|log_2 FC| > 1$ and $p - value \leq 0.05$ are used as the limits for the differential expression of a gene. Results of GLS are presented in heatmaps. Nine different statistical methods were utilized to identify the prevalent expression trend within a pathway. Based on their prevalent trend and p-value, pathways were classified and ranked into five groups, namely "distinct up", "mixed up", "non-directional", "mixed down" and "distinct down" according to their given "p-value" Each column represents one of the clusters. Each row corresponds to a pathway. Colour scale denotes statistical significance (-log10(p-value)).

3.3. Identification of pathways affected by known steatogenic compounds

The known steatogens, used *in vitro*, were integrated into the platform in a two-step manner. Firstly, they were input to the DrugBank (Wishart et al., 2006) and the GSEA-MSigDB (Liberzon et al., 2015) databases to identify their targets and the pathways affected, forming the "steatogenic compounds" pathways group (Table 4).

Drug/Compound	Gene	Pathway
Valproic acid	ACADSB	Fatty acid metabolism
		Metabolism of amino acids and derivatives
	OGDH	Metabolism of amino acids and derivatives
	PPARA	PPAR signalling
		Nuclear receptors
		Metabolism of lipids and lipoproteins
		Fatty acid, triacylglycerol and ketone body metabolism
	PPARD	PPAR signalling
		Nuclear receptors
	PPARG	PPAR signalling
		Nuclear receptors
		NFAT transcription factor
		Metabolism of lipids and lipoproteins
		Fatty acid, triacylglycerol and ketone body metabolism
Amiodarone	ADRB1	Endocytosis
	PPARG	PPAR signalling
		Nuclear receptors
		NFAT transcription factor

		Metabolism of lipids and lipoproteins
		Fatty acid, triacylglycerol and ketone body metabolism
Tetracycline	-	-
Tamoxifen	ESR1	Activating Transcription Factor 2
		Activator Protein 1
	EBP	Metabolism of lipids and lipoproteins
Oleic acid	PPARA	PPAR signalling
		Nuclear receptors
		Metabolism of lipids and lipoproteins
		Fatty acid, triacylglycerol and ketone body metabolism
	PPARD	PPAR signalling
		Nuclear receptors
	PPARG	PPAR signalling
		Nuclear receptors
		NFAT transcription factor
		Metabolism of lipids and lipoproteins
		Fatty acid, triacylglycerol and ketone body metabolism
	RXRA	PPAR signalling
		Metabolism of lipids and lipoproteins
		Fatty acid, triacylglycerol and ketone body metabolism
Palmitic acid	PPT1	Lysosome
	PPARA	PPAR signalling
		Nuclear receptors
		Metabolism of lipids and lipoproteins
		Fatty acid, triacylglycerol and ketone body metabolism

Table 4 Pathways affected that belong to the intersection of the "Clinical Data Pathway Group" and "Steatogenic Compounds Pathway Group"

3.4. Identification of repositioned compounds

The known steatogens were also introduced to the Connectivity Map (cMap) (Broad Institute) (Lamb, 2006; Subramanian et al., 2017) to identify FDA-approved compounds with similar or opposite gene signature (repositioned compounds). The DrugBank and MSigDB databases were

again used to identify the pathways that these repositioned compounds affect, to form a group of "DR compounds' pathways" (Table 5-12).

Tables presenting the drugs inferred by cMap for every "signature question'. For each steatogenic compound used as "signature question" two tables are presented, one for compounds that have opposite gene signatures and one for compounds with similar gene signatures. N represents the number of independent experiments included in cMap for each compound. Enrichment score takes values [-1,1] and is a meter of similarity of the gene expression of cell lines included in cMap after treatment with a compound. Positive values correspond to similar gene expression, whilst negative values correspond to opposite gene expression. Specificity value is a meter for evaluating the singularity of the given compound with the "signature question", the higher the value the most common the connection between the two compounds. The %Non-null value signifies the percentage of experiments for a given compound in accordance with the enrichment score.

Amiodarone

Cmap name	Mean	n	Enrichment Score (ES)	P	Specificity	% non null
Timolol	-0.510	4	-0.792	0.00376	0.0224	100
Ketoconazole	-0.408	4	-0.689	0.0213	0.0000	75
Aminoglutethimide	-0.280	3	-0.781	0.02157	0.0179	66
Fusidic acid	-0.406	4	-0.666	0.02801	0.0063	75

Table 5 Drugs with reverse gene signature compared to Amiodarone.

Cmap name	Mean	n	Enrichment Score (ES)	P	Specificity	% non null
Ethisterone	0.432	6	0.698	0.00205	0.0000	8
Meclizine	0.412	5	0.709	0.00491	0.0131	80
Omeprazole	0.379	4	0.739	0.00889	0.0642	100
Trimetazidine	0.338	4	0.720	0.01243	0.0078	100
Prednisone	0.282	5	0.607	0.02842	0.0526	80
Amiloride	0.340	5	0.597	0.03276	0.0167	80
Ursodeoxycholic acid	0.390	3	0.725	0.04054	0.0517	100

Table 6 Drugs with similar gene signature compared to Amiodarone.

Cmap name	Mean	n	Enrichment Score (ES)	P	Specificity	% non null
Estropipate	-0.374	4	-0.816	0.00209	0.0000	50
Physostigmine	-0.391	4	-0.804	0.00284	0.0000	75
Cefmetazole	-0.312	4	-0.708	0.01514	0.0142	50
Carbimazole	-0.396	3	-0.792	0.01827	0.0480	66
Sulindac	-0.342	7	-0.524	0.02499	0.0388	57
Diflorasone	-0.376	4	-0.668	0.02731	0.0186	50

Table 7 Drugs with reverse gene signature compared to Tamoxifen.

Cmap name	Mean	n	Enrichment Score (ES)	P	Specificity	% non null
Mefloquine	0.476	5	0.933	0.00000	0.0098	100
Sirolimus	0.215	44	0.330	0.00012	0.2590	65
Acepromazine	0.360	4	0.810	0.00241	0.0074	100
Disulfiram	0.357	5	0.709	0.00495	0.0694	80
Clomifene	0.415	4	0.748	0.00778	0.0517	100
Ivermectin	0.321	5	0.651	0.01442	0.1075	80
Clomipramine	0.410	4	0.705	0.01578	0.1105	100
Albendazole	0.390	3	0.793	0.01815	0.0116	100
Rimexolone	0.333	4	0.689	0.01950	0.0840	100
Gallamine triethiodide	0.314	5	0.616	0.02453	0.0081	80
Clotrimazole	0.263	5	0.597	0.03258	0.1333	80
Pimozide	0.325	4	0.642	0.03995	0.1708	75
Fenoterol	0.326	3	0.723	0.04202	0.0692	100
Isoetarine	0.230	4	0.637	0.04227	0.0400	75
Progesterone	0.282	4	0.632	0.04579	0.0307	75
Mifepristone	0.241	4	0.629	0.04768	0.0584	75

Table 8 Drugs with similar gene signature compared to Tamoxifen.

Tetracycline

Cmap name	Mean	n	Enrichment Score (ES)	P	Specificity	% non null
Raloxifen	-0.380	7	-0.519	0.02697	0.0520	57
Oxprenolol	-0.502	4	-0.650	0.03529	0.0594	75
Dipivefrin	-0.519	4	-0.641	0.03965	0.0526	75
Metoprolol	-0.417	4	-0.624	0.04953	0.0534	75

Table 9 Drugs with reverse gene signature compared to Tetracycline.

Cmap name	Mean	n	Enrichment Score (ES)	P	Specificity	% non null
Aceclofenac	0.720	4	0.727	0.01108	0.0137	75
Cefotaxime	0.675	5	0.592	0.03475	0.0136	80
Cortisone	0.701	3	0.738	0.03523	0.0826	100

Table 10 Drugs with similar gene signature compared to Tetracycline.

Valproic acid

Cmap name	Mean	n	Enrichment Score (ES)	P	Specificity	% non null
Naftifine	-0.558	4	-0.698	0.01751	0.0196	75
Ketoprofen	-0.370	6	-0.525	0.04660	0.0385	50
Pralidoxime	-0.320	4	-0.625	0.04864	0.0526	75

Table 11 Drugs with reverse gene signature compared to Valproic acid.

Cmap name	Mean	n	Enrichment Score (ES)	P	Specificity
Resveratrol	0.294	9	0.655	0.00026	0.1226
Nabumetone	0.267	4	0.722	0.01182	0.0130
Mepacrine	0.330	2	0.860	0.03941	0.0677

Table 12 Drugs with similar gene signature compared to Valproic acid.

3.5. Eleven (11) pathways-to-target and 46 compounds were identified based on network similarity between the clinical and *in vitro* data

The comparison between the "clinical data pathways" and the pathways targeted by the steatogenic compounds used *in vitro*, or the "steatogenic compounds' pathways", revealed 11 significant deregulated pathways in NAFL/NASH, hence the pathways-to-target (Figure 9). Namely, these pathways were: the nuclear-receptors pathway (BIOCARTA, M16393), the endocytosis pathway (KEGG, ko04144), the PPAR-signalling pathway (KEGG, HSA-03320), the fatty acid metabolism pathway (KEGG, map01212), the activator-protein-1 pathway (PID, M167), the activating-transcription factor 2 pathway (PID, M166), the NFAT transcription factor pathway (PID, M60), the fatty acid triacylglycerol and ketone body metabolism pathway (REACTOME, R-HSA-188467), the metabolism of amino acids and derivatives pathway (REACTOME, R-HSA-71291) and the metabolism of lipids and lipoproteins pathway (REACTOME, R-HSA-556833) (Fabregat et al., 2018; Jassal et al., 2020; Kanehisa and Goto, 2000; Mootha et al., 2003; Nishimura, 2004; Schaefer et al., 2009). For both datasets, volcano plots, provided in Supplementary Figure S5, depict the DEGs in the identified pathways-to-target.

Approximately 20'000 approved and experimental compounds were screened *in silico* for their ability to affect the aforementioned pathways. Forty-six (46) compounds were found to interfere with both the "DR compounds' pathways" and the identified pathways-to-target and were therefore proposed as promising for the treatment of hepatic steatosis. Figure 9 illustrates the repositioned compounds, as well as the genes and pathways affected by these compounds. Details can be found in Table 13.

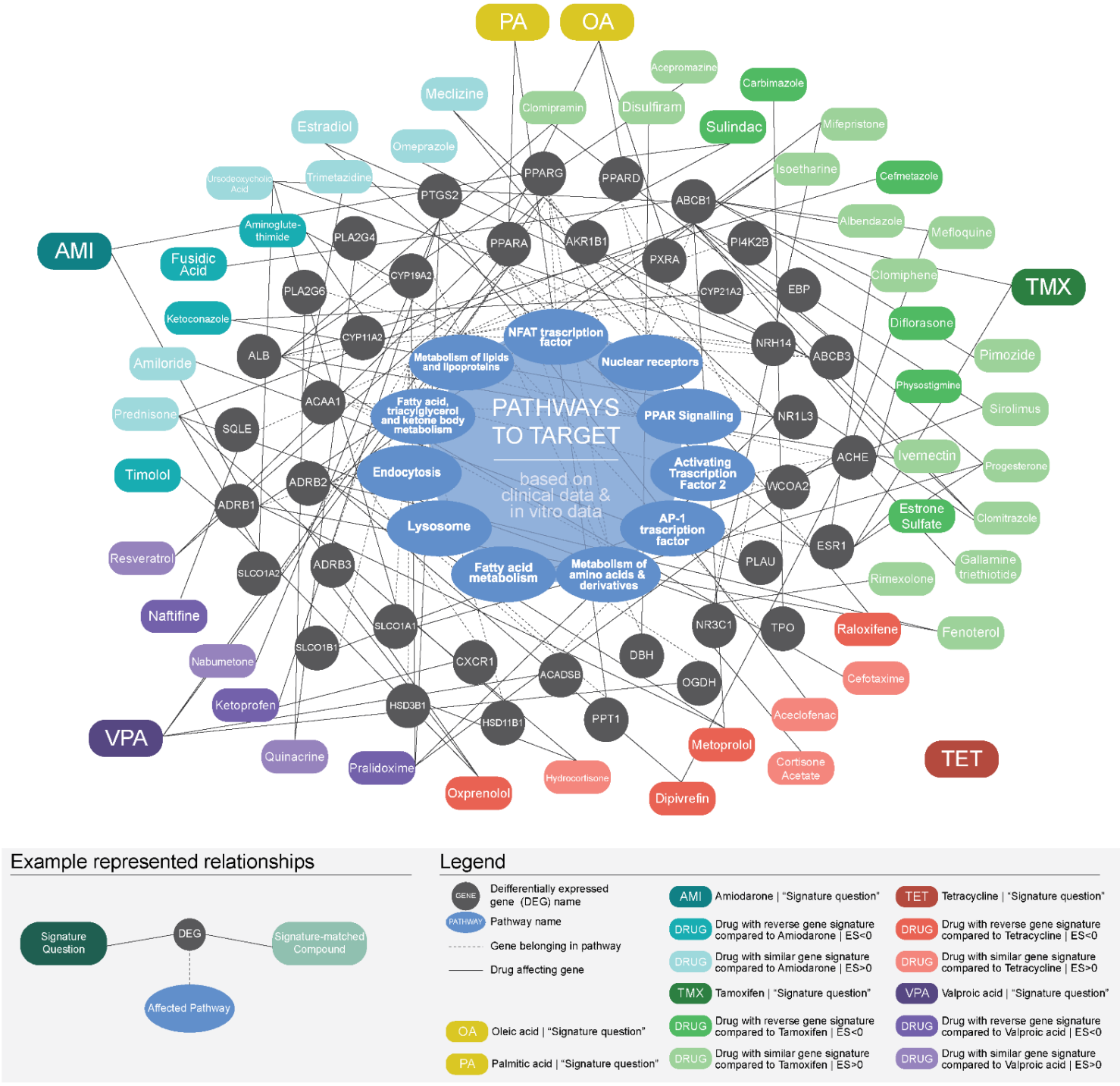


Figure 9 Network representation of genes and pathways affected by the steatosis-inducing compounds used in vitro and the compounds proposed by the DR platform. *The target-pathways depicted in blue in the centre of the graph belong at the intersection of the "Clinical Data Pathway Group" and "Steatogenic Compounds Pathway Group". The differentially expressed genes (DEGs) and the pathways affected by each compound are illustrated in grey circles. Each steatosis-inducing compound was used as a "signature question" to cMap. The cMap tool compares two-sample distributions using the Kolmogorov-Smirnov (K-S) statistical test and calculates an Enrichment Score that takes values in the interval [-1,1]. ES>0 signifies that two drugs present*

similar gene signature, while ES<0 means that two drugs have reverse gene signatures. The steatosis-inducing compounds are depicted in rectangles of different colours. Every steatosis-inducing compound drugs with ES>0 or ES<0 is illustrated with rectangles of the same colour scale. Lines connect each compound and drug with their target-genes and the pathways they affect.

#	Drug/Compound	Gene	Pathway
1	**Naftifine**	SQLE	Metabolism of lipids and lipoproteins
2	**Ketoprofen**	PTGS2	NFAT transcription factor
		CXCR1	Endocytosis
3	**Pralidoxime**	ACHE	Activating Transcription Factor 2
			Metabolism of lipids and lipoproteins
4	**Resveratrol**	PTGS2	NFAT transcription factor
		PI4K2B	Metabolism of lipids and lipoproteins
		NR1L3	Nuclear receptors
		PPARA	PPAR signalling
			Nuclear receptors
			Metabolism of lipids and lipoproteins
			Fatty acid, triacylglycerol and ketone body metabolism
		PPARG	PPAR signalling
			Nuclear receptors
			NFAT transcription factor
			Metabolism of lipids and lipoproteins
			Fatty acid, triacylglycerol and ketone body metabolism
5	**Nabumetone**	PTGS2	NFAT transcription factor
6	**Quinacrine**	PLA2G6	Metabolism of lipids and lipoproteins
		PLA2G4	
7	**Timolol**	ADRB1	Endocytosis
8	**Ketoconazole**	CYP21A2	Metabolism of lipids and lipoproteins
		NR1L3	Nuclear receptors
9	**Aminoglutethimide**	CYP19A1	Metabolism of lipids and lipoproteins
		CYP11A1	
10	**Fusidic acid**	ABCB11	Nuclear receptors
			Metabolism of lipids and lipoproteins
11	**Meclizine**	NR1L3	Nuclear receptors
12	**Estradiol**	ESR1	Activating Transcription Factor 2
			Activator Protein 1
		WCOA2	Metabolism of lipids and lipoproteins
			Fatty acid, triacylglycerol and ketone body metabolism
13	**Omeprazole**	ABCB3	Nuclear receptors
			Metabolism of lipids and lipoproteins

14	**Trimetazidine**	ACAA1	PPAR signalling
			Metabolism of lipids and lipoproteins
			Fatty acid, triacylglycerol and ketone body metabolism
15	**Prednisone**	NR3C1	Activator Protein 1
		HSD11B1	Metabolism of lipids and lipoproteins
		ABCB1	Nuclear receptors
		SLCOA2	Metabolism of lipids and lipoproteins
16	**Amiloride**	PLAU	Activator Protein 1
			Activating Transcription Factor 2
17	**Ursodeoxycholic acid**	ABCB1	Nuclear receptors
			Metabolism of lipids and lipoproteins
		SLCO1A1	Metabolism of lipids and lipoproteins
		SLCO1A2	
		NR1H4	Nuclear receptors
18	**Raloxifene**	ESR1	Activating Transcription Factor 2
			Activator Protein 1
19	**Oxprenolol**	ADRB1	Endocytosis
		ADRB2	
		ADRB3	
20	**Mifepristone**	NR3C1	Activator Protein 1
		ABCB1	Nuclear receptors
21	**Fenoterol**	ADRB1	Endocytosis
		ADRB2	
22	**Dipivrefin**	ADRB2	Endocytosis
		ACHE	Activating Transcription Factor 2
			Metabolism of lipids and lipoproteins
23	**Metoprolol**	ADRB1	Endocytosis
		ADRB2	Endocytosis
		ABCB1	Nuclear receptors
			Metabolism of lipids and lipoproteins
24	**Aceclofenac**	PTGS2	NFAT transcription factor
25	**Cefotaxime**	ALB	Metabolism of lipids and lipoproteins
26	**Hydrocortisone**	HSD3B1	Metabolism of lipids and lipoproteins
		SLCO1A2	
27	**Cortisone acetate**	NR3C1	Activator Protein 1
28	**Estrone sulfate**	ESR1	Activating Transcription Factor 2
			Activator Protein 1
29	**Physostigmine**	ACHE	Activating Transcription Factor 2
			Metabolism of lipids and lipoproteins
30	**Cefmetazole**	ALB	Metabolism of lipids and lipoproteins

	Carbimazole	TPO	Metabolism of amino acids and derivatives
31	**Sulindac**	AKR1B1	Metabolism of lipids and lipoproteins
		PPARD	PPAR signalling
			Nuclear receptors
32	**Diflorasone**	NR3C1	Activator Protein 1
33	**Mefloquine**	ACHE	Activating Transcription Factor 2
			Metabolism of lipids and lipoproteins
		ABCB1	Nuclear receptors
			Metabolism of lipids and lipoproteins
34	**Sirolimus**	SLCO1B1	Metabolism of lipids and lipoproteins
		ABCB1	Nuclear receptors
			Metabolism of lipids and lipoproteins
35	**Acepromazine**	ALB	Metabolism of lipids and lipoproteins
36	**Disulfiram**	DBH	Metabolism of lipids and lipoproteins
37	**Clomifene**	ESR1	Activating Transcription Factor 2
			Activator Protein 1
		ABCB1	Nuclear receptors
			Metabolism of lipids and lipoproteins
38	**Ivermectin**	ABCB1	Nuclear receptors
			Metabolism of lipids and lipoproteins
39	**Clomipramin**	ALB	Metabolism of lipids and lipoproteins
		ABCB1	Nuclear receptors
			Metabolism of lipids and lipoproteins
40	**Albendazole**	ABCB1	Nuclear receptors
			Metabolism of lipids and lipoproteins
41	**Rimexolone**	NR3C1	Activator Protein 1
42	**Gallamine triethiotide**	ACHE	Activating Transcription Factor 2
			Metabolism of lipids and lipoproteins
43	**Pimozide**	ABCB1	Nuclear receptors
			Metabolism of lipids and lipoproteins
44	**Clotrimazole**	NR1L3	Nuclear receptors
		ABCB1	Nuclear receptors
			Metabolism of lipids and lipoproteins
		ABCB3	Nuclear receptors
			Metabolism of lipids and lipoproteins
45	**Progesterone**	ESR1	Activating Transcription Factor 2
			Activator Protein 1
		ABCB1	Nuclear receptors
			Metabolism of lipids and lipoproteins
		SLC10A1	Metabolism of lipids and lipoproteins

46	**Isoetharine**	ADRB1 ADRB2	Endocytosis

Table 13 The target-genes and affected pathways significantly differentiated in NAFLD/NASH, as proposed by the DR platform, of the repositioned compounds.

3.6. Experimental validation of candidate compounds resulting from *in silico* selection revealed 6 with anti-steatotic effect

The 46 candidate compounds were reviewed on the ToxDB and the LiverTox databases. Twenty-five (25) were documented to be hepatotoxic or to induce steatosis *in vitro* or *in vivo* and were, thus, eliminated. The remaining 21 moved onto screening to investigate their capacity to reduce steatosis and oxidative stress *in vitro*. Those were: Acepromazine, Cefmetazole, Clomifene, Diflorasone, Estradiol, Estrone sulfate, Fenoterol, Fusidic acid, Gallamine triethiotide, Ivermectin, Mefloquine, Naftifine, Pimozide, Pralidoxime, Quinacrine, Raloxifene, Resveratrol, Rimexolone, Sirolimus (or Rapamycin) and Timolol.

High-content screening of the 21 compounds was performed on HepG2 cells. Verified compounds would demonstrate the capacity to reduce intracellular lipid accumulation and ROS production in steatotic cell cultures. Pimozide (PIM), Clomifene (CLO) and Mefloquine (MEF) led to a significant increase (p-value<0.05) in the cells' intracellular lipid loading and ROS production (Figure 11). Conversely, HepG2 cells, co-treated with FFAs 200uM and either Sirolimus (SIR), Resveratrol (RES), Diflorasone (DIF), Fenoterol (FEN), Pralidoxime (PRA) or Gallamine triethiotide (GAL) were found to effectively ameliorate the steatotic phenotype at 10uM, while none reduced cell viability below 80%. In detail, all six co-treatments led to a significant (p-value<0.05) reduction in lipid loading and oxidative stress, when compared to the FFA-treated control (Figure 10). Equally important, lipid loading and oxidative stress were not significantly increased in cells treated solely with the six DR compounds (Figure 12).

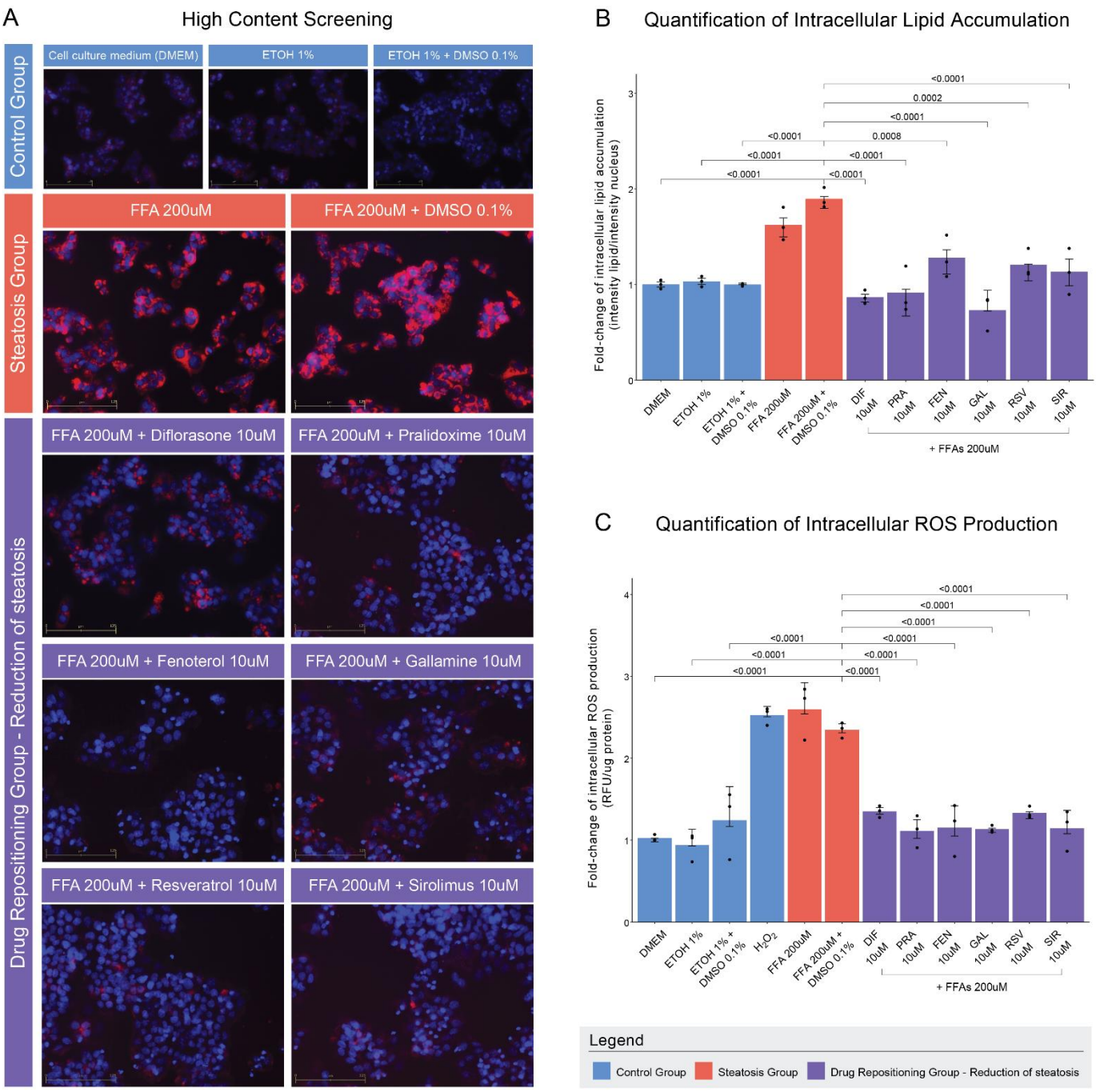


Figure 10 Reduction of intracellular lipid accumulation and ROS production in HepG2 cells, after treatment with the repositioned compounds. *A) Intracellular lipid accumulation observed via HCS-based fluorescent microscopy with Nile Red staining. Hoechst33342 was used for staining the nuclei. Images were acquired under 20x optical magnification. B) Quantification of lipid accumulation via MATLAB-based image analysis. Bars represent the FC of lipid droplet intensity per cell in treated cells over respective controls. C) FC of intracellular ROS production compared to controls. H_2O_2 was used as a positive control. (B, C) Data expressed as mean±SEM of n=3 independent experiments, and the p-value is denoted by brackets.*

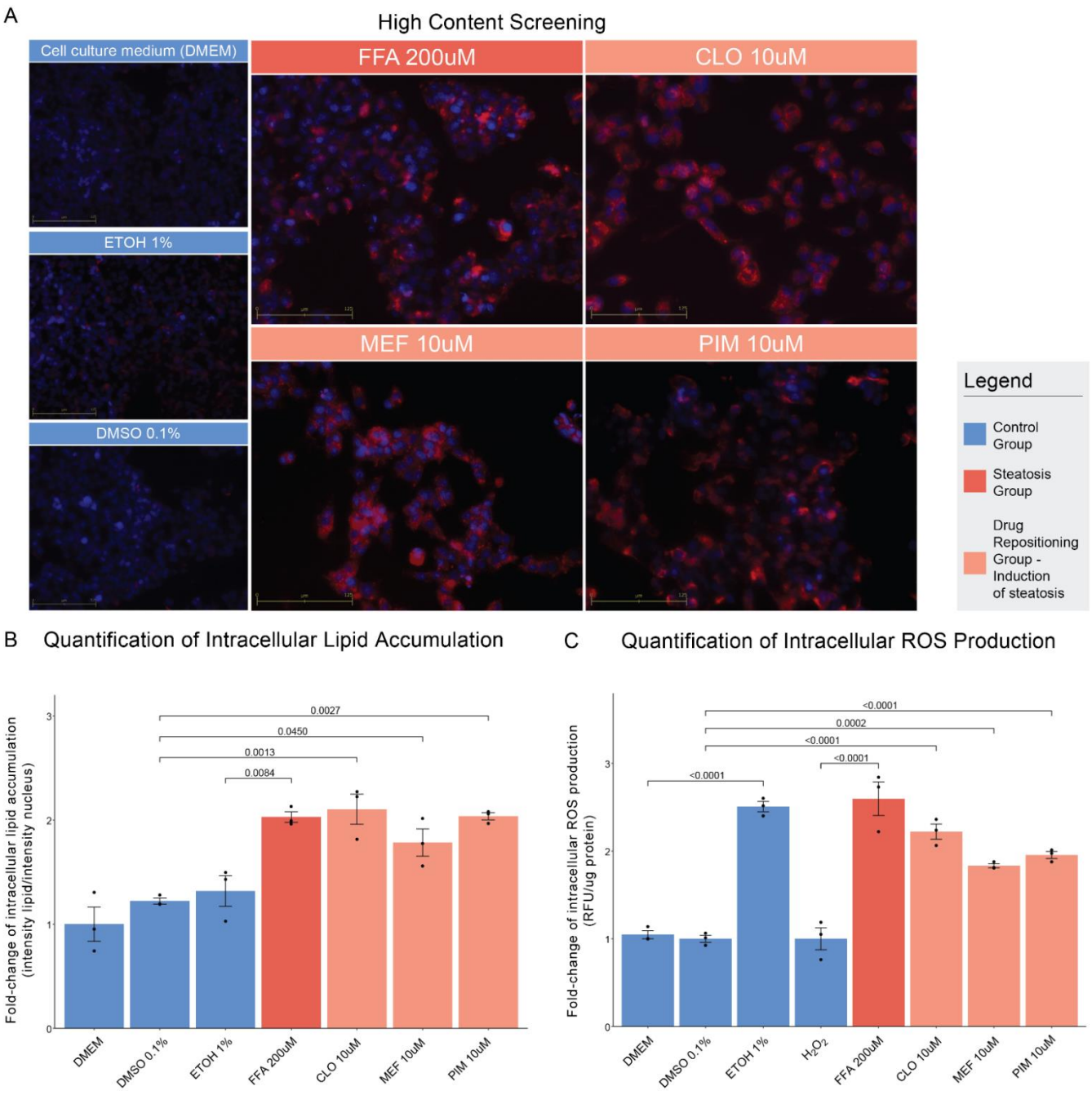


Figure 11 Formation of intracellular lipid droplets and increase of ROS production in HepG2 cells, after treatment with FFA, CLO, MEF and PIM. *A) Intracellular lipid accumulation observed via HCS-based fluorescent microscopy with Nile Red staining. Hoechst33342 was used for staining the nuclei. Images were acquired under 20x optical magnification. B) Quantification of lipid accumulation via MATLAB-based image analysis. Bars represent the FC of lipid droplet intensity per cell in treated cells over respective controls. C) FC of intracellular ROS production compared to controls. H_2O_2 was used as a positive control. (B, C) Data expressed as mean±SEM of n=3 independent experiments, and the p-value is denoted by brackets.*

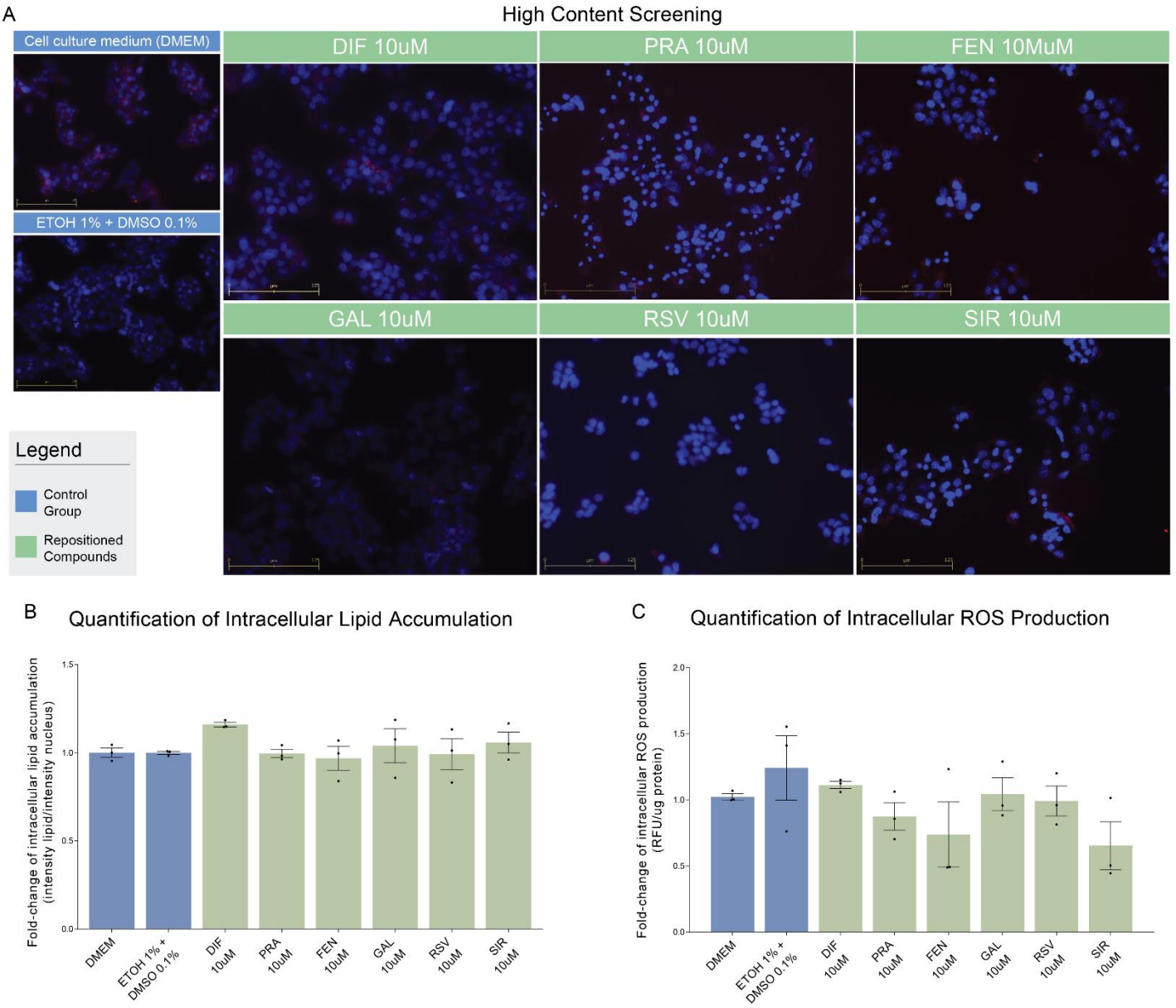


Figure 12 Treatment with the repositioned compounds alone does not increase intracellular lipid accumulation and oxidative stress. *A) Intracellular lipid accumulation observed via HCS-based fluorescent microscopy with Nile Red staining. Hoechst33342 was used for staining the nuclei. Images were acquired under 20x optical magnification. B) Quantification of lipid accumulation via MATLAB-based image analysis. Bars represent the FC of lipid droplet intensity per cell in treated cells to the respective controls. C) FC of intracellular ROS production compared to controls. H_2O_2 was used as a positive control. (B, C) Data expressed as mean±SEM of n=3 independent experiments.*

3.7. Signalling-based clustering reveals the anti-steatotic efficacy of repositioned compounds

The measurements on the phospho-protein and cytokine-release levels are summarized in Figure 13A. All compounds were investigated for their shared motifs at the protein signalling level.

Principal Component Analysis (PCA) on the whole of the experimental data, followed by k-means clustering (Figure 13B), revealed 4 clusters, distinguished by their anti-steatogenic mode of action. All of the negative controls (DMEM, ETOH 1%, ETOH 1% and DMSO 0.1%) were appropriately clustered together ("negative control" cluster). All FFA-treated samples (FFA 200uM, FFA 200uM and DMSO 0.1%) were grouped into a second, "steatosis" cluster. Sole administration of DIF, GAL, PRA and FEN fell into a third distinct cluster. Importantly all the rest of the DR co-treatments, except for DIF, were grouped with SIR+FFAs, as well as RSV+FFAs; the DR compounds that have been already proven effective *in vitro* or *in vivo* for the treatment of NAFL. The DIF+FFAs co-treatment failed to cluster with the DR candidates and was clustered into the steatosis cluster.

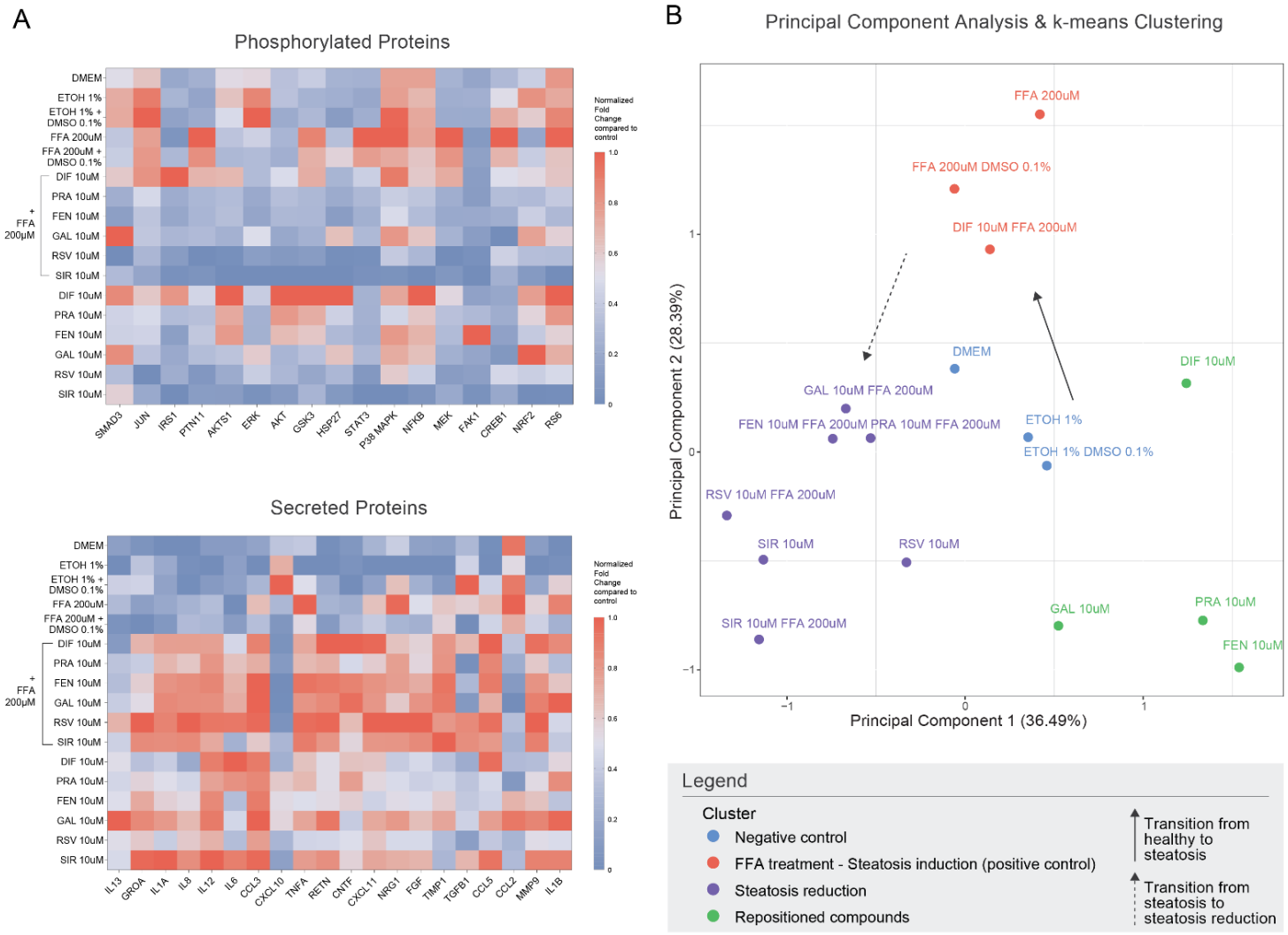


Figure 13 Proteomic profiling of the effect of the compounds. *(A) Heatmap of the normalized fold change of phosphorylated proteins and secreted cytokines compared to controls. Each column corresponds to a protein and each row to a cell treatment. Data in each column were fraction-normalized to [0-1] scale*

for better representation. The colour scale represents the value of normalized fold change compared to the control. (B) PCA and k-means clustering was performed on proteomic, lipid accumulation and intracellular ROS production data. The axes denote the first two principal components and the percentage of variability they contribute. k-means was used to deduce the clusters of the resulting profile of the compound treatment. The 4 different clusters formed are denoted with different colours and were named after the majority of treatments included. The solid arrow represents the transition from control to steatosis state, whereas the dashed arrow represents the transition from steatosis to an amelioration of steatosis state.

3.8. Statistical analysis of the signalling proteins' expression

Bar graphs of phospho-proteomic and cytokine release measurements per repositioned compound and the results of the statistical analysis conducted are summarized in Figures 14-25. Fold change of the Median Fluorescent Intensity (MFI) of each treatment to the respective controls was calculated and results were analyzed using regular two-way ANOVA with Tukey's multiple comparisons test.

Significant changes in phosphorylation levels of STAT3, PTN11 and CREB1 were observed across all treatments. pPTN11 and pSTAT3 were significantly increased in both FFAs treatments compared to the control group (FC>2.00). pCREB1 was found significantly upregulated in FFA treatment, but not in FFAs + DMSO treatment, although in both cases FC was higher than 2.00. Contrary, co-treatment of the cells with FFAs and the DR compounds, and the DR compounds alone, led to a significant reduction of the phosphorylation levels of these two proteins when compared to the steatosis group. In this case, pSTAT3 and pPTN11 levels were similar (FC≈1.00) to the control group, whereas pCREB1 was reduced up to 0.5fold (p-value<0.05). Regarding pCREB1, it should be noted that RSV was the only compound that both in co-treatment or when administered alone reduced the FC to ≈1.00. A similar phosphorylation pattern was observed for MEK in all compound treatments. Treatment with FFAs led to an over 1.5fold increase in the phosphorylation of MEK (p-value<0.05), while co-treatment of FFAs with the DR compounds and treatment with the DR compounds alone, significantly reduced the FC to FC≈1.00.

Levels of pJUN were also increased in FFA treated cells compared to the respective negative controls (FC≈1.20). Treatment with DIF and FEN, as well as co-treatment with those compounds and FFAs led to a significant reduction in levels of those proteins compared to both FFAs+DMSO and the corresponding negative control samples (FC≈0.60). GAL and PRA led to a significant reduction only when in co-treatment with FFAs (FC≈0.75). Differing, a significant reduction to FC≈0.50 was observed when SIR was administered alone, and although a trend in reduction was observed in co-treatment with FFAs, it was not found to be of statistical significance.

pGSK3 has likewise presented a trend in the increase in the FFAs-treated group although it was not found to be statistically significant in the comparisons made. Nonetheless, co-treatment with FFAs and either FEN, GAL or PRA led to a significant reduction in pGSK3 levels compared to the respective FFA-treated samples. Interestingly only FEN was found to reduce pSGK3 levels when administered alone with a p-value<0.05.

On the contrary, FFAs+DMSO treatment significantly reduced phosphorylation levels of RS6 and ERK (FC≈0.70). As to pRS6, co-treatment with FFAs with DIF and SIR further reduced its phosphorylation levels (p-value<0.05) compared to FFA-DMSO and control, FC≈0.50), which was also observed in cells treated with DIF and SIR alone. FEN also led to a significant reduction of pRS6 when in co-treatment with FFAs, but not when administered alone. GAL and RSV did not demonstrate any effect at RS6 phosphorylation levels in this experimental setting. pERK was also reduced in cells co-treated with FFAs and DIF and SIR (p-value<0.05 compared to FFA-DMSO and control group, FC≈0.50), while no reduction resulted from co-treatment with FEN, GAL, PRA and RSV. Treatment with SIR and DIF alone showed a greater reduction of pERK compared to FFA-DMSO.

Phosphorylated AKT and AKTS1 were likewise reduced in FFA-DMSO treated HepG2 cells (FC≈0.75). Regarding pAKTS1, the same statistically significant reduction was observed in co-treatments with all the repurposed compounds, whereas co-treatment with FFAs and FEN further reduced its phosphorylation levels (p-value<0.05). These changes were found significant in comparison with both the control group and the FFA treated cells. Co-treatment with PRA also led to pAKTS1 reduction, but when administered alone no significant change to FFA-DMSO treatment or the negative controls was observed. Furthermore, FEN, GAL and RSV led to a significant reduction of AKT phosphorylation levels compared to FFA-DMSO treated cells and their respective negative controls. Contrary, GAL and SIR appeared to not affect pAKT (FC≈1.00), whereas co-treatment with RSV+FFAs and RSV alone led to a significant increase in pAKT (FC≈1.50 and FC≈2.00 respectively)

Although pNFKB, pFAK1, pP38-MAPK and pSMAD3 were not significantly reduced in FFA-treated cells, co-treatment with re repurposed compounds led to alterations in the phosphorylation patterns. Co-treatment with DIF, FEN and SIR reduced pFAK1 with p-value<0.05 both compared to the control group. Interestingly, SIR significantly reduced pFAK1 in both co-treatment with FFAs and when administered alone compared to FFAs+DMSO and the negative control group. Likewise, cells treated with PRA, FEN and FFAs presented lower pNFKB with p-value<0.05 compared to the negative control group and the FFA-DMSO treated cells. SIR also significantly reduced NFKB levels

compared to FFA_DMSO treatment and the respective controls. It should be noted that only DIF and SIR alone significantly reduced pNFKB levels compared to the negative control group. SIR was also reduced by 0.5fold pP38-MAPK with p-value<0.05, though in co-treatment FC remained close to 1.00. Concerning pSMAD3 levels, they were significantly altered to FC≈1.2 only when FEN was administered with FFAs.

Focusing on secreted cytokines, a limited number of fluctuations was observed overall. Treatment with FFAs+DMSO demonstrated a trend in the increase of IL12, and a slight decrease in CCL2 and IL8 though no change was statistically significant.

Co-treatment with FFAs and either RSV, SIR, FEN or GAL led to a significant decrease in CCL2 secretion levels compared to FFA-treated cells but reaching the same levels of FC as FFAs+DMSO treated cells (FC≈0.75). It should be noted that the reduction observed in co-treatment with SIR was found statistically significant when compared to the etOH+DMSO treatment.

Secretion of CCL3 on the other hand was found significantly increased in co-treatment with DIF, FEN or SIF and FFAs compared to both the FFA+DMSO-treated cells and the respective negative control. GAL's secretion was also significantly increased when in co-treatment with FFAs but only compared to the negative control. Conversely, PRA and RSV led to an increase in CCL3 secretion only when administered alone.

Another significant fluctuation across treatments with all the repositioned compounds was observed in IL12. The increase of IL12 in treatmeant with FFAs and DMSO was found significant in most of the comparisons made. Co-treatment with FFAs and RSV, GAL DIF or SIR significantly increased IL12 (FC≈1.5) compared to the negative control samples, though co-treatment with SIR was found significant and in comparison with FFAs and DMSO-treated cells. In general treatment with all six compounds alone significantly increased IL12 secretion compared to the negative control.

On the contrary, although DIF or FEN alone led to a significant decrease in treatment alone, they were found to increase IL8 secretion when in co-treatment with FFAs+DMSO compared to the corresponding controls. GAL and PRA followed the same reduction pattern administered alone, but the levels were increased to those of FFA-treated samples in co-treatment. Finally, IL8 was significantly increased in treatment with SIR and FFAs only compared to its negative control.

Regarding the less altered secreted proteins, GROA, although not altered in the FFA-treatment group, was found increased in co-treatment with DIF and further increased when administered alone compared to FFAs and DMSO treated cells. Treatment with FEN, GAL and RSV alone also

increased GROA secretion compared to the negative controls, reaching an FC≈1.50 in the case of RSV. MMP9 was also increased in co-treatment of FFAs with DIF and SIR compared to etOH+DMSO-treated cells. SIR alone also increased MMP9 secretion. The same pattern was observed with RETN and FGF. RETN was found increased by GAL ad SIR alone compared to the respective negative control, as well as in co-treatment with FFAs and SIR that was observed as significant in comparison with both the negative controls and the FFA-treatment group. Secretion levels of FGF were likewise increased by RSV and SIR alone while co-treatment with SIR was significant compared only to the negative control group. Finally, SIR also increased NRG1 secretion.

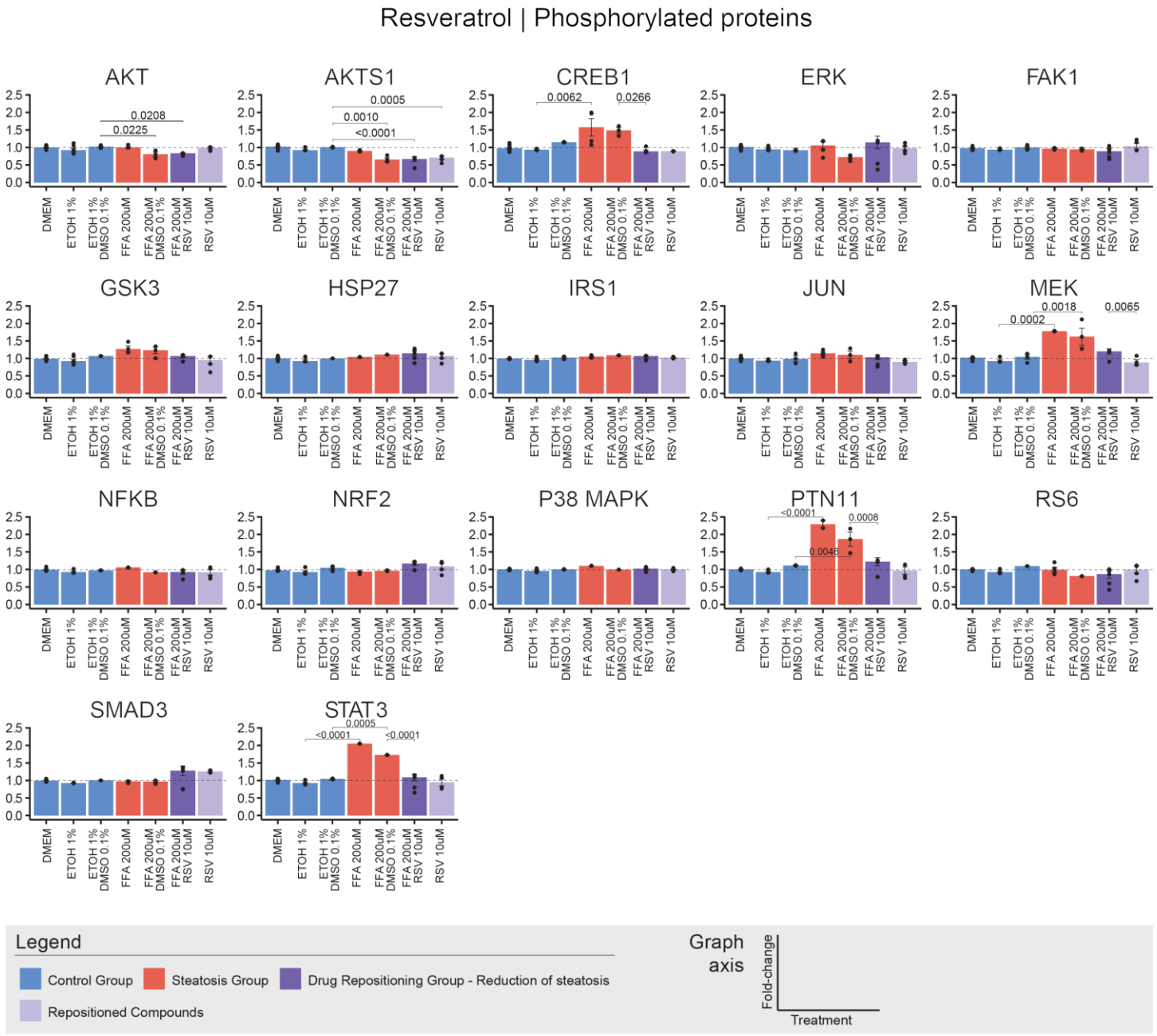


Figure 14 Statistical analysis of the effect of Resveratrol on the phosphorylated proteins measured. *Bars represent the FC of median fluorescent intensity per protein in treated cells over respective controls. Data expressed as mean±SEM of at least n=3 independent experiments, and the p-value is denoted by brackets.*

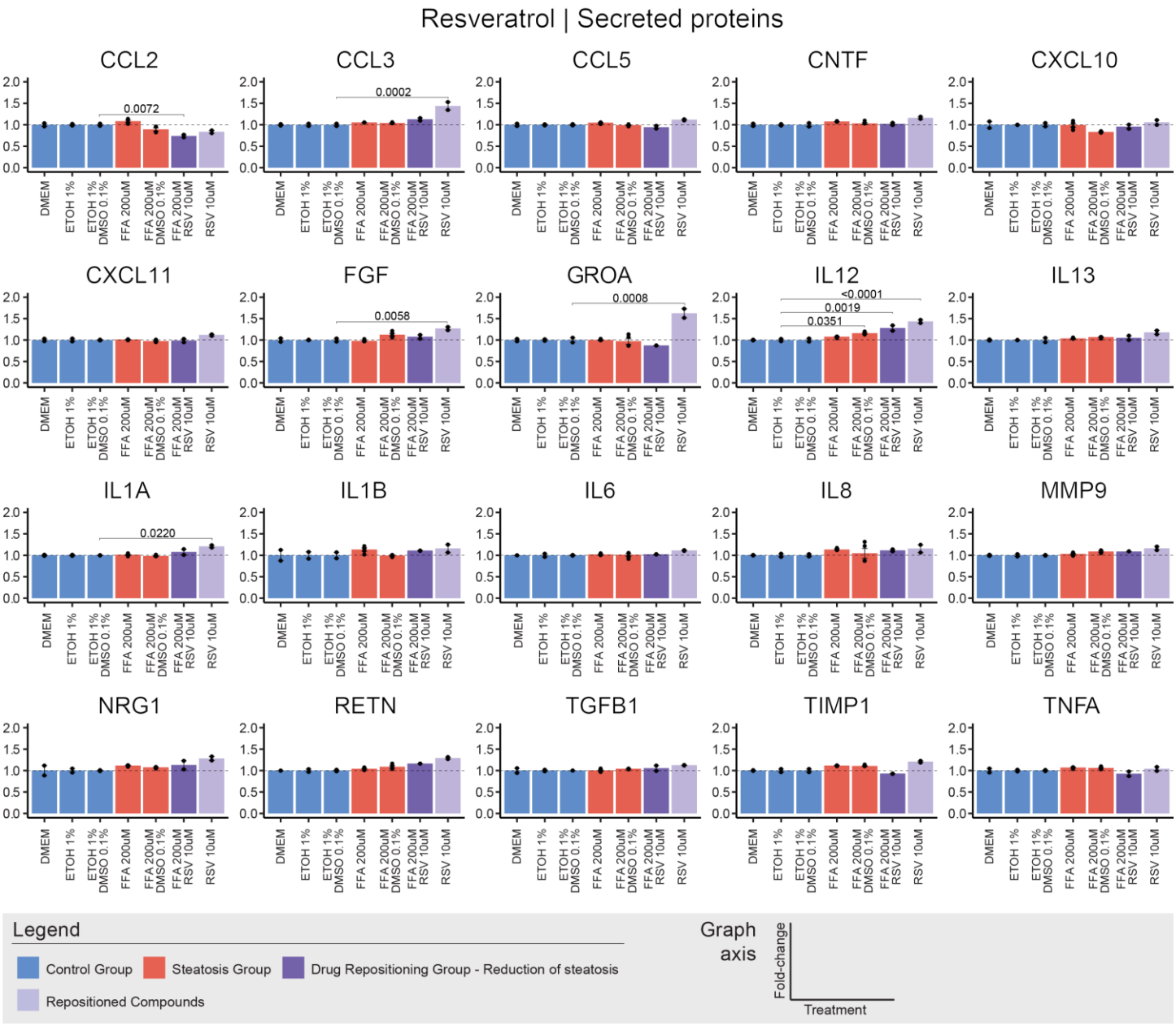


Figure 15 Statistical analysis of the effect of Resveratrol on the secreted proteins measured. *Bars represent the FC of median fluorescent intensity per protein in treated cells over respective controls. Data expressed as mean±SEM of at least n=3 independent experiments, and the p-value is denoted by brackets.*

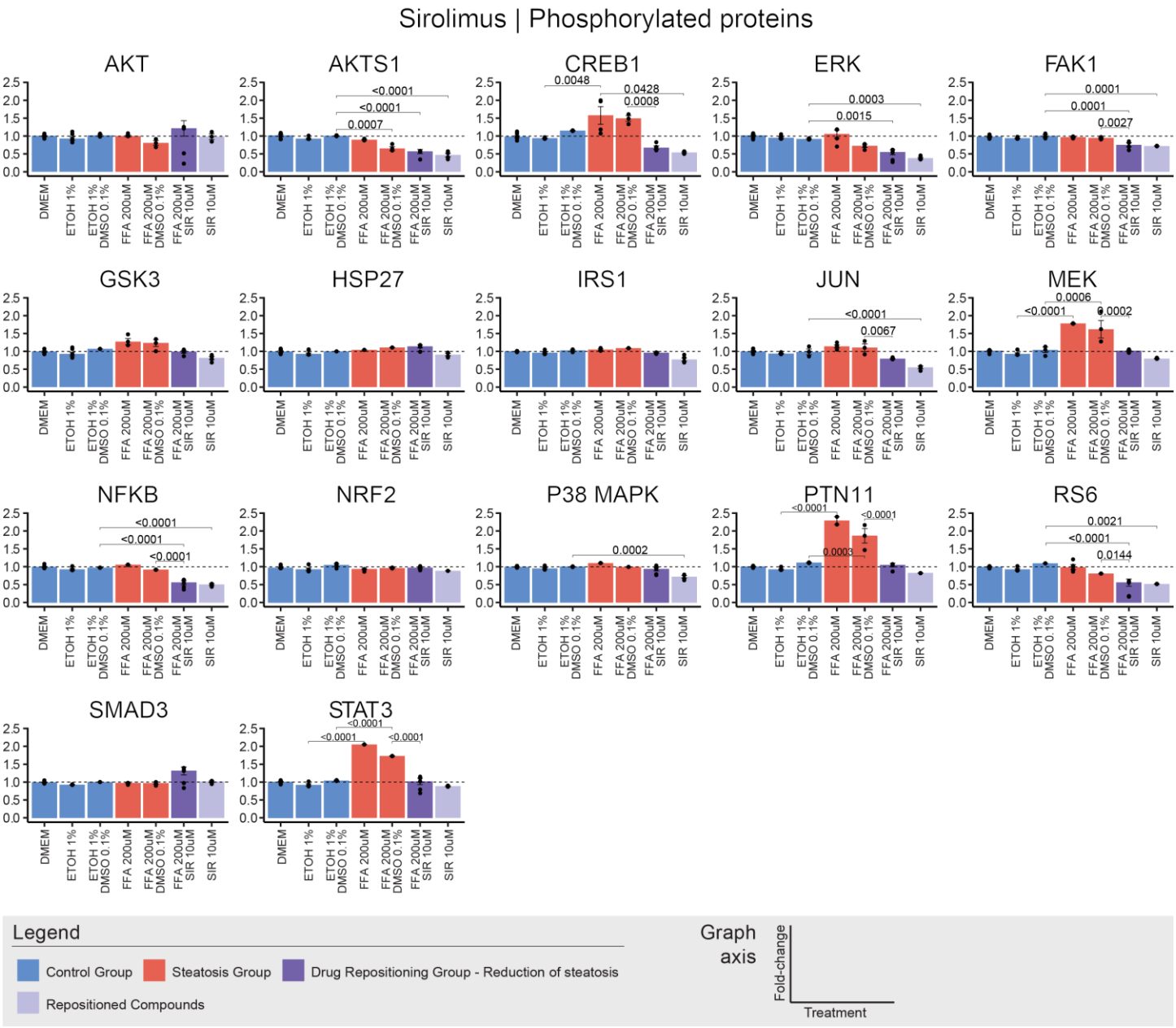


Figure 16 Statistical analysis of the effect of Sirolimus (rapamycin) on the phosphorylated proteins measured. *Bars represent the FC of median fluorescent intensity per protein in treated cells over respective controls. Data expressed as mean±SEM of at least n=3 independent experiments, and the p-value is denoted by brackets.*

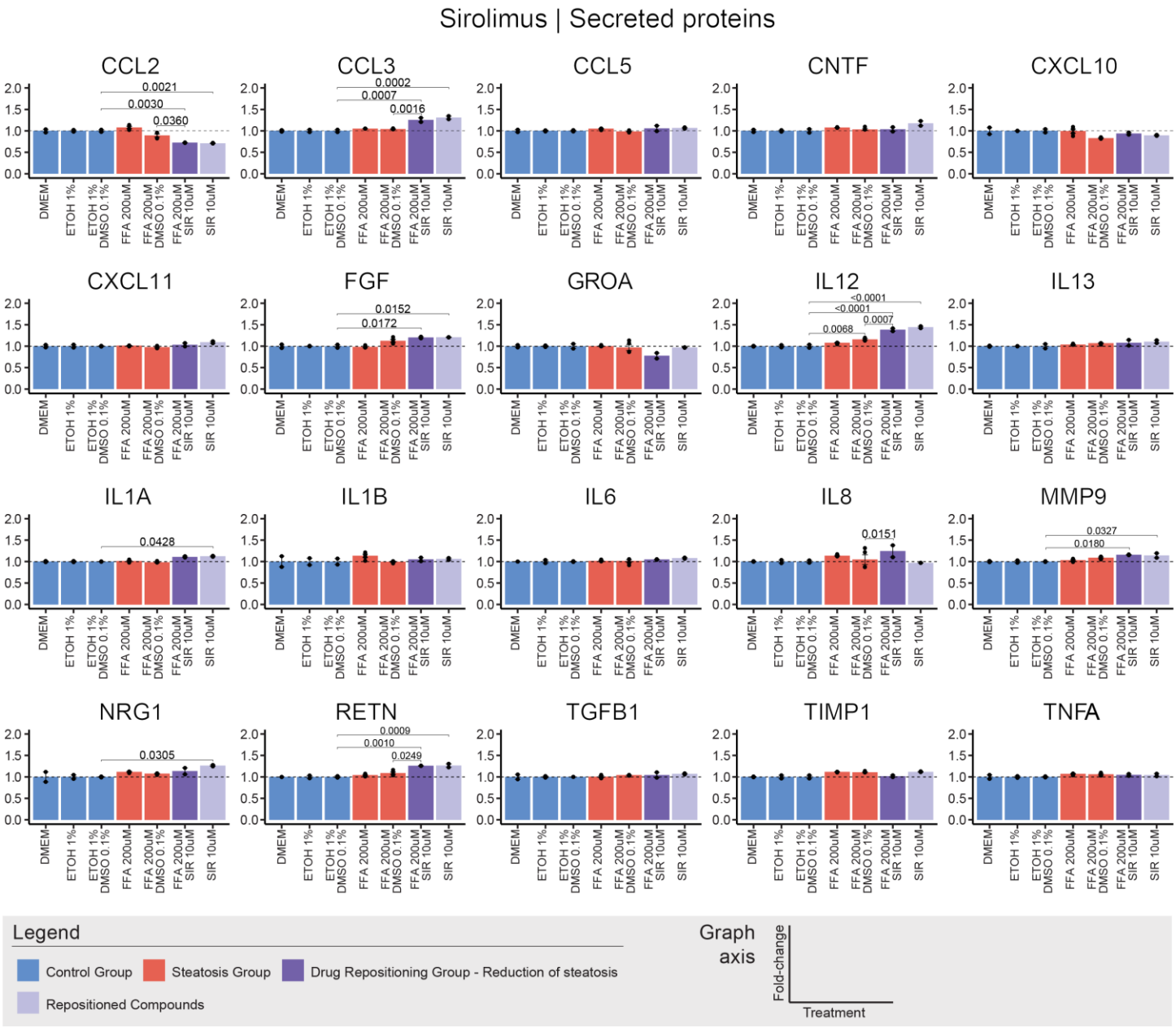


Figure 17 Statistical analysis of the effect of Sirolimus (rapamycin) on the secreted proteins measured. *Bars represent the FC of median fluorescent intensity per protein in treated cells over respective controls. Data expressed as mean±SEM of at least n=3 independent experiments, and the p-value is denoted by brackets.*

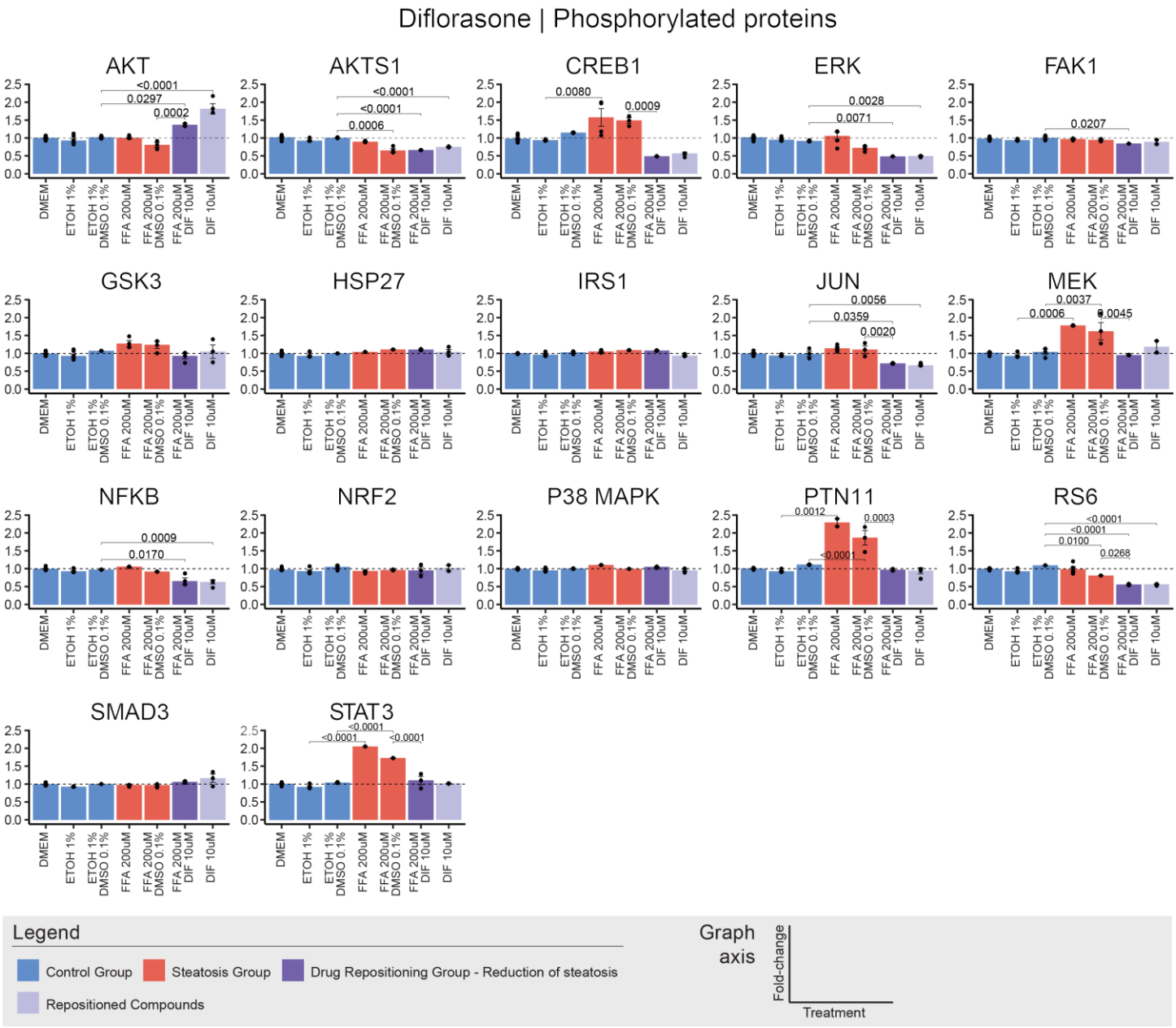


Figure 18 Statistical analysis of the effect of Diflorasone on the phosphorylated proteins measured. *Bars represent the FC of median fluorescent intensity per protein in treated cells over respective controls. Data expressed as mean±SEM of at least n=3 independent experiments, and the p-value is denoted by brackets.*

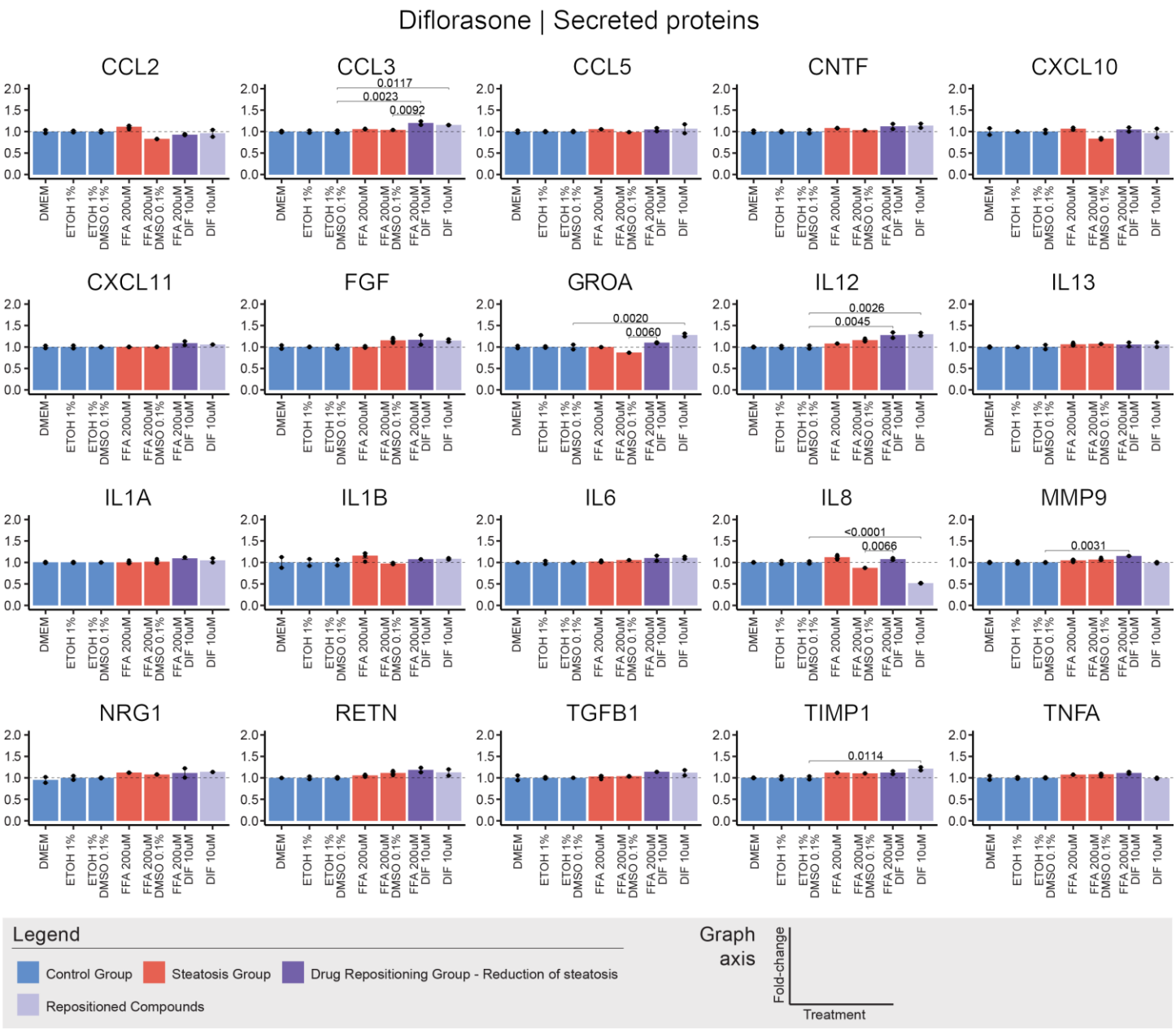


Figure 19 Statistical analysis of the effect of Diflorasone on the secreted proteins measured. *Bars represent the FC of median fluorescent intensity per protein in treated cells over respective controls. Data expressed as mean±SEM of at least n=3 independent experiments, and the p-value is denoted by brackets.*

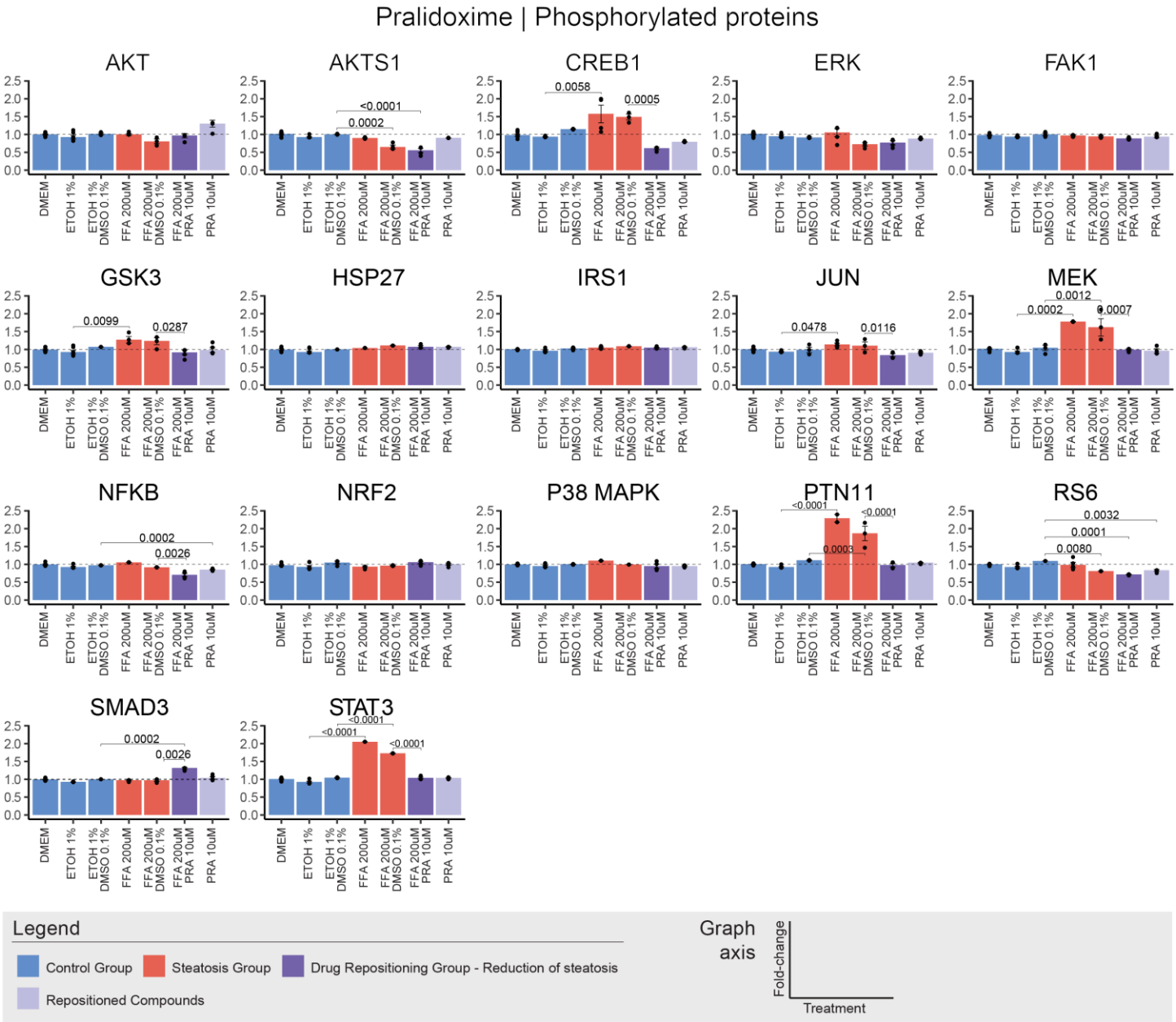


Figure 20 Statistical analysis of the effect of Pralidoxime on the phosphorylated proteins measured. *Bars represent the FC of median fluorescent intensity per protein in treated cells over respective controls. Data expressed as mean±SEM of at least n=3 independent experiments, and the p-value is denoted by brackets.*

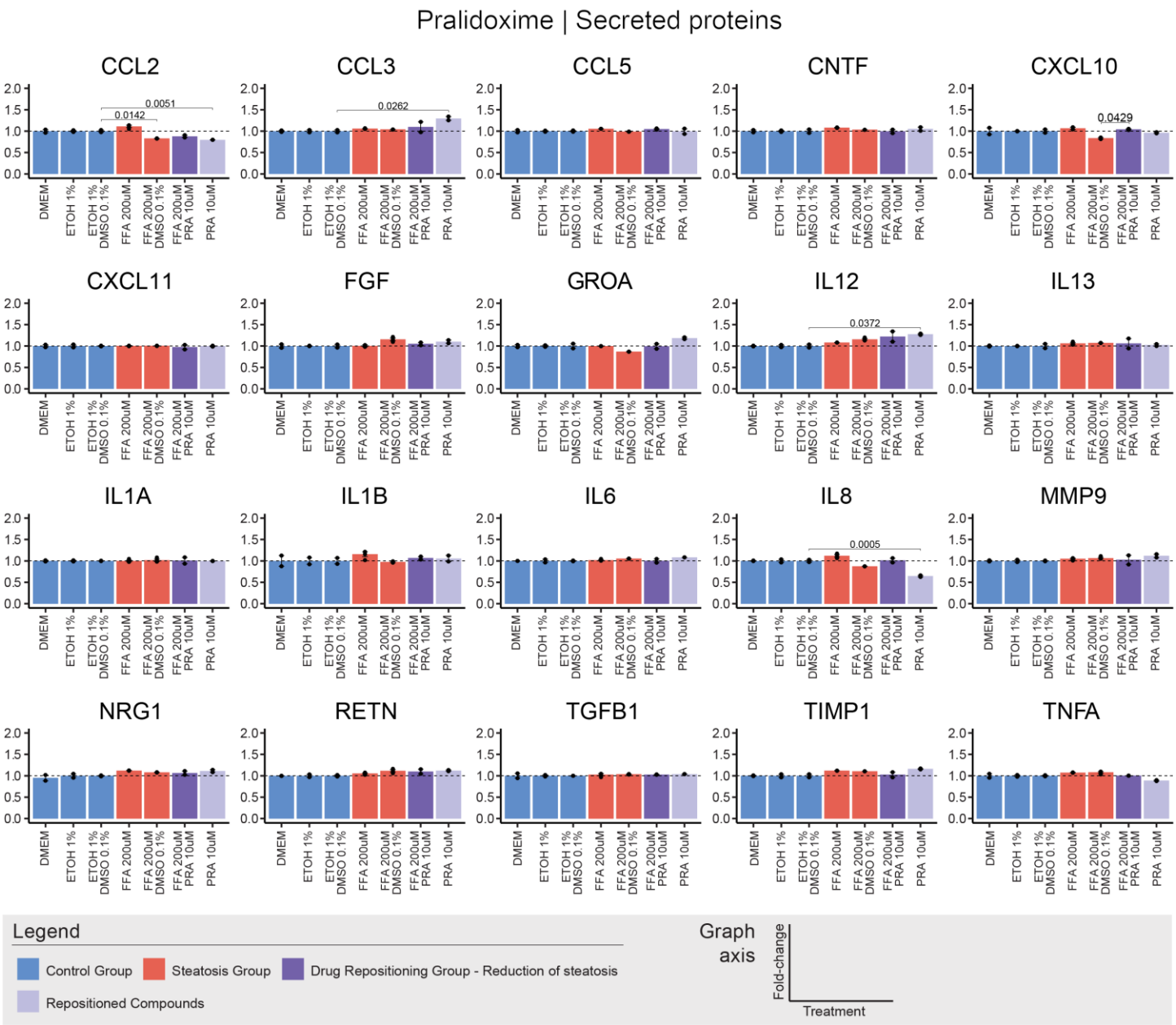


Figure 21 Statistical analysis of the effect of Pralidoxime on the secreted proteins measured. *Bars represent the FC of median fluorescent intensity per protein in treated cells over respective controls. Data expressed as mean±SEM of at least n=3 independent experiments, and the p-value is denoted by brackets.*

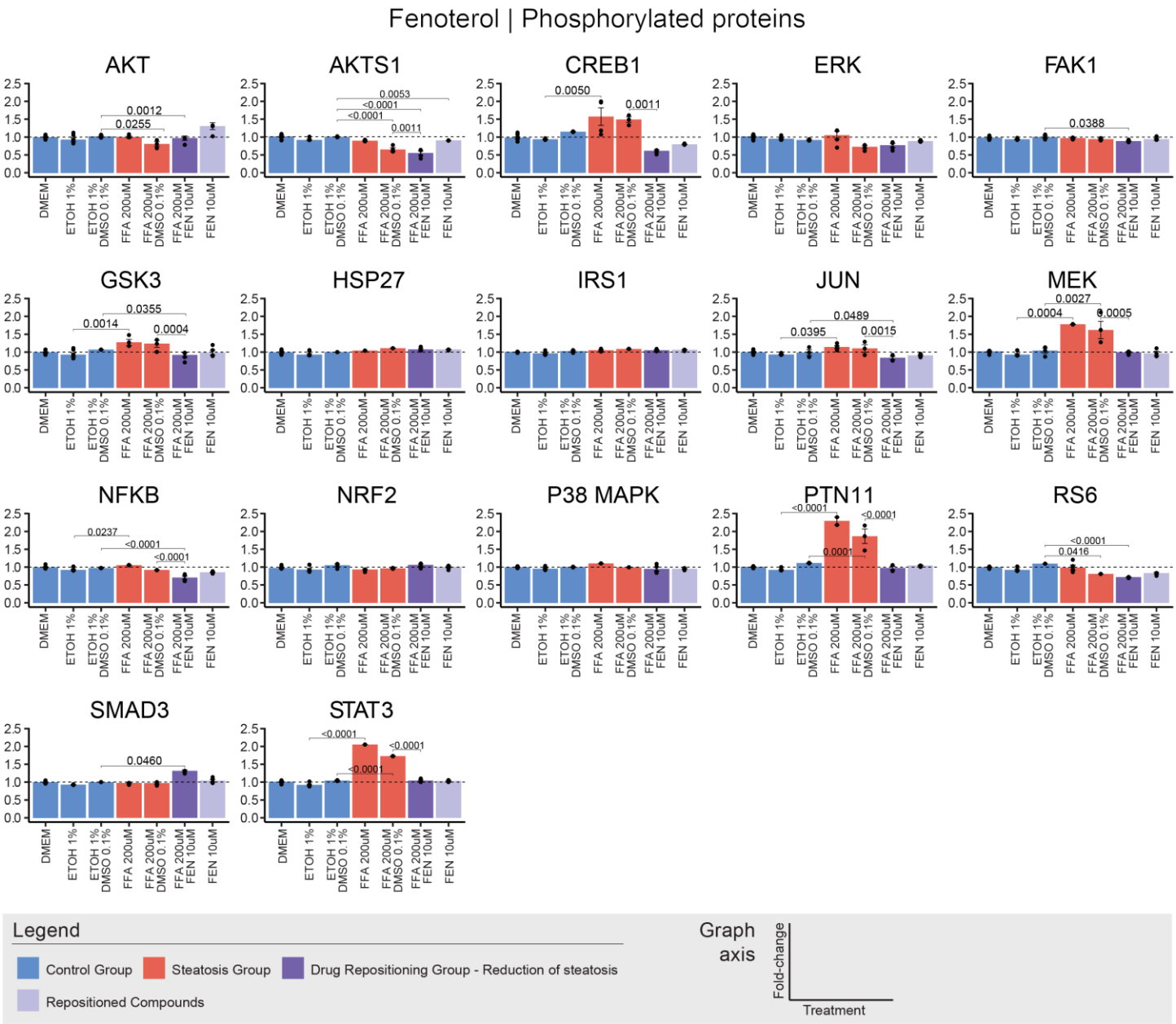


Figure 22 Statistical analysis of the effect of Fenoterol on the phosphorylated proteins measured. *Bars represent the FC of median fluorescent intensity per protein in treated cells over respective controls. Data expressed as mean±SEM of at least n=3 independent experiments, and the p-value is denoted by brackets.*

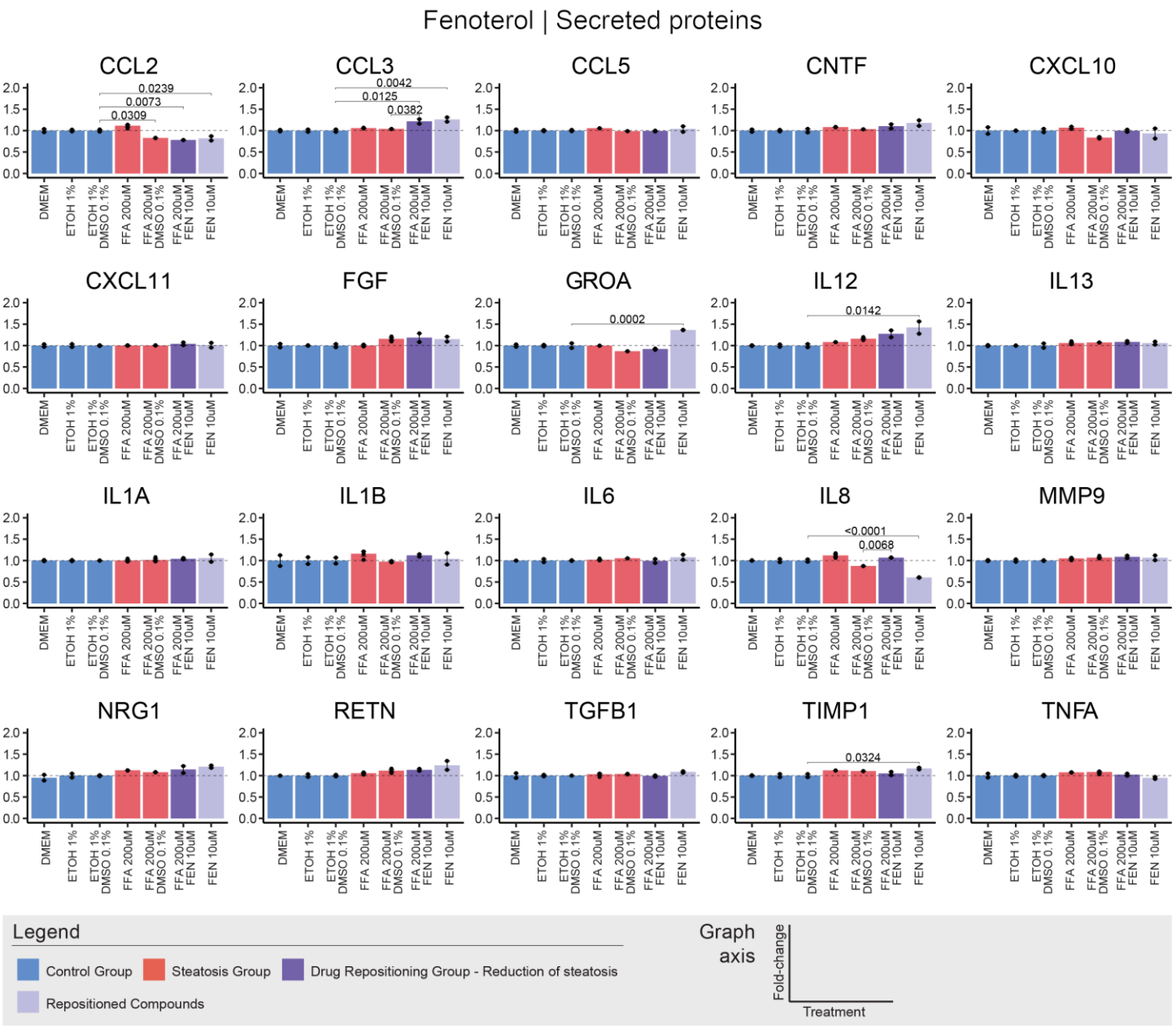


Figure 23 Statistical analysis of the effect of Fenoterol on the secreted proteins measured. *Bars represent the FC of median fluorescent intensity per protein in treated cells over respective controls. Data expressed as mean±SEM of at least n=3 independent experiments, and the p-value is denoted by brackets.*

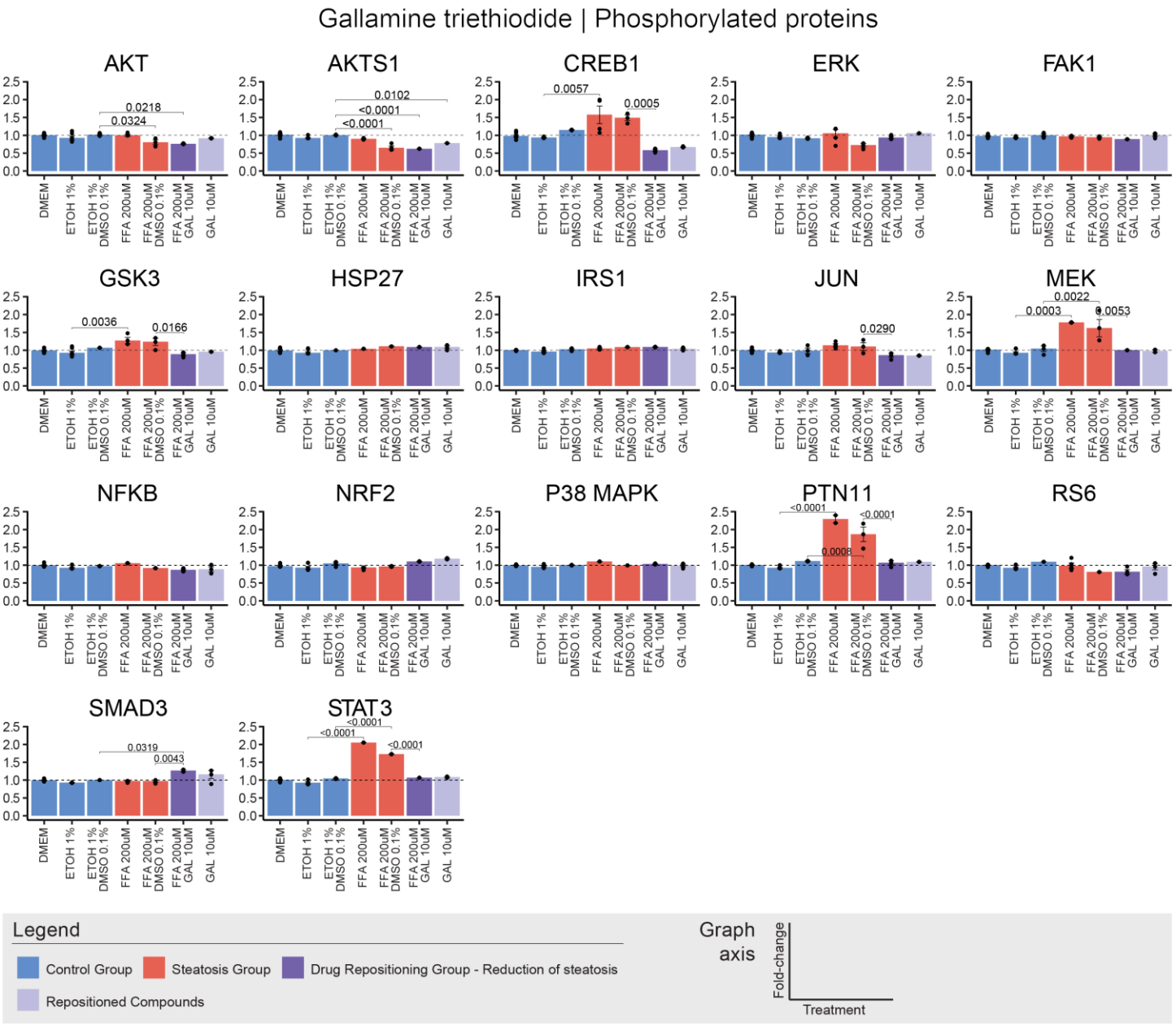


Figure 24 Statistical analysis of the effect of Gallamine triethiodide on the phosphorylated proteins measured. *Bars represent the FC of median fluorescent intensity per protein in treated cells over respective controls. Data expressed as mean±SEM of at least n=3 independent experiments, and the p-value is denoted by brackets.*

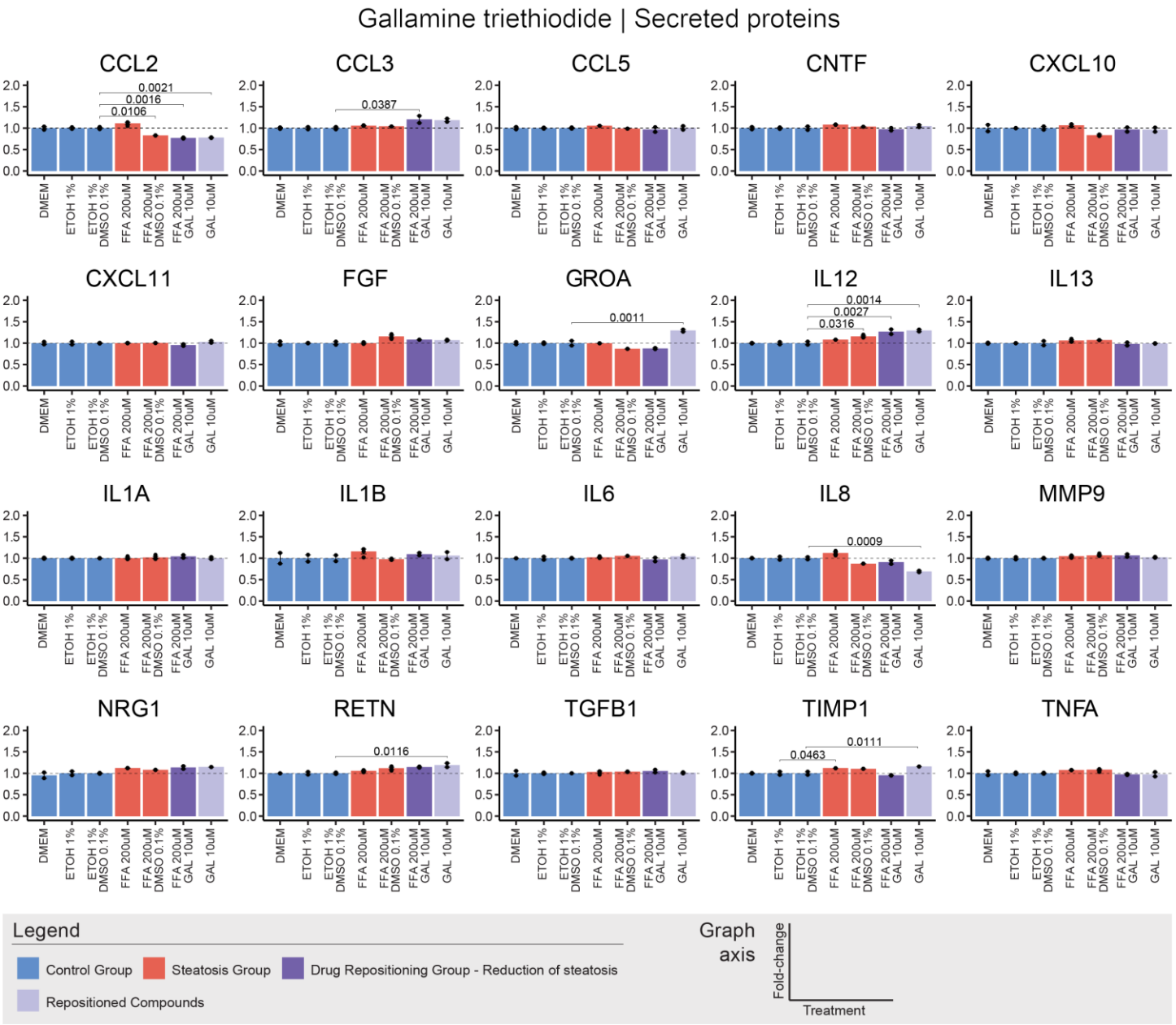


Figure 25 Statistical analysis of the effect of Gallamine triethiodide on the secreted proteins measured. *Bars represent the FC of median fluorescent intensity per protein in treated cells over respective controls. Data expressed as mean±SEM of at least n=3 independent experiments, and the p-value is denoted by brackets.*

4 | DISCUSSION

4. DISCUSSION

In this work, a compound-selection framework for NAFL/NASH that capitalizes on the effective combination of *in silico* speed and *in vitro* efficacy validation has been developed. Applied to a set of 20'000 compounds, this approach successfully selected two compounds with documented efficacy and revealed 19 new and 2 known compounds with anti-steatogenic potential. Several of the novel predictions were experimentally confirmed using high-throughput bioassays and *in vitro* steatosis models, and demonstrated the framework's efficiency in discovering anti-steatogenic compounds for the amelioration of NAFL.

In contrast to other cDR methods, the proposed *in silico* selection of compounds was integrated with existing *in vitro* models, hence directed towards compounds that could be validated *in vitro*. For that, the differential gene expression between healthy and NAFL/NASH samples was compared to the gene expression profile of compounds used for *in vitro* steatosis models. Just as important, on the eDR front, the development of *in vitro* models and their incorporation in the *in silico* analysis allowed for the development of a high-throughput drug-screening setup for the validation and efficacy testing of the computational predictions, reaching a very high *in vitro* screening hit rate of 28.5% (out of the 21 screened compounds, 6 showed positive anti-steatotic results).

4.1. The experimental and computational drug screening pipeline

For the experimental drug-screening pipeline, four hepatic cell lines and primary human heptacytes were treated with known *in vitro* and *in vivo* steatogens to create *in vitro* steatosis models and successfully recapitulated the steatotic phenotype. Consistent with previous *in vitro* studies, all the compounds were found to increase intracellular lipid accumulation and oxidative stress across all cell lines except HuH7. In fact, as the phenotype was recapitulated at lower-than-IC_{10} concentrations, the steatogenic compounds were suitable for the in silico platform.

Pathways affected by 20’000 FDA-approved and investigational compounds, available on the cMap database, were then compared to the pathways identified *in silico* from clinical and *in vitro* data. The eleven proposed pathways-to-target and/or their main components have already been studied extensively for their implication in NAFL/NASH development and have been proposed as possible targets for drug development.

4.1.1. Proposed pathways to target

Nuclear-receptors pathway (BIOCARTA, M16393)

Nuclear receptors (NRs) consist of a superfamily of transcription factors that are activated by ligands to perform a variety of biological processes including metabolism, cell growth and differentiation, as well as apoptosis. Ligands of NRs include hormones, biomolecules (lipids), vitamins, bile acids, metabolites, medicines, and xenobiotic toxins are among other numerous natural and synthetic molecules that sensitize NRs. NRs are divided into six subfamilies based on sequence homology, with 48 NRs identified in humans. Many illnesses, including liver disease, Parkinson's disease, and osteoarthritis, are directly or indirectly connected to NR signalling, and some NRs have become popular therapeutic targets (López-Velázquez et al., 2012).

The significance of NRs in liver disorders has been extensively reported. NRs have important roles in the start and development of a variety of hepatic disorders, including lipid and glucose metabolism, bile acid balance, drug detoxification, inflammation, regeneration, fibrosis, and tumour formation. These functions are explained by a complex transcriptional network that allows them to maintain cellular nutritional homeostasis, protect against toxins by limiting their absorption and enabling their metabolism and excretion, and participate in a variety of important stages of inflammation and fibrosis. Increasing data suggest that certain NRs are involved in the development of NAFLD, which may lead to innovative approaches for NAFLD diagnosis and therapy (Tanaka et al., 2017).

The role of the NR1 subfamily in NAFL/NASH has been extensively reported. These ligand-activated NRs form a heterodimer with NR2B1-3, the RXR a/b/g, and regulate matching target genes in the nucleus. The NR1 subfamily includes:

- NR1C1-3, peroxisome proliferator-activated receptor (PPAR): is comprised of PPARa, PPARb/d, and PPARg, and play a substantial role in the control of lipid homeostasis as will be discussed further below (Yang et al., 2020).
- NR1H2-3, the liver X receptor (LXR) a/b: is a family that regulates the homeostasis of lipids ad cholesterol, a direct regulator of hepatic lipogenesis, with LXRa mainly expressed in the liver and LXRb at a lower level in other tissues. LXR regulates SREPB1c and ChREBP expression in NAFLD hence controlling lipogenesis and lipid metabolism. Several studies highlight the potential implication of LXRa in NAFL/NASH progression through the regulation of lipogenesis and inflammation. Activation of LXR has been shown to have anti-inflammatory effects (TNFa reduction, JNK and Pi3K pathway inhibition, iNOS induction) in steatosis and could alleviate

liver injury along with reducing cholesterol accumulation in the liver. Interestingly, hepatic expression of LXRa displayed an abnormal increase in NAFLD patients highlighting the importance of its inhibition ad a possible treatment strategy. Moreover, oleanolic acids have shown positive hepatoprotective results through a decrease in LXR promoter activity. To conclude, this contradicting evidence pinpoints LXR as the main determinant of liver steatosis and the need for further studies for its implications in NAFL/NASH (Becares et al., 2019; Yang et al., 2020).

- NR1H4, the farnesoid X receptor (FXR): is considered a bile acid receptor that regulates their homeostasis, along with the homeostasis of lipids and glucose, and has been shown to be implicated in NAFLD development. Bile acids are products of the catabolism of cholesterol, hence implicated in lipid metabolism, that in high concentrations induce oxidative stress, inflammation, fibrosis and chirossis leading to NAFLD development. They are also important components of the gastrointestinal tract, linking the gut microbiota to hepatic metabolism that has been also implicated in NAFL/NASH pathogenesis and progression. FXR transcriptionally controls the synthesis, influx, efflux and detoxification of bile acids. Moreover, FXR is critical for the homeostasis of cholesterol, and a number of FXR agonists have been studied for the treatment of NASH (i.e., obeticholic acid, cilofexor, fexaramine, cycloastragenol) along with strategies of targeting the gut microbiota-bile acid-FXR mechanism (Xiao et al., 2021).
- NR1I3, the constitutive androstane receptor (CAR): has been reported as a key mediator for the protection against hepatosteatosis by suppressing gluconeogenesis and lipogenesis, while it has been shown to induce fatty acid b-oxidation (Yang et al., 2020).
- NR1I2, the pregnane X receptor (PXR): regulates genes related to the expression of drug-metabolizing enzymes, as well as the homeostasis of endobiotics (lipids, bile acids, glucose, bilirubin, retinoic acid), and is expressed in the onset of liver diseases via regulation of lipids, glucose and bile acids homeostasis. In detail, PXR aggravates hepatic steatosis by increasing the synthesis of lipids and fatty acid uptake, whilst suppressing genes implicated with fatty acid b-oxidation (PPARa, thiolase, JNK, SLC13A5). Moreover, PXR is considered a physiological sensor of bile acids regulating detoxification-involved and metabolism genes (CYP3A), and studies have reported that the FGF21-PXR pathway is involved in the decrease of the metabolic activity of CYP3A4 in NAFLD (Xiao et al., 2021).

Endocytosis pathway (KEGG, ko04144)

Hepatocytes are responsible for a variety of physiological activities including the absorption and subsequent metabolism or processing of different proteins, lipids, pathogens, or toxins. In reality,

vesicle trafficking is one of the most common activities carried out by the hepatocyte. Moreover, the liver's primary function is to regulate lipid homeostasis, which includes cholesterol production and very-low-density lipoprotein secretion, as well as turning ingested lipids into stored energy - a process that, if overdone, can result in hepatic steatosis.

It has been proposed that lipids and/or proteins internalized at the plasma membrane and trafficked along an endocytic pathway might influence lipid droplet formation and function. Lipid droplets are the central fat storage organelles and their accumulation is evident in steatosis, yet little is known about their origin, formation and breakdown. Regarding their breakdown, it was recently hinted that lipid droplets might interact with components and compartments of the endocytic trafficking pathway, based on the discovery that the breakdown of LDs is mediated by autophagic mechanisms. The physiologically most relevant form of autophagy with respect to LD breakdown is, however, macroautophagy - or more specifically, a special subtype of it termed lipophagy (Schroeder and McNiven, 2014).

Another noteworthy point involves the endo-lysosomal trafficking network, which is essential for signal transduction, protein degradation, and lipid/cholesterol metabolism. This network includes endocytosis, cargo sorting, intracellular membrane fusion and fission, and lysosomal degradation. Lysosomes, the cell's degradation centre, provide critical degradative tasks such as the breakdown of membrane lipids, polysaccharides, and protein components into their respective molecular building blocks: free fatty acids, monosaccharides, and amino acids. The function of the lysosome and its trafficking are linked. Endocytosis transports extracellular or cell surface cargos to lysosomes, while autophagy transports intracellular components. Moreover, endocytosis is affected by abnormal cholesterol trafficking since vesicular transport involves the interaction of endosomes, the ER and lysosomes and is crucial for the maintenance of cholesterol homeostasis (J. Du et al., 2020).

PPAR-signalling pathway (KEGG, HSA-03320)

A mentioned previously, PPARs are a ligand-activated family of transcription factors that play an important role in the regulation of lipid homeostasis and is comprised of PPARa, PPARb/d, and PPARg. The PPAR family is expressed differently in different tissues; PPARa is highly expressed in the liver, kidney, heart, and intestine, PPARg is most highly expressed in adipose tissues, and PPARb/d is more widely expressed in various tissues such as the liver, brain, kidney, heart, and adipose tissues. Free fatty acids, eicosanoids, and other complex lipids have all been proposed as endogenous PPAR ligands, while environmental and pharmaceutical compounds are examples of exogenous ligands.

Upon activation by a ligand, PPARs form a heterodimer with RXR so to regulate the expression of genes encoding proteins involved in beta-oxidation, fatty acid absorption, adipogenesis, and adipocyte differentiation. Given the importance of the PPARs as regulators of lipid and glucose metabolism, it is not unexpected that they have been suggested for the treatment of metabolic disorders such as NAFLD (Liss and Finck, 2017).

PPARa is expressed in all organs, but it is most abundant in the liver, where it regulates fatty acid absorption, beta-oxidation, ketogenesis, bile acid production, and triglyceride turnover. PPARa is also considered to have anti-inflammatory effects through complicated control of NF-kB, in addition to its function in metabolic regulation. Increased hepatic expression of PPARa and PPARa target genes involved in fatty acid oxidation has been linked to the administration of a high-fat diet in wild-type mice, and it has been proposed that this is an adaptive or protective response by PPARa. Hepatic expression of PPARa is reduced in individuals with NAFLD, although it has been observed to rise in tandem with NAFLD histological improvement as a result of lifestyle changes or bariatric surgery. Furthermore, increasing fatty acid turnover and catabolism through PPARa activation is considered to reduce triglyceride buildup. PPARa in the human liver is able to effectively induce the expression of genes involved in lipid transport and catabolism, notably mitochondrial and peroxisomal fatty acid b-oxidation. PPARa was also identified as a key regulator of adiponectin homologue novel osmotin protection against obesity/diabetes-induced NAFLD mouse models, which was associated with activation of the PPARa/AMPK/SIRT1 axis. Consequently, PPARa agonists have been proposed as potential candidates for NAFL/NASH therapy. Interestingly, although PPARa agonists have positive functions in animal models of NAFLD, there was no obvious improvement in NAFLD patients through assessing serum biochemical index and grade of steatosis, inflammation and fibrosis (Atherton et al., 2009; Yang et al., 2020).

The PPARb/d isoform is found in skeletal muscle, adipose tissue, and the skin, although it is most abundant in muscle, where it regulates mitochondrial metabolism and fatty acid beta-oxidation. PPARd is highly expressed in hepatocytes in the liver, but it is also found in Kupffer cells and hepatic stellate cells, suggesting a possible role in inflammation and fibrosis. Several in vitro and in vivo studies have evaluated the efficacy of PPARd agonists for the treatment of NAFL/NASH. GW501516 administration was linked with increased hepatic expression of genes involved in fatty acid b-oxidation, but its effect on the production of inflammatory cytokines was varied, and it was not connected with higher adiponectin levels in mice fed a high-fat diet. The experimental PPAR agonist GW0742 enhanced insulin signalling, reduced hepatic steatosis, and lowered inflammatory gene expression in mice. Finally, MBX-8025, a new PPAR agonist, was studied in a short,

randomized, double-blind, placebo-controlled trial, which revealed that therapy with MBX-8025 improved lipid profiles and reduced GGT, a marker of liver damage. Nonetheless, the implication of PPAR in carcinogenesis is still debated, since there are contradictory findings in vitro, preclinical, and clinical investigations (Liss and Finck, 2017; Yang et al., 2020).

PPARg is most abundant in adipose tissue, where it regulates adipocyte differentiation, adipogenesis, and lipid metabolism, whereas hepatic PPAR expression is elevated in NAFLD patients and experimental animals. Furthermore, PPARg is a key regulator of inflammatory responses and lipid storage, and it is well understood that aberrant lipid-mediated hepatic inflammatory immunological dysfunction and persistent low-grade inflammation are key contributors to the development of NAFLD. Thiazolidinediones (TZDs) are the most frequently studied PPARg agonists in the front of NAFL/NASH drug development. Activation of PPAR by TZDs leads to increased synthesis of adipokines, particularly adiponectin, which boosts hepatic fatty acid oxidation. PPAR activation also increases fat storage in adipocytes while reducing adipose tissue lipolysis, lowering the number of fatty acids delivered to the liver. TZDs are considered to reduce inflammation and cytokine production in individuals with metabolic syndrome, in addition to their metabolic benefits. (Liss and Finck, 2017; Yang et al., 2020).

Activator-protein-1 pathway (PID, M167)

Activator protein 1 (AP-1) proteins are prototypic oncogenes that control cell proliferation, differentiation, and transformation in numerous organs during development and in adults. Jun (c-Jun, JunB, JunD), Fos (c-Fos, FosB, Fra-1, Fra-2), activating transcription factor (Atf), and musculoaponeurotic fibrosarcoma (Maf) proteins structure AP-1, a dimeric transcription factor. C-Jun, in particular, has been demonstrated to be important in a variety of aspects of liver function and pathology (Schulien et al., 2019). To this end, c- Jun-/- knockout mice die around day E13 of embryonic development and had higher hepatoblast apoptosis, showing that c-Jun expression is required for liver development (Eferl et al., 1999). Conditional knockout mice with hepatocyte-specific c-Jun deletion around birth were able to avoid this phenotype. Furthermore, during fulminant immune-mediated hepatitis and chemically induced ER stress, c-Jun increases hepatocyte survival. AP-1, namely c-Jun, may be implicated in the development of metabolic liver disease, according to many lines of evidence. It was recently shown that overexpression of Fra-1 and Fra-2 protects against NAFL and NASH caused by HFD by inhibiting PPARg transcription via inhibitory c-Jun/Fra-1 or c-Jun/Fra-2 heterodimers (Hasenfuss et al., 2014). Furthermore, the Jun kinases Jnk1 and Jnk2, which work upstream of c-Jun and regulate its activity, are functionally connected to a slew of NASH-related pathways, including insulin resistance, the ER stress

response, and autophagy. The severity of NASH in MCDD-fed mice, a well-established animal model of NASH and consequent fibrosis, was dramatically decreased in Jnk1-/- mice, who also had lower hepatic c-Jun expression and phosphorylation (Schattenberg et al., 2006, p. 2). Furthermore, feeding a NASH-inducing western diet to wild-type mice resulted in increased c-Jun expression as well as significant changes in c-Jun-dependent gene expression (Dorn et al., 2014). The molecular influence of c-Jun on NASH development, however, is yet unknown.

Activating-transcription factor 2 pathway (PID, M166)

The term activating transcription factor (ATF) refers to a collection of proteins that bind to the core sequence CGTCA on the promoter of an adenoviral gene. ATF/CREB proteins can act as sensors in response to extracellular fluctuations in nutrient concentrations, hormone levels, and energy status, and function as transcription factors or cofactors to play critical roles in the regulation of systemic homeostasis in key metabolic tissues, particularly the liver, according to extensive research. The nuclear protein cAMP-responsive element-binding protein (CREB) binds to the cAMP-responsive element on the promoter of the neuropeptide somatostatin. The ATF/CREB transcription factor family has been found to regulate several cellular processes involved in glucose metabolism, particularly gluconeogenesis and insulin sensitivity. Members of the ATF/CREB family are involved in hepatocyte growth and proliferation in response to various environmental stimuli, as well as glucose and lipid metabolism. However, the method by which extracellular signals modify these proteins, allowing them to contribute to cell metabolism and growth balance, remains unknown. Furthermore, how chronic overnutrition affects these homeostatic processes under physiological settings, resulting in excess fat storage in the liver, altered hepatic cytokine production, hyperinsulinemia, and eventually type 2 diabetes, NAFLD, and HCC, is a hot topic of research right now.

ATF2 is induced by JNK activation triggered by the AAR pathway, which is independent of the ATF4 pathway. ATF2 activation increases cell proliferation and may contribute to the development of HCC. The effects of ATF2 on cell proliferation are backed up by evidence of enhanced hepatocyte regeneration when ATF2 is present. MKK7, JNK-1, and GSTP1/2 have been shown to directly activate ATF2, which increases cell cycle gene expression and hepatocyte proliferation. In addition, MAPK phosphorylates ATF2 to enhance cell survival and proliferation. Activation of c-Jun/ATF2, on the other hand, suppresses p38 phosphorylation by activating MAPK phosphatases, providing a negative feedback mechanism to prevent hyperactivation of p38 activity and abnormal cell survival and proliferation (Cui et al., 2021).

NFAT transcription factor pathway (PID, M60)

The nuclear factor of the activated T-cells (NFAT) family consists of 5 members (NFATc1, NFATc2, NFATc3, NFATc4, NFAT5), and was first recognized to play an important role in the differentiation of T cells. Subsequent studies demonstrate that NFAT also plays an important role in nonimmune cells. In addition to its established role in immune cells targeted disruption of the calcineurin-regulated NFAT members has further illuminated the role of NFAT in multiple biological processes, including cardiac morphogenesis and neural pathfinding. Whether NFAT contributes to obesity, however, has yet to be established (Yang et al., 2006).

Recent studies have indicated that NFAT also plays a role in adipocyte differentiation. NFAT interacts with transcription factor C/EBP to form a composite element to regulate the PPARg2 gene. Given that NFAT regulates cytokine gene expression in immune cells, NFAT may also modulate adipokine gene expression and contribute to glucose and insulin homeostasis. Recent evidence also indicates a role for NFATc as a metabolic sensor in adipocyte differentiation and cardiovascular complications associated with, for example, obesity and diabetes. To this end, it was discovered that hepatic NFATc4 activation accelerates the progression of NASH by suppressing PPARa signalling and increasing OPN expression, hence genetic or pharmacological inhibition of NFATc4 may have the potential for future therapy of NASH (M. Du et al., 2020; Yang et al., 2006).

Metabolism of amino acids and derivatives pathway (REACTOME, R-HSA-71291)

A number of amino acid metabolic pathways has been shown to be deregulated in the presence of NAFLD.

The risk of developing hyperglycemia, insulin resistance, metabolic syndrome, and diabetes has been associated with tyrosine metabolism. The causes and mechanisms of tyrosine metabolism dysregulation in hepatic steatosis are yet unknown. Tyrosine can enter the ketogenic route and be metabolized directly to acetyl-CoA by ketogenesis, according to one theory. As a result, a high dietary tyrosine consumption combined with a calorie surplus may promote fatty acid production and lipid deposition in the liver. Increased tyrosine levels in the serum of individuals with NAFLD have been observed often, while the aromatic amino acids have been linked to the frequency of liver disorders. This is not unexpected as the liver metabolizes aromatic amino acids, and lipid accumulation causes the hepatic metabolism of these amino acids to be impaired. Another probable reason is the down-regulation of the aromatic amino acid transporter (SLC16A10), which transports these amino acids from the bloodstream to the liver, as seen in NASH patients.

Several additional important amino acids, such as tryptophan, BCAA, glycine, serine, alanine, and threonine, have been found to be deregulated in the presence of hepatic steatosis. Because the liver is so important for maintaining amino acid balance, the abnormalities might be the result of an aberrant liver activity.

BCAAs (leucine, isoleucine, valine) have been the most extensively studied and shown to be elevated in obese and/or NAFLD patients. In addition, the activity of mitochondrial BCAA-catabolizing enzymes, branched-chain aminotransferase (BCAT) and branched-chain ketoacid dehydrogenase (BDKDH), was shown to be reduced in obese people. However, it's unclear if this increase is caused by insulin resistance. Even though increased plasma BCAA levels and deregulated BCAA metabolism are shown in insulin resistance and type 2 diabetes, the function of BCAA in the aetiology of NAFLD is unknown.

The evidence supporting altered tryptophan, glycine, serine, alanine, and threonine metabolism in NAFLD is extremely minimal, and this is an area that will be investigated more in the future. Patients with NAFLD had lower levels of glutamine, serine, and glycine than healthy controls. These amino acids act as direct or indirect precursors of glutathione (GSH), one of the most essential antioxidants. Glutamine has been found to reduce oxidative stress in NAFLD rats' livers by boosting GSH production and improving hepatic steatosis (Chashmniam et al., 2019; Gaggini et al., 2018; Jin et al., 2016).

Fatty acid, triacylglycerol and ketone body metabolism pathway (REACTOME, R-HSA-188467)

Hepatocytes are responsible for the hepatic metabolism of fatty acids (FA) and their neutral storage form, triglycerides (TG). The liver processes a considerable quantity of FA on a daily basis under normal conditions, but only retains a tiny amount in the form of TG, with steady-state TG levels of less than 5%. This is because FA absorption from the plasma and de novo synthesis inside the liver are counterbalanced by FA oxidation and secretion into the plasma as TG-enriched very-low-density lipoprotein (VLDL-TG). The TG deposited in the liver is found in cytoplasmic lipid droplets in relatively modest amounts, while excess TG buildup in the liver is a symptom of NAFLD.

FA originate from either dietary or endogenous sources in the liver. Following hydrolysis by pancreatic lipase, dietary TG is emulsified by bile acids inside the intestinal lumen, yielding sn-2-monoacylglycerols and free FA as products. These lipid molecules are absorbed by enterocytes and resynthesized into TG after emulsification. TG is packed into chylomicrons, which are secreted into the lymphatic system before reaching the plasma. Because lipoprotein lipase, which is expressed on the luminal surfaces of capillary endothelial cells in these tissues, is active, a large

portion of the chylomicron TG is taken up by muscle and adipose tissue. When these particles are taken up by receptor-mediated endocytosis, TG remains inside the chylomicron remnants, while FA is released during lysosomal processing.

When in an abundance of carbohydrates, the liver turns glucose to FA, a process known as de novo lipogenesis (DNL). DNL is mainly regulated by transcription. Plasma insulin stimulates the sterol regulatory element-binding protein 1C (SREBP1c), a membrane-bound transcription factor in the endoplasmic reticulum that translocates to the nucleus and upregulates all genes in the FA biosynthesis pathway. Excess plasma glucose stimulates the nuclear translocation of carbohydrate response element-binding protein (ChREBP), a transcription factor that also upregulates transcription of the majority of FA biosynthetic genes, as well as pyruvate kinase, increasing citrate available for FA synthesis.

Direct plasma absorption is another significant source of FA. During fasting, FA from plasma is the primary source of hepatic triglycerides in human individuals. When plasma insulin concentrations are low, a lipolytic program in white adipose tissue is begun, increasing the plasma FA pool that is accessible for absorption by the liver. Within the circulation, FA are mostly albumin-bound. Hepatic FA absorption involves many stages. FA dissociation from albumin, transit through the hepatocyte plasma membrane, binding to intracellular proteins, and esterification to coenzyme A are all examples of these processes (CoA). In both fasting and fed states, plasma FA are the primary source of VLDL-TG.

FA are esterified to glycerol-3-phosphate (G3P) and cholesterol in the hepatocyte to produce TG and cholesteryl esters, respectively. These neutral lipids might be retained in cytoplasmic lipid droplets (LDs) or released as VLDL particles into the circulation. FA can also be utilized to make other complex lipids, such as phospholipids, within the liver (PL). FA are employed as a local energy source as well as a substrate for the synthesis of ketone bodies during fasting. Overall, numerous interconnected transcriptional and signalling mechanisms govern hepatic FA metabolism, and these pathways are still being investigated.

FA are taken up by hepatocytes via plasma membrane-associated proteins, despite their capacity to diffuse across a lipid bilayer. As previously mentioned, a number of proteins have been linked to long-chain FA transport, including plasma membrane FABP, CD36, caveolin-1, and extremely long-chain acyl-CoA synthetases.

Hepatic DNL pathways appear to be used rarely in humans under normal conditions. Increased DNL, in addition to increased FA absorption, can contribute significantly to hepatic steatosis. ATP-

citrate lyase (ACLY) catalyzes the first step in the DNL pathway, converting citrate to acetyl-CoA, which is then carboxylated to malonyl-CoA by acetyl-CoA carboxylase (ACC). Following the ACC, a series of reactions convert malonyl-CoA to palmitate. The primary rate-limiting enzyme in palmitate production is fatty acid synthase (FAS), and its activity is controlled by a variety of processes. Elongases and desaturases can alter palmitate to produce a variety of FA species.

The activation of long-chain FA molecules by thioesterification to CoA to produce fatty acyl-CoA molecules is a necessary step in their metabolism. Members of the long-chain acyl-CoA synthetase (ACSL) family of enzymes catalyze this process. Different ACSL isoforms are involved in the partitioning of fatty acyl-CoAs into various metabolic pathways. The activities of acyl-CoA thioesterases reverse the process mediated by ACSL isoforms (ACOTs). The intracellular balance of fatty acyl-CoAs and FA, intracellular and intra-organelle CoA concentrations, and the availability of lipid substrates for several metabolic pathways might all be controlled by ACSL and ACOT enzymes. The mechanisms through which ACSL and ACOT enzymes regulate FA metabolism in hepatocytes are unknown, and whether their aberrant regulation contributes to insulin resistance and associated metabolic disorders is unknown.

Long-chain FAs and their acyl-CoA derivatives are engaged in a variety of intracellular activities, including protein palmitoylation, intracellular signalling, and transcription factor activation, in addition to being lipid synthesis and oxidation substrates. It's only natural that the intracellular quantities and location of free FA and fatty acyl-CoAs are closely controlled, given the diversity of cellular activities, relative insolubility, and potential cytotoxicity. This is made possible in part by the actions of lipid-binding proteins, which have been linked to the regulation of intracellular concentrations and partitioning of long-chain FA and acyl-CoA inside hepatocytes. The liver FABP1, acyl-CoA binding protein (ACBP), and sterol carrier protein-2 are among them (SCP2). Because these intracellular lipid-binding proteins are linked to the metabolic fates of FA and acyl-CoA molecules, it's tempting to speculate that they could be used to manipulate the flux of lipids between anabolic and catabolic pathways to protect against FA-mediated lipotoxicity, which contributes to the pathogenesis of NAFLD.

The primary mechanism through which the liver accumulates and exports FA is through the formation of TG molecules. The liver retains little TG under normal circumstances, but it exports a significant quantity in the form of VLDL particles, which transport FA to muscle and fat tissue depending on nutritional status. Previously, it was thought that excess TG stores in NAFLD led to lipotoxicity, but new research suggests that increased TG storage and VLDL production are protective against FA-mediated hepatotoxicity.

The G3P pathway is the primary route for TG synthesis in most mammalian cell types, accounting for approximately 90% of total TG synthesis. The esterification of long-chain acyl-CoA to G3P, which is mediated by mitochondrial and microsomal G3P acyltransferase (GPAT) enzymes, is the first and rate-limiting step in this process. The acylglycerol-3-phosphate acyltransferases (AGPAT) located in the ER membrane acylate the lysophosphatidic acid (LPA) molecules generated in this process to create phosphatidic acid (PA). PA can be converted to CDP-DG (cytidine diphosphate diacylglycerol), a substrate for the production of glycerolphospholipids and cardiolipins. It may also be dephosphorylated by phosphatidate phosphohydrolase (Lipin) to produce DG, which is a precursor molecule for the production of TG, phosphatidylcholine (PC), and phosphatidylethanolamine (PEA) (PE). The acylation of DG, which is the final step in TG production, is catalyzed by DG acyltransferase (DGAT). Newly generated TG molecules are subsequently directed to create cytosolic LDs from the ER lipid bilayer.

The method by which FA are exported from the liver and transported to muscle for oxidation and adipose tissue for storage is represented by TG-rich VLDL particles. Hepatic steatosis can be caused by a decrease in VLDL secretion. This happens when apoB100 and MTP genes are mutated, resulting in hypobetalipoproteinemia and abetalipoproteinemia, respectively.

The rate-limiting step in TG lipolysis within adipocytes is the LD-associated adipose triglyceride lipase (PNPLA2). The hormone-sensitive lipase (HSL) hydrolyzes the resultant DG molecules to liberate monoglycerides (MG). The monoacylglycerol lipase (MGL) cleaves MG into glycerol and FA in the last phase. Hepatic ATGL is necessary for the lipolysis of TG stored in LDs, regulates substrate availability for FA oxidation, and affects the development of hepatosteatosis, according to gain and loss of function studies. Co-activation by comparative gene identification-58 is required for full ATGL activation (CGI-58). Ablation of CGI-58 in the liver causes NAFLD phenotypes in mice, including hepatic steatosis and fibrosis. Lipolysis mediated by hepatic ATGL is reduced in the presence of hepatic steatosis when PLIN5 binds competitively to CGI-58, displacing ATGL. Higher rates of lipolysis within white adipose tissue are a key contribution to increased FFA plasma levels and hepatic steatosis in the context of insulin resistance. The role of TG hydrolysis in hepatocytes in abnormal lipid buildup in the liver, on the other hand, is less apparent (Alves-Bezerra and Cohen, 2017; Fletcher et al., 2019).

While hepatocyte cytoplasmic lipid metabolism anomalies are frequent in NAFLD, the function of mitochondrial metabolism, which regulates oxidative and terminal fat "disposal," in NAFLD pathogenesis is less apparent. Nonetheless, the majority of researchers believe that aberrant mitochondrial metabolism occurs in NAFLD and contributes to it. Ketogenesis can eliminate up to

two-thirds of the fat that enters the liver. As a result, ketone body metabolism imbalance might play a role in NAFLD aetiology. Hepatic ketogenesis is triggered when fatty acid levels are high and glucose availability is low, as well as when circulating insulin levels are very low. Ketogenic processes inside hepatic mitochondria condense acetyl-CoA generated from -oxidation into the ketone molecules acetoacetate (AcAc) and hydroxybutyrate (OHB). Ketogenesis recycles 2 moles of free coenzyme A (CoASH) every mole of ketone produced while also disposing of acetyl-CoA produced in excess of the liver's own energy demands. Due to the low expression of the fate-committing ketogenic enzyme mitochondrial 3-hydroxymethylglutaryl-CoA synthase (HMGCS2) in normal circumstances, robust ketogenesis is limited to hepatocytes. In contrast, oxidative ketone body disposal is almost universal, as all cells except hepatocytes express the ketone body oxidation fate-committing enzyme succinyl-CoA 3-oxoacid CoA transferase (SCOT). OHB is oxidized to AcAc in the mitochondria of extrahepatic tissues and subsequently directed to the TCA cycle for terminal oxidation via a process mediated by SCOT. Ketone body metabolism has been ignored as a possible treatment target in NAFLD despite its great capacity as a disposal route for hepatic fatty acids. Obesity-associated hyperinsulinemia inhibits ketogenesis, resulting in relative ketogenic insufficiency and hypoketonemia in obese animal models and people as compared to lean controls, through unknown processes (Cotter et al., 2014).

Fatty acid metabolism pathway (KEGG, map01212)

Numerous studies on the relationship between NAFLD and lipid disorders have demonstrated that disruption of the lipid metabolism balance in the liver causes lipid accumulation and consequently, hepatotoxicity, and NAFLD. More specifically, the process of lipid uptake in the liver in both physiological and pathological conditions is mediated through liver FA binding protein (FABP1) and CD36. Abnormal protein regulation may lead to excessive hepatic accumulation of non-esterified FAs (NEFAs) and TG, causing cytotoxicity and resulting in NAFLD.

CD36 is a key receptor implicated in long-chain FA transport and TG storage that is found in a variety of cells, including macrophages and monocytes, as well as organs including the liver, heart, and adipose tissue. It detects changed lipoproteins such as oxidized LDL, stimulates the development of lipid-laden foam cells, and regulates lipid consumption processes. CD36 has recently been discovered to have an important function in the liver, engaging in FA absorption, storage, and TG secretion. CD36 regulates FFA uptake in a variety of tissues, and FA uptake plays a key role in hepatic steatosis. As a result, CD36 anomalies may contribute to hepatic steatosis. The bulk of NEFAs in the blood are linked to carrier proteins (mostly albumin), and their absorption

requires CD36-mediated carrier protein dissociation. NAFLD progresses as a result of an excessive hepatic buildup of NEFAs and TG, which causes cytotoxicity.

The liver, gut, and kidney all have significant amounts of FABP1. FABP1 is involved in the absorption, transport, and metabolism of long-chain FAs and other lipid ligands in cells, in addition to CD36. A novel method for preventing hepatic steatosis and liver damage has been proposed by the means of downregulation of FABP1.

Lipid abnormalities are of many types and many have been implicated with the development of NAFL/NASH. For example, lipotoxicity is defined as abnormal cellular lipid composition that leads to the accumulation of toxic lipids, organelle dysfunction, cellular damage, and chronic inflammation and is caused by increased FA uptake. Briefly, FA accumulation causes mitochondrial damage that further aggravates FA accumulation and results in insulin resistance and, finally, liver inflammation and fibrosis (Pei et al., 2020).

Metabolism of lipids and lipoproteins pathway (REACTOME, R-HSA-556833)

The liver's primary metabolic function is to maintain plasma glucose within strict physiological limits, independent of the dietary status. When there is a surplus of energy, glucose is converted to fatty acids, which are then utilized to make triglycerides. Hepatocytes can store triglycerides as lipid droplets, or they can be integrated into very-low-density lipoproteins (VLDL) and released into the bloodstream. Once in the bloodstream, lipoprotein lipase (LPL) reduces the triglyceride content of these particles, resulting in intermediate-density lipoproteins (IDLs) and low-density lipoproteins (LDL) with a relatively high cholesterol content. By binding to the LDL receptor, LDL circulates and is absorbed by the liver (Perla et al., 2017).

In order to maintain hepatic lipid homeostasis, patients with insulin resistance increase VLDL production. Insulin resistance is linked to aberrant lipoprotein concentrations, increased VLDL synthesis, and an increase in plasma LDL. Plasma LDL levels were also reported to be elevated in patients with NAFLD (Perla et al., 2017).

The link between fatty liver and high levels of small dense LDL (sdLDL) is now well established. After triglycerides are gradually eliminated from LDL, tiny, dense LDL, the most atherogenic subtype of LDL, develops as the triglyceride-rich VLDL enters plasma at a faster rate. This process is thought to be aided by two enzymes. First, CETP promotes the transfer of triglycerides from VLDL to LDL (and cholesteryl esters from LDL to VLDL), and second, hepatic lipase enhances the lipolysis of triglyceride-rich LDL, leading to the production of sdLDL. CETP, in conjunction with HL, remodels VLDL in circulation, enriching it in cholesterol and favouring the development of sdLDL.

Hepatic steatosis patients had higher CETP activity. Smaller particles have a lesser affinity for the LDL receptor, thus they stay in the circulation longer. Low lipoprotein lipase activity or a high level of apolipoprotein C-3 (APOC-3), a lipoprotein lipase inhibitor, can aggravate hyperlipidemia. Polymorphisms in the APOC-3 gene have been linked to fatty liver in humans (M.-R. Li et al., 2014).

Unlike simple steatosis, steatohepatitis is linked to abnormal VLDL production and secretion. The inhibition of hepatic VLDL secretion causes triglyceride buildup in the liver. The production of VLDL in the liver requires the microsomal triglyceride transfer protein (MTTP). Human MTTP polymorphisms are linked with increased intracellular triglyceride buildup and reduced MTTP activity and VLDL export. Overall, this has an effect on NASH development, potentially via altering the postprandial lipid profile. Enhanced oxidative stress has been associated with the postprandial period, and increased lipid peroxidation has been connected to NASH development. Hepatic stellate cells are important in NASH development and can be activated by oxidized LDL.

Sortilins, or apolipoprotein B 100 (APO-B 100) intracellular sorting receptors, are novel actors in lipoprotein metabolism. Sortilin 1 (Sort 1) gene was linked to LDL metabolism in genome-wide association studies (GWAS) of common genetic variants. Several results suggest that sortilin 1 is involved in the hepatic metabolism of APO-B-containing lipoproteins, however, the specific process remains unknown.

The relative rates of synthesis and clearance by LDL receptor govern the plasma levels of LDL, the main cholesterol-carrying lipoprotein in humans. The route of LDL receptor-mediated endocytosis was described by Goldstein and Brown, and its control by cholesterol-dependent negative feedback kexin type 9 increases LDLR degradation, resulting in plasma low-density lipoprotein accumulation. PCSK9 binds to the EGF-A domain of the low-density lipoprotein receptor (LDLR), allowing it to be targeted to endosomes/lysosomes and degraded. Inhibiting PCSK9's effect on LDLR has emerged as a promising new treatment for hypercholesterolemia. PCSK9 loss has recently been demonstrated to give resistance to hepatic steatosis; however, this impact appears to be independent of LDLR (Fon Tacer and Rozman, 2011; Heeren and Scheja, 2021).

4.1.2. Compounds proposed for drug repositioning

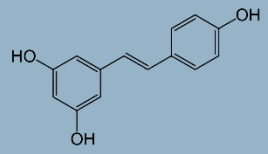
Therefore, the cDR platform identified pathways significantly affected both in NAFL/NASH patients and in the *in vitro* models and suggested 46 compounds as capable of interfering with the disease's pathways. Out of these, 25 were eliminated from further analysis as known hepatotoxic.

The remaining 19 compounds were examined for their capacity in reducing intracellular lipid accumulation and oxidative stress with a high-content screening setup. For this purpose, only HepG2 cells were used, as they showed the highest stability compared to primary human hepatocytes and the other cell lines. In fact, primary human hepatocytes presented with low viability in co-treatment, and differentiated when sequential treatment was attempted due to the prolonged treatment time.

For the screening setup, only FFAs were administered as a steatosis induction mechanism. In fact, the concurrent administration of any other steatogens with the DR compounds resulted in low cell viability and was therefore abandoned. Sequential administration of FFAs and the DR candidates was also attempted. However, aside from the longer treatment, removing the FFAs might have been sufficient to reduce intracellular lipid loading and so, mask a DR compound's anti-steatogenic potential. Hence, the co-administration of FFAs and the DR compounds was selected.

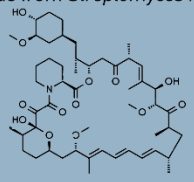
Six (6) compounds (Sirolimus, Resveratrol, Gallamine triethiotide, Fenoterol, Pralidoxime and Diflorasone) out of the 21 screened *in vitro* succeeded in effectively ameliorating the steatotic phenotype *in vitro* (Figure 27). In all co-treatments with FFAs and the identified compounds, cells showed reduced oxidative stress and lipid loading, not at the expense of cell viability. Specifically, on Sirolimus and Resveratrol, the platform succeeded in confirming the documented *in vitro* and *in vivo* anti-steatogenic effects (Ali et al., 2015; Berman et al., 2017; Bujanda et al., 2008; Charytoniuk et al., 2017; C. Wang et al., 2014; Wang, 2010). As the *in silico* analysis was non-directional, it also identified compounds that induce or deteriorate steatosis *in vitro*. In detail, Pimozide, Clomifene and Mefloquine underwent a verification process but were found to aggravate steatosis *in vitro* (Figure 28). Nonetheless, as all tests were performed at a compound concentration of 10uM, further investigation is required towards an optimal, compound-specific concentration.

A) **Resceratrol (RES)**
NF-kB supressor
Polyphenolic phytoalexin | Produced in plants



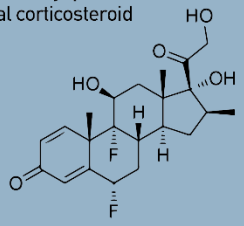
Differentially expressed genes:
PPARA, PTGS2, PI4K2B, NR1L3
Differentially affected pathways:
Metabolism of lipids and lipoproteins, NFAT, PPAR signalling, Nuclearrs, Fatty Acid Triacylglycerol nad ketone body metabolism

B) **Sirolimus (SIR)** (OR Rapamycin)
Blocks transcription activation of cytokines (IL2,IL4, IL15)
Macrolide from *Streptomyces hygroscopius*



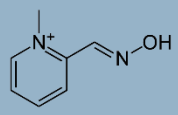
Differentially expressed genes:
ABCB1, SLC01B1
Differentially affected pathways:
Metabolism of lipids and lipoproteins, Nuclearrs pathway

C) **Diflorasone (DIF)**
Induction of phospholipase A2 ihnibitory proteins
Topical corticosteroid



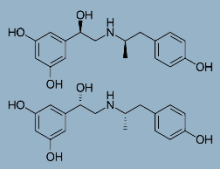
Differentially expressed genes:
NR3C1
Differentially affected pathways:
Apolipoprotein 1 pathway

D) **Pralidoxime (PRA)**
Inactivates acetylocholinesterase
Antidote to organophosphate pesticides and chemicals



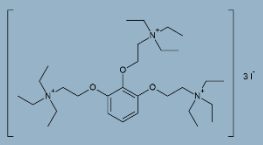
Differentially expressed genes:
ACHE
Differentially affected pathways:
Metabolism of lipids and lipoproteins, ATF2 pathway

E) **Fenoterol (FEN)**
Adrenergic-beta agonist
Used as brochondilator and tocolytic



Differentially expressed genes:
ADRB1, ADRB2
Differentially affected pathways:
Endocytosis pathway

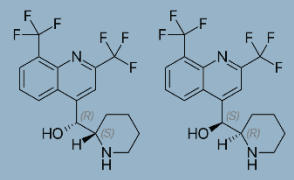
F) **Gallamine triethiotide (GAL)**
Competes with acetylocholine and binds to muscarinic acetylocholine receptors
Synthetic nondepolarizing drug



Differentially expressed genes:
ACHE
Differentially affected pathways:
Metabolism of lipids and lipoproteins

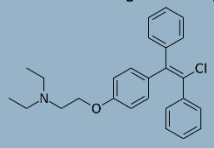
Figure 26 Promising compounds for drug repositioning: chemical structure, differentially expressed genes via GLS analysis, and differentially affected pathways via GSA analysis.

A) **Mefloquine (MEF)**
Phospholipid-interacting antimalarial drug



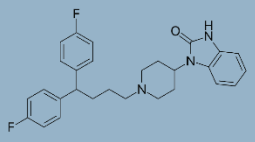
Differentially expressed genes:
ACHE, ABCB1
Differentially affected pathways:
Metabolism of lipids and lipoproteins, Nuclearrs pathway

B) **Clomifene (CLO)**
Estrogen agonist or antagonist depending on target
Stimulates release of gonadotropines



Differentially expressed genes:
ABCB1, ESR1
Differentially affected pathways:
Apolipoprotein 1 pathway, ATF2 pathway, Nuclearrs pathway

C) **Pimozide (PIM)**
Dopaminergic blocker
D2 receptor inhibitor
Antypsyxhotic agent



Differentially expressed genes:
ABCB1
Differentially affected pathways:
Nuclearrs pathway

Figure 27 Compounds found to aggrevate steatosis: chemical structure, differentially expressed genes via GLS analysis, and differentially affected pathways via GSA analysis.

Resveratrol

Resveratrol, or 3,5,4'-trihydroxystilbene, is a phytoalexin that is rich in polyphenols. Resveratrol, inhibits the reproduction of herpes simplex virus types 1 and 2 in a dose-dependent and reversible manner, as well as NF-kB activation, while it acts as anti-inflammatory and antioxidant. (Wishart et al., 2018).

Moreover, SIRT1, one of the mammalian versions of the sirtuin family of proteins, is activated by resveratrol. SIRT1 is a deacetylase that deacetylates histones and nonhistone proteins, such as transcription factors. Metabolism, stress resistance, cell survival, cellular senescence, inflammation-immune function, endothelial functions, and circadian rhythms are all affected by the SIRT1-regulated pathway. Because resveratrol has been proven to activate SIRT1, it is expected to help illnesses involving aberrant metabolic regulation, inflammation, and cell cycle abnormalities. Resveratrol has an effect on the NFKB signaling system, which controls inflammation, the immunological response to infection, and cellular responses to stimuli. Furthermore, it has been demonstrated to strongly block the IGF-1R/Akt/Wnt pathways while activating p53, influencing cell growth and cancer. Resveratrol can also block the PI3K/AKT pathway, which controls cell differentiation, growth, and proliferation, among other things.

Many have anticipated that resveratrol might be a viable therapeutic option for NAFLD since it has been proven to reduce inflammation, activate SIRT1, and imitate the effects of calorie restriction. However, there can be no conclusive deductions about resveratrol's efficacy as a therapy for NAFLD because it has only been evaluated in a small number of clinical trials, however some tendencies can be noticed. When a lower amount of resveratrol was given over a shorter period of time, it was proven to be helpful to patients with NAFLD, but when patients were given large dosages of resveratrol for prolonged periods of time, however, no positive effects were found. Overall, these trials appear to demonstrate that, while resveratrol may be a potential therapy for individuals with NAFLD, it may be most beneficial under certain settings and as a complement to lifestyle modifications (Berman et al., 2017; Charytoniuk et al., 2017).

Sirolimus

Sirolimus, a macrocyclic lactone generated by *Streptomyces hygroscopicus*,is a powerful immunosuppressant with antifungal and antineoplastic effects. Sirolimus prevents the transcriptional activation of cytokines and it is only bioactive when attached to immunophilins. It suppresses T cell activation and proliferation in response to antigenic and cytokine (IL-2, IL-4, and IL-15) stimulation by a mechanism unique from those of other immunosuppressants. Sirolimus

also suppresses the formation of antibodies and interacts with the immunophilin FK Binding Protein-12 (FKBP-12) in cells, forming an immunosuppressive complex. The complex of sirolimus: FKBP-12 does not affect calcineurin activity, but binds to and suppresses the activation of the important regulatory kinase mammalian Target Of Rapamycin (mTOR). This inhibitor inhibits cytokine-driven T-cell proliferation by preventing the cell cycle from progressing from the G1 to the S phase (Wishart et al., 2018).

Sirolimus has anti-lipid deposition effects in non-alcoholic fatty liver disease (NAFLD), although the mechanisms by which it reduces hepatic steatosis remain unknown.It has been indicated that sirolimus induces NAFLD in mice bu it has also been suggested that sirolimus is neither necessary nor sufficient to attenuate hepatic steatosis. Nonetheless, the vast majority of studies have demonstrated that rapamycin indeed alleviates hepatic lipid accumulation through down-regulating some important lipid metabolic enzymes including SREBP1c, SREBP2, fatty acid synthase, ACC, SCD1, and low-density lipoprotein receptor (Wang, 2010).

Diflorasone

Diflorasone is a topical corticosteroid that is used to treat the symptoms of inflammatory skin diseases such as erythema, pruritus, and pain. Diflorasone, like other topical corticosteroids, has anti-inflammatory, antipruritic, and vasoconstrictive effects. Topical corticosteroids, once absorbed via the skin, follow the same pharmacokinetic routes as systemically given corticosteroids. The specific mechanism of topical steroids' anti-inflammatory action in the treatment of steroid-responsive dermatoses, in general, is unknown. Corticosteroids, on the other hand, are hypothesized to function via inducing phospholipase A2 inhibitory proteins known as lipocortins. It is thought that these proteins regulate the production of powerful inflammatory mediators like prostaglandins and leukotrienes by limiting the release of their common precursor arachidonic acid. Phospholipase A2 liberates arachidonic acid from membrane phospholipids (Wishart et al., 2018).

Pralidoxime

Pralidoxime is a cholinesterase reactivator used in the treatment of organophosphate pesticides and chemicals poisoning. It is also used to avoid anticholinesterase drug overdosage in patients with myasthenia gravis. The purpose of pralidoxime is to reactivate cholinesterase, mostly outside of the central nervous system, that has been inactivated by phosphorylation caused by an organophosphate pesticide or similar chemical. The breakdown of stored acetylcholine can then begin, and neuromuscular connections can resume normal function. Pralidoxime reactivates the

enzyme cholinesterase within 24 hours of organophosphate exposure by cleaving the phosphate-ester link established between the organophosphate and acetylcholinesterase. Acetylcholinesterase inhibition allows acetylcholine to accumulate at synapses, causing cholinergic fibres throughout the nervous system to be stimulated continuously. Pralidoxime also slows the "ageing" process of phosphorylated cholinesterase to a non-reactivatable state and detoxifies some organophosphates through a direct chemical reaction. The drug's most important effect is the relief of respiratory muscular paralysis. Because pralidoxime is less efficient in alleviating respiratory centre depression, atropine is always administered concurrently to counteract the impact of accumulating acetylcholine at this location. Pralidoxime reduces muscarinic symptoms such as salivation and bronchospasm, although this effect is rather insignificant because atropine is enough for this purpose (Wishart et al., 2018).

Fenoterol

Fenoterol is a bronchodilator and beta-2 adrenergic agonist that is used to treat asthma symptoms by widening the airways to the lungs and lowering bronchoconstriction. The activation of beta(2)-receptors in the lung causes smooth muscle relaxation, bronchodilation, and increased bronchial outflow (Wishart et al., 2018).

Gallamine triethiotide

Gallamine triethiodide is a non-depolarizing blocking drug (NDMRD) that inhibits the cardiac vagus and can induce sinus tachycardia, hypertension, and increased cardiac output. The sensitivity of different muscle groups to various types of relaxants varies, with ocular muscles being the most sensitive, followed by muscles of the neck, mouth, limbs, and abdomen, while the diaphragm is the muscle that is least susceptible to NDMRDs. NDMRDs have a delayed start of action but a longer duration of action, making them more suited to preserving neuromuscular relaxation throughout major surgical procedures. This drug competes with acetylcholine (ACh) molecules and binds to muscarinic acetylcholine receptors on the motor endplate's post-synaptic membrane. It works by interacting with cholinergic receptor sites in a muscle and competitively inhibiting acetylcholine transmitter activity. It inhibits ACh activity and hinders the activation of the muscular contraction mechanism. It can also block the release of ACh by acting on nicotinic presynaptic acetylcholine receptors (Wishart et al., 2018).

Proposed compounds that induced steatosis

Mefloquine

Mefloquine is an antimalarial drug used to prevent and cure mild to moderate cases of malaria caused by *Plasmodium falciparum* and *Plasmodium vivax*. Mefloquine's mechanism of action is not entirely known. According to some research, mefloquine particularly targets the Plasmodium falciparum 80S ribosome, reducing protein synthesis and generating schizonticidal effects. There are several studies in the literature with little in vitro data on the mechanism of action of mefloquine (Wishart et al., 2018).

Clomifene

Clomifene is classified as an orally administered, non-steroidal, ovulatory stimulant that acts as a selective estrogen receptor modulator (SERM), used mainly in female infertility due to anovulation. Clomifene, as previously stated, has both estrogenic and anti-estrogenic characteristics, although the specific mechanism of action has yet to be established. Clomifene appears to stimulate the production of gonadotropins, follicle-stimulating hormone (FSH), and luteinizing hormone (LH), which leads to ovarian follicle growth and maturation, ovulation, and subsequent formation and activity of the corpus luteum, culminating in pregnancy. Clomifene does not appear to have any progestational, androgenic, or antiandrogenic effects, and it does not appear to interact with pituitary-adrenal or pituitary-thyroid function. It can interact with organs that have estrogen receptors, such as the hypothalamus, pituitary, ovary, endometrial, vagina, and cervix. It may compete with estrogen for estrogen-receptor-binding sites and may cause a delay in the replenishment of intracellular estrogen receptors. Clomifene causes a cascade of endocrine processes, culminating in a spike of preovulatory gonadotropin and subsequent follicular rupture. The first endocrine event that occurs in response to clomifene treatment is an increase in pituitary gonadotropin secretion that causes steroidogenesis and folliculogenesis, which leads to ovarian follicle development and a rise in the circulating amount of estradiol. Plasma progesterone and estradiol levels increase and fall after ovulation, just like they would in a normal ovulatory cycle (Wishart et al., 2018).

Pimozide

Pimozide is an orally administered diphenylbutylpiperidine that serves as an antipsychotic and an alternative to haloperidol in the treatment of Tourette syndrome individuals who have not responded to conventional therapy. Although the precise mechanism of action is unknown, it has been hypothesized that postsynaptic dopamine receptor blockade is involved. Pimozide's ability to

suppress motor and phonic tics in Tourette's syndrome is considered to be largely due to dopaminergic inhibiting action in the central nervous system. Pimozide binds to and inhibits the dopamine D2 receptor in the central nervous system. However, receptor blockage is frequently followed by a variety of secondary changes in central dopamine metabolism and function, which may contribute to both the therapeutic and adverse effects of pimozide. Furthermore, pimozide, like other antipsychotic medications, has a variety of effects on other central nervous system receptor systems that are not fully understood. Pimozide also has a lower propensity for causing drowsiness and hypotension than other neuroleptic drugs because it has more selective dopamine receptor blocking action (Wishart et al., 2018).

4.2. Proteomic profiling of the repositioned compounds

Proteomic profiling with a diverse panel of phosphorylated proteins and secreted cytokines was performed to deduce the compounds' signalling motifs. PCA and k-means clustering led to the formation of four clusters, named "Negative control", "FFA induction", "Steatosis reduction" and "Repositioned compounds" after the majority of treatments that comprised each of them. Firstly, this clustering revealed a distinct difference between all of the negative control samples (DMEM, etOH 1%, etOH 1% & DMSO 0.1%) and the FFA-treated samples, underlining that the variables of the analysis were able to distinguish between the healthy and the steatotic phenotype, based on their respective signalling mode of action. Most importantly, all co-treatments with the repositioned compounds, except for DIF, were found in the same cluster with the SIR+FFAs and RSV+FFAs, thus highlighting GAL's, FEN's and PRA's potential in ameliorating *in vitro* steatosis at the pathway level. Regarding DIF, when used in co-treatment with FFAs, it is clustered together with the FFA-treated controls, a finding that comes into conflict with DIF's observed capacity to lower lipid loading and oxidative stress. DIF's variability in the proteomic measurements is the primary reason for the misclassification, suggesting either a different mode of action than the one captured with the used proteomic panel, or a dose that was insufficient to reverse steatosis at the pathway level. The fourth cluster consisted of samples treated with the repositioned compounds alone, except for RSV and SIR that cluster with the anti-steatosis group, suggesting a high impact of RSV's and SIR's mode of action on this cluster. This impact can be attributed to the effect of these drugs on the proteins selected in the multiplex panel.

A more detailed statistical analysis of the results revealed significant variations on the phosphorylation levels of a number of proteins as well as on the excretion of cytokines both in steatosis samples and on the repositioned compounds' group.

The significant reduction of CREB1's phosphorylation upon treatment with the repurposed compounds compared to the steatosis group is in accordance with previous research. In three distinct insulin-resistant mouse models, lowering CREB expression in the liver reduced fasting plasma glucose and insulin concentrations substantially. In a T2DM rat model with fatty liver and hepatic insulin resistance, CREB inhibition enhanced hepatic insulin sensitivity and decreased plasma glucose concentrations (Erion et al., 2009). Moreover, it was reported that in the livers of rats abruptly exposed to PCB126, decreased phosphorylation of CREB is a critical event in the deregulation of gluconeogenesis and glycogenolysis (Gadupudi et al., 2016). CREB1's phosphorylation reduction is a characteristic of cAMP-stimulated signalling, which is required for the activation of diverse gene expression programs across various cell types in response to various hormonal stimuli. In response to feeding and fasting cues, CREB functions as a metabolic sensor that regulates the expression of many genes in the liver. Phosphorylation allows for a reversible method to control genes involved in gluconeogenesis and fatty acid oxidation, ensuring metabolic homoeostasis of carbohydrate, lipid, and amino-acid nutrition (Cui et al., 2021).

Phosphorylation levels of pSTAT3 and pPTN11 were also found increased in the steatosis sample group but were subsequently reduced in co-treatment with the repositioned compounds, as reported before in the literature. In fact, because of its well-documented role in the pathophysiology of NAFL/NASH, STAT3 has previously been recommended as a therapeutic target. Pharmacological inhibition of STAT3 activity, for example, was found to decrease lipid accumulation and modify STAT3-responsive microRNAs, including miR-21, in lipid-overloaded HepRG cells. Long-term metformin treatment decreased phospho-STAT3-miR-21 levels in the liver of C57/BL6 mice, protecting them from ageing-related hepatic vesicular steatosis, as seen in vivo (Belloni et al., 2018, p. 3). Moreover, a retrospective study showed that severe lobular inflammation, severe ballooning degeneration, and progressive fibrosis were all linked to pSTAT3 expression in hepatocytes and hepatic stellate cells (Choi et al., 2018, p. 3). Regarding pPTN11, Luo et. Al. have previously reported that deficiencies in both Shp2 and Pten Increased cJun expression/activation, as well as increased ROS and inflammation in the hepatic milieu, promote non-alcoholic steatohepatitis and the formation of liver tumour-initiating cells (Luo et al., 2016).

Phosphorylated levels of JUN have also been reported to increase in NAFL/NASH, per the findings reported in this study. Although the AP-1 transcription factor JUN is a key regulator of hepatic stress responses, its role in disease development is somewhat unclear. Nonetheless, a novel mouse model developed by Dorn et al., demonstrated significant pathological abnormalities similar to those seen in human NASH, indicating that c-Jun/AP-1 activation is a key regulator of hepatic alterations, and hypothesized that the presence of c-Jun in NAFLD aids in the establishment and

progression of NASH (Dorn et al., 2014). In another example, the function of c-Jun in NASH development was investigated mechanistically in mutant mice given a diet low in methionine and choline (MCDD) as increased c-Jun expression in hepatocytes was linked to NASH patients. They concluded that c-Jun expression in humans is associated with disease progression from steatosis to NASH, and it has cell-type-specific activities in mice as it increased cell survival in hepatocytes, reducing ductular response and fibrogenesis (Schulien et al., 2019). In this study, co-treatment with diflorasone, fenoterol, pralidoxime and gallamine triethiodide succeded in significantly reduced pJUN levels. On the contrary, resveratrol displayed no significant association with JUN phosphorylation levels, whereas sirolimus significantly reduced pJUN only when administered alone.

GSK3 showed a tendency in the rise in the FFA-treated group as well, albeit it was not statistically significant in the comparisons conducted. GSK3, one of the main kinases implicated in the development of fatty liver, reduces glycogen storage by phosphorylating glycogen synthase 2 and therefore inhibiting glycogen storage. Hepatic lipid accumulation and lipoapoptosis are both aided by GSK3 activation (Zhang et al., 2016, p. 3). It was reported that by inhibiting GSK3 phosphorylation of PPAR serine 73, biliverdin reductase A reduces hepatic steatosis (Hinds et al., 2016, p. 3). Furthermore, glycocoumarine has been proposed as a new active component against NAFL, since it inhibits hepatocyte lipoapoptosis via autophagy activation and suppression of the ER stress/GSK-3-mediated mitochondrial pathway (Ibrahim et al., 2011, p. 3). Following these findings, co-treatment with FFAs plus either fenoterol, gallamine triethiodide, or pralidoxime resulted in a substantial decrease in pGSK3 levels, whereas fenoterol had the same effect when administered alone as well.

FFA treatment on the contrary was found to significantly reduce RS6 and ERK phosphorylation levels. Regarding pRS6, this might come in contrast with previous research although its implication in NAFL/NASH is not yet understood or well documented. Immunohistochemistry for total RPS6 and phosphorylation of RPS6 in patient liver samples revealed that while all patients expressed RS6, patients with steatosis had higher RPS6 phosphorylation (Sapp et al., 2014). Furthermore, immunostaining on patient samples revealed a negative association between the development of liver disease and the phosphorylation of RS6. This protein is an important kinase substrate downstream of mTORC1 and AMPK (Scagliola et al., 2021). In the experimental process followed in this study, o-treatment with FFAs and diflorasone or sirolimus decreased its phosphorylation levels much further, which was also seen in cells treated with these compounds alone. When combined with FFAs, fenoterol resulted in a substantial decrease in pRS6, but not when used alone.

In this experiment, neither GAL nor resveratrol showed any influence on RS6 phosphorylation levels.

Phosphorylated ERK was likewise decreased in cells co-treated with FFAs, diflorasone and sirolimus, but co-treatment with fenoterol, gallamine triethiodide, pralidoxime, and resveratrol displayed no effect in its phosphorylation levels. When compared to the FFA treated group, treatment with sirolimus and diflorasone alone resulted in a higher reduction in pERK. In general, despite the fact that ERK1/2 has been linked to hepatic metabolism and is elevated in obese people, other studies show that ERK1/2 activity is unaffected. Furthermore, it is unknown if ERK1/2 activity is changed in obese people who proceed to NAFLD or NASH (Lawan and Bennett, 2017). In a study by Choi et al., pERK was expressed in hepatic stellate cells in all of the hepatic steatosis cases studied, and their findings suggest that pERK in hepatic stellate cells may be related to the development of hepatic steatosis (Choi et al., 2018). pERK has previously been linked to the development of hepatic steatosis and steatohepatitis in rat liver tissue. In earlier investigations, pERK signalling in activated hepatic stellate cells was found to be pro-fibrogenic in NAFLD and AFLD (Bai et al., 2017; Mahli et al., 2018).

In FFAs-treated HepG2 cells, phosphorylated AKT and AKTS1 were also decreased. In the case of pAKTS1, co-treatments with all of the repurposed compounds resulted in the same statistically significant decrease, but co-treatment with FFAs and fenoterol significantly lowered its phosphorylation levels. In comparison to both the control group and the FFA-treated cells, these alterations were determined to be substantial. When pralidoxime was given in combination with FFAs and DMSO, pAKTS1 was reduced as well, but when pralidoxime was administered alone, there was no significant difference between the FFAs treatment and the negative controls. Furthermore, when compared to FFA treated cells and their respective negative controls, fenoterol, gallamine triethiodide, and resveratrol resulted in a significant reduction in AKT phosphorylation levels. Gallamine triethiodide and sirolimus, on the other hand, seemed to have no effect on pAKT, whereas co-treatment with resveratrol and FFAs or resveratrol alone resulted in a significant increase in pAKT.

AKTS1 (or PRAS40) is a direct substrate of AKT. AKT, along with SGK1 and PKCa, are the most well-studied mTORC2 substrates. AKT activity is mediated by mTORC2, which phosphorylates AKT S473 and T450 cotranslationally in response to growth factor stimulation. mTORC2 indirectly regulates mTORC1 in response to growth factor signalling because AKT regulates it through phosphorylation of TSC2 and AKTS1. The mTOR signalling pathway regulates lipid homeostasis and adipogenesis among others (Lamming and Sabatini, 2013). Horst et al. evaluated the lipogenic

mTORC1 pathway, with its insulin-dependent (through AKTS1) in obese control subjects and obese patients with NAFLD. They reported that compared with fructose, glucose ingestion stimulated AKTS1 phosphorylation in control subjects but not in subjects with NAFLD (Horst et al., 2021). Furthermore, mounting data suggests that hepatocyte dysregulation of the PI3K/AKT pathway is a frequent molecular event linked to metabolic dysfunctions such as obesity, metabolic syndrome, NAFLD. This pathway is also one of the upstream the mTOR pathway, along with AMPK and RAS/RAF/MEK/ERK signalling pathways. mTOR is a key hub in the regulation of autophagy, that its regulation is liked with a multitude of liver diseases including NAFL. In the PI3k/AKT pathway PTEN overexpression has been found to suppress insulin signalling, including reduced AKT activity and GLUT4 translocation to the cell membrane, indicating a role in the development of IR and eventually NAFLD. Downregulation of PTEN, on the other hand, causes enhanced glucose absorption in response to insulin. PTEN deficiency, on the other hand, induces NAFLD and HCC. This paradox's mechanism has yet to be discovered (Calvisi et al., 2011; Matsuda et al., 2013; Wang et al., 2019).

Although phosphorylation patterns of pNFKB, pFAK1, pP38-MAPK, and pSMAD3 were not significantly altered in FFA-treated cells, co-treatment with repurposing drugs did. In comparison to the control group, co-treatment with diflorasone, fenoterol, and sirolimus decreased pFAK1. In contrast to FFAs and the negative control group, sirolimus substantially decreased pFAK1 in both co-treatment with FFAs and when delivered alone. FAK has not been studied extensively for its implication in NAFLD, but it is supported to have a key role in promoting hepatic stellate cells (HSCs) activation in vitro and liver fibrosis progression in vivo. It is found to be involved in the stimulation of hepatic stellate cells (HSCs) in vitro and the development of liver fibrosis in vivo. In fibrotic living tissues, FAK activation is linked to increased expression of aSMA and collagen. FAK activation is induced by TGF-1 in a dosage and time-dependent manner. In TGF-1 treated HSCs, inhibiting FAK activation limits the production of aSMA and collagen, as well as the creation of stress fibres. In addition, inhibiting FAK activation decreases HSC migration and small GTPase activation, as well as causing apoptosis in TGF-1 treated HSCs. Notably, FAK inhibitor decreases collagen and -SMA expression in an animal model of liver fibrosis and attenuates liver fibrosis in vivo. These findings show that FAK is required for HSC activation and the development of liver fibrosis and that the FAK signalling pathway might be a viable target for liver fibrosis (Zhao et al., 2017).

Similarly, as compared to the negative control group and the FFA treated cells, cells treated with pralidoxime, fenoterol, and FFAs had reduced pNFKB. In comparison to the FFAs-treated group and the corresponding controls, sirolimus dramatically decreased NFKB levels. It's worth noting

that only diflorasone and sirolimus lowered pNFKB levels when compared to the negative control group. NFKB has been implicated in various aspects of NAFL, and its downregulation has been proposed as an anti-steatotic target. It is well documented that, FFAs and ROS can initiate the activation of NFKB, which leads to increased pro-inflammatory cytokines production, and insulin resistance in the liver. NFKB activation can boost the production of inflammatory cytokines, and inflammatory substances can boost NF-B activity, even more, exacerbating the inflammation. As mentioned before, PPARa is considered to have anti-inflammatory effects through complicated control of NFKB. It is supported that adiponectin activates which in turn could inhibit the NFKB pathway, and PPARa agonists are considered prominent candidates for NAFL/NASH therapy. Inflammatory cytokines can activate the JNK, p38, and NFKB signalling pathways, which can lead to an increase in the production of inflammatory cytokines. Furthermore, by activating AMP kinase, adiponectin can limit fatty acid production and boost fatty acid beta-oxidation, preventing steatosis and increasing insulin sensitivity, but it can also activate PPARa and block the NFkB pathway. Another aspect includes the inhibition of AMPK activity by resistin in the liver and skeletal muscle, obstructs insulin signalling via the PI3K/Akt pathway, and induces nuclear transcription of the NFKB gene (Zeng et al., n.d.).

On the same front and contrast with other findings, phosphorylation of p38-MAPK was found unaltered in this study. Only administration of sirolimus alone was found to significantly reduce pP38-MAPK. MAPKs, which comprise cell ERKs, JNK, and p38-MAPK, are a kind of serine/threonine protein. Under stress conditions, p38 signalling pathways are engaged in cell inflammatory responses and apoptosis, which are linked to the production of a range of inflammatory cytokines following activation. To investigate the mechanism of action of an antioxidant in high-fat-diet rats, Sinha-Hikim et al. examined the phosphorylation of JNK and p38 signal levels in high-fat-diet-induced NAFLD rats, finding a significant increase in both phospho-JNK and pp38-MAPK levels compared to the normal control group, which was significantly reduced after antioxidant treatment, implying that oxidative stress can cause NAFLD (Zeng et al., n.d.). Both in the cases of p38-MAPK and NFKB, it is possible that the concentration of the administered FFAs was insufficient to induce an inflammatory process.

Concerning pSMAD3 levels, they were significantly elevated only when fenoterol was administered with FFAs. SMAD proteins have been widely investigated as TGF-1's intracellular effectors and transcription factors. SMAD3 and SMAD4 are pro-fibrotic in hepatic fibrosis, but SMAD2 and SMAD7 are protective. Type I collagen expression is inhibited and the epithelial-myofibroblast transition is blocked when SMAD3 is deleted. SMAD2 disruption, on the other hand, increases type I collagen expression. By increasing SMAD3 responsive promoter activity, SMAD4 plays an important role in

fibrosis disease, whereas SMAD7 inhibits SMAD3-induced fibrogenesis. Because SMAD3 plays such an important part in fibrosis, inhibiting SMAD3 signalling might be a promising target for fibrotic treatment (Xu et al., 2016). Taking this into account, fenoterol could induce fibrotic mechanisms in higher concentrations.

Overall, a small number of variations in secreted cytokines were identified. This can be attributed to the fact that HepG2 cells independent of other cells such as Kupffer or HSCs, participate in a differential and lower cytokine response, which differs according to the compound and the time of exposure. Treatment with FFAs resulted in an increase in IL12 and a small reduction in CCL2 and IL8, however, no statistically significant difference was seen.

When cells were co-treated with FFAs plus either resveratrol, sirolimus, fenoterol, or gallamine triethiodide, CCL2 secretion was significantly reduced compared to FFA-treated cells, while their levels were comparable to FFAs-treated cells. When compared to the control group, the reduction observed in co-treatment with sirolimus was found to be statistically significant. In contrast to that, CCL2 has been found to increase in the livers of animals with NASH caused by a high-fat diet. CCL2's pathogenic component, stimulation of PPARa, directly led to fat buildup in hepatocytes. In numerous animal models of metabolic disorders, pharmacological therapies blocking the CCL2/CCR2 pathway has been reported to reduce obesity, insulin resistance, hepatic steatosis, and inflammation in the adipose tissue. Only a few human research have looked at the function of CCL2 in the pathology of NAFLD, and it is supported that patients with NAFLD exhibited low-grade systemic inflammation and had greater CCL2 levels in their blood than healthy people. Several findings show that CCL2 plays a significant role in the development of mild steatosis to NASH, and although the significance of CCL2 in metabolic disorders has to be investigated further, these results show that CCL2 may have a direct role in NAFLD and, in particular, NASH (Pan et al., 2020; Vincent Braunersreuther and ois Mach, 2012). Finally, CCL2 is predominantly secreted by macrophages and, with a smaller amount secreted by activated endothelial cells and HSCs but not by hepatocytes. This might could resonate with the absence of CCL2 secretion by HepG2 cells observed in this study.

CCL3 secretion, on the other hand, was shown to be considerably enhanced when cells were co-treated with diflorasone, fenoterol, or sirolimus with FFAs, as compared to FFA-treated cells and the respective negative control. When gallamine was co-treated with FFAs, CCL3’s secretion was likewise considerably enhanced, but only when compared to the control group. Pralidoxime and resveratrol, on the other hand, only increased CCL3 secretion when given alone. In contrast to these findings, CCL3 and CCL4 are increased in mouse liver fibrogenesis models and human

fibrosis samples, promote mouse liver fibrosis by increasing HSC proliferation and migration and are linked to stellate cell activation and liver immune cell infiltration, in contradiction to these results (Chen et al., 2018). Hence, similar to the case of CCL2 the absence of CCL2 secretion by HepG2 cells observed can be attributed to the fact that this chemokine is mainly secreted by other liver cell types.

IL12 showed yet another substantial variation between treatments with all of the repositioned drugs and was found increased in the FFAs group. In comparison to the negative control samples, co-treatment with FFAs and resveratrol, gallamine triethiodide, diflorasone, or sirolimus substantially elevated IL12, however only co-treatment with sirolimus was found significant in comparison to FFAs-treated cells. When compared to the negative control, treatment with all six substances alone raised IL12 secretion substantially. According to the literature, IL-12 has a link to the severity of NAFLD, and IL12 in the circulation can be used as one of the supporting techniques in evaluating the severity of NAFLD (Darmadi and Ruslie, 2021).

In contrast, whereas diflorasone or fenoterol alone resulted in a substantial decrease in IL8 secretion when compared to the control group, they were shown to enhance it when co-treated with FFAs. IL8 levels in gallamine triethiodide and pralidoxime levels were reduced in the same way when provided alone, but in co-treatment, they were raised to those of FFA-treated samples. Finally, when cells were treated with sirolimus and FFAs, IL8 was considerably higher than the control group. There is minimal evidence that it has a role in NAFLD. When compared to hepatosteatosis or a healthy control group, serum levels of IL8 were substantially greater in NASH patients. Furthermore, IL8 serum levels were linked to NASH on their own. Another research, on the other hand, found no evidence of this link or any substantial changes in serum IL8 levels (Vincent Braunersreuther and ois Mach, 2012).

In terms of the less altered secreted proteins, GROA was shown to be increased in co-treatment with diflorasone and further enhanced when provided alone as compared to FFA treated cells. When compared to the negative controls, fenoterol, gallamine triethiodide, and resveratrol treatment alone enhanced GROA secretion. GROA has been implicated in the development of HCC along with CCL2, but sufficient data on its role in NAFLD pathogenesis exist so far (Vincent Braunersreuther and ois Mach, 2012). LCN2, GROA, and CXCL9 were found overexpressed and localized differences in the liver of fatty liver of mice and hypothesized that these proteins play an important role in the development of NASH (Semba et al., 2013).

In comparison to the control group, MMP9 was likewise enhanced when FFAs were co-treated with diflorasone and sirolimus. MMP9 secretion was similarly enhanced by sirolimus alone.

Contradicting to this, using data mining and gene expression analysis, MMP9 levels were found as predictors of poor prognosis in patients with NAFLD. The proteins of two major genes, FABP4 and MMP9, were measured in the blood and utilized as non-invasive prognostic indicators for NAFL and NASH development, according to this study (Coilly et al., 2019).

RETN and FGF both presented with a similar trend. Gallamine triethiodide and sirolimus alone were shown to increase RETN when compared to the respective negative control, as well as when combined with FFAs, and the decrease aster treatment with sirolimus was found to be significant when compared to both the control group and the FFA-treatment group. Resveratrol and sirolimus alone led to an increase of FGF secretion levels, but in co-treatment with sirolimus this decrease was significant only when compared to the control group. Finally, NRG1 secretion was increased by SIR.

RETN is produced in liver cells amongst others, and its implication in NAFLD pathogenesis is unclear. It is indicated that its synthesis appears to rise as liver damage progresses. In histologically verified NAFLD investigations, individuals with simple fat buildup or non-alcoholic steatohepatitis had greater circulating levels of RETN than controls. However, studies have found that people with simple steatosis had similar RETN levels to those with steatohepatitis (Colica and Abenavoli, 2018). Several members of the FGF family, including FGF1, FGF19, and FGF21, have been demonstrated to control hepatic lipid metabolism by targeting FGF receptors. As a result, changes in FGF signalling may play a role in the development of NAFLD. Although several clinical trials are now underway to assess the safety and effectiveness of FGF-mimetics in the treatment of NAFLD, the mechanisms through which FGFR activity modulates hepatic lipid metabolism remain largely unknown (He et al., 2017). Finally, it has been recently reported that NRG1 may have a protective role in the development of NAFLD by modulating the activation of the PI3K-AKT pathway through ErbB3 phosphorylation (Meng et al., 2021, p. 1).

All in all, cytokines and chemokines are mainly secreted by macrophages and, with a smaller amount secreted by activated endothelial cells and HSCs but not by hepatocytes. This might could resonate with the absence of secretion of cytokines by HepG2 cells observed in this study.

4.3. Limitations of the proposed pipeline

The present study comes with certain limitations. The use of 2D *in vitro* models, although sufficient for screening, cannot recapitulate the complexity met in the *in vivo* processes (Müller and Sturla, 2019). Limitations imposed by the simplified 2-dimensional steatosis models can be surpassed with additional *in vivo* and *in vitro* studies on representative and physiologically relevant 3D liver

models. Lastly, the clustering of treatments can only be indicative of the HCS-based screening and the proteomic measurements, hence a broader proteomic panel and additional biochemical measurements could be sufficient to shuffle the clusters. Nevertheless, this study is contributing to the integration of an *in silico* and an *in vitro* screening pipeline for DR and acknowledges that additional experiments are needed to decipher the compounds' mode of action and anti-steatogenic effect.

4.4. Conclusion

The implemented drug repurposing approach successfully identified 6 compounds, including the known anti-steatogenic drugs Resveratrol and Sirolimus. In short, Gallamine triethiotide, Diflorasone, Fenoterol and Pralidoxime ameliorate steatosis similarly to Resveratrol/Sirolimus. Moreover, since the computational analysis was non-directional, Clomifene, Mefloquine and Pimozide were found to aggravate steatosis in accordance with the existing literature. Proteomic profiling of the repositioned compounds successfully identified CREB1, PTPN11 and STAT3 as potential targets in ameliorating liver steatosis, as confirmed by previous studies.

In conclusion, this framework allows for the evaluation of a great number of compounds, at the early stages of drug discovery, by combining the large compound-examination capacity, offered by the *in silico* models, with the rigor of the *in vitro* validation. The attempted implementation actively saves up screening time, as several candidates were eliminated *in silico*, long before their verification *in vitro*. Albeit the gap between *in vitro* validation and clinical efficacy, the proposed framework enables the exploration of the large chemical space and delivers promising compounds for subsequent *in vivo* efficacy studies. This strategy provides a basis for evaluating the repositioning potential of widely used drugs, even beyond NAFL, and, as such, the illustrated framework holds significant potential in assisting the treatment of several diseases.

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