SUPPLEMENTAL MATERIALS

Antropometric measures

Height and weight were measured and body mass index (BMI) calculated as weight (kg)/stature $(m)^2$. Waist circumference was measured at the part of the trunk midway between the most caudal part of the lateral costal arch and the iliac crest in the morning before breakfast, after lavatory visit with the person standing with feet about 25-30 cm apart. The circumference was measured to the nearest 0.5 cm, at the end of a normal expiration. Hip circumference was measured as the maximal circumference over the buttocks.

Dual X-Ray Absorptiometry

Body composition was assessed by dual X-ray absorptiometry (DEXA), using a Lunar DPX-L scanner (Lunar Corporation, WI, USA). Scanning mode was determined from the body mass index by the scanner software (version 1.3).

Blood samples analyses

Peripheral blood samples were drawn at 8:00 a.m. after an overnight fast. Plasma glucose, glycated hemoglobin (HbA1c), transaminases, γGlutamyl transferase, lipid profile and blood count were measured by routine analysis. Insulin was measured by ARCHITECT Insulin assay (Abbott Laboratories), a chemiluminescent microparticle immunoassay (CMIA).

Proteomics

Monocytes, isolated from 3 mL of heparinized blood, or HSC obtained from liver biopsies, were mixed with lysis buffer, containing 100µL of 5mM Tris-HCl buffer at pH 8.0, 0.1M Triton X-100 and protease inhibitor cocktail, for 30 minutes on ice. Cell lysates were centrifuged at 4˚C for 10 minutes at 1,500 rpm. Supernatants were stored in aliquots at -80˚C until analyses and pellet discharged. Pierce™ BCA Protein Assay Kit measured the protein concentrations. One-hundred µg of protein per sample were digested by filter-aided sample preparation (1).

Trypsin-catalyzed 16O/18O labelling was performed according to Qian et al. (2) and Petritis et al. (3) iTRAQ labelling was performed using the reagents and protocols supplied in the 8Plex Multiplex kit. Each dried iTRAQ-labelled peptide sample was dissolved in 15 µl of 0.1% ammonium formate and then separated by nano-LC-MS/MS using an Eksigent ekspert TM nano-LC 425 system coupled with a TripleTOF 6600 System (SCIEX). Peptides were eluted at a flow rate of 300 nl/min into a reverse phase C18 column using a linear gradient of acetonitrile (3–36%) in 0.1% formic acid with a running time of 120 minutes. Mass spectra and tandem mass spectra were recorded. The nanospray needle voltage was 2,300 V. The mass window for precursor ion selection of the quadrupole mass analyzer was ±2 m/z. We used Proteome Discoverer 1.4 with the SEQUEST algorithm to search the original MS/MS protein data in the specified non-redundant databases. The differential protein abundance in monocytes and in HSCs between liver fibrosis and absence of liver fibrosis was calculated by ANOVA. Q values were computed adjusting the p-values found using an optimized False Discovery Rate (FDR) approach with parameters set with a strict target FDR at <0.0001 (4).

Human Primary hepatocytes isolation

Hepatocytes from obese, NAFLD subjects and healthy subjects were processed and collected in an icebox at 4 °C under aseptic conditions, as described elsewhere (5). Tissue obtained during percutaneous liver biopsy, approximatively 3 cm length, 2 cm were diced into smaller pieces, <3 mm each, and washed in HBSS to remove excess blood. Tissue was then transferred to a tube containing pre-warmed EGTA buffer (HBSS, 0.5 mM EGTA, 0.5% fatty acid free bovine serum albumin (BSA)) and agitated (100 rpm) in a water bath with shaking bed for 10 min, 37 °C. After three washes, to remove the remaining blood and EGTA, the tissue was placed in pre-warmed digestion buffer (HBSS, 0.05% collagenase IV, 0.5% fatty acid free BSA, 10 mM CaCl2) and agitated (100 rpm) in a water bath with shaking bed for 30 min, 37 °C. The digested tissue was filtered through 100 µm cell strainer while the remaining tissue

was again digested in fresh digestion buffer. Cell suspensions were pooled and centrifuged at 80 g for 5 min, 4 °C and the supernatant discarded. Cells were grown on cover slips until confluent and used for immunofluorescence analysis or directly stained with PLIN2 for flow cytometry analysis.

Human Primary Hepatic Stellate cells isolation

Hepatic stellate cells from obese, NAFLD subjects and healthy subjects were isolated from resected liver human tissue as described elsewhere (6). Briefly, liver biopsy was placed in a Petri dish, washed 5 times with a sterile phosphate-buffered saline (PBS) solution to remove residual blood from the tissue surface and finely minced into small pieces of less than 3 mm each. The liver was then digested using pronase and collagenase solution (Merck, Darmstadt, DE) followed by density gradient centrifugation (1,380g for 17 min at 4 °C without brake) using Nicodenz (Merck, Darmstadt, DE) to remove non-parenchymal cells. Isolated cells were grown on cover slips until confluence and used for immunofluorescence or directly stained with RAB14 for flow cytometry analysis.

Cell viability and cell purity after isolation

To assess cell viability, hepatocytes and hepatic stellate cells were stained with propidium iodide and analyzed by flow cytometry. Cell purity was assessed by flow cytometry by staining primary hepatocytes with GLUT2 Alexa Fluor® 488-conjugated antibody and hepatic stellate cells with α-Smooth Muscle Actin PE-conjugated antibody. Flow cytometry analysis for both primary hepatocytes and hepatic stellate cells are reported in supplemental figure 7 A-D.

Isolation of Peripheral Blood Mononuclear Cells

Blood (3 ml) from subjects with obesity and NAFLD and from healthy subjects, was collected using EDTA as an anticoagulant and processed within 2 hours. Peripheral blood mononuclear cells (PBMCs) were obtained from whole blood by standard gradient centrifugation over lympholyte®-h cell separation media. In brief, blood was diluted 1:1 (vol/vol) in PBS, carefully

layered on lympholyte®-h cell separation media and centrifuged at room temperature for 25 min at 1,600 × g. PBMCs were washed, resuspended in PBS and used for flow cytometry. CD14+CD16- monocytes were isolated from PBMCs using classical monocyte isolation kit, following manufacturer's instruction. Using a cyto-centrifuge (Hettich, Tuttlingen, DE), cells were deposited on the slides and used for immunofluorescence analysis.

Immunofluorescence

Nile red staining of neutral lipids was used to identify lipid droplets accumulation in primary hepatocytes. Hepatocytes were fixed in 4% paraformaldehyde for 10 min at room temperature and stained with Nile Red (100 ng/mL) for 10 minutes at room temperature. Nuclear staining was performed with DAPI. PLIN2 staining was performed on primary hepatocytes and monocytes, while RAB14 staining was performed on primary HSCs and monocytes. Cells were fixed in 4% paraformaldehyde for 10 min at room temperature, permeabilized with PBS containing 0.1% Triton X-100 and blocked with 1% BSA. Primary antibodies were incubated overnight at 4 °C while secondary antibodies were incubated for 1 hour at room temperature in the dark. Nuclear staining was performed with DAPI and slides were mounted on ProlongGold.

Flow Cytometry

All flow cytometry data were acquired on 9-color/3-laser Cytoflex flow cytometers (Beckman Coulter, Brea, CA) and were analyzed with Kaluza software (Beckman Coulter, Brea, CA). CytoFLEX Daily QC beads were run as quality control to verify optical alignment and fluidics system. Fluorescence target channels were met for all channels by using CytoFLEX Daily QC beads as well, and the acquisition settings were defined by using VersaComp Antibody Capture Kit.

Gating strategies for both primary hepatocytes and PBMCs are reported in supplemental figure 8 A,B. The technician doing flow cytometry measurements was blinded to the sample identity.

The levels of PLIN2 and RAB14 in monocytes are reported as Median Fluorescence intensity (MFI). MFI represents the number of antibodies that recognize and attach to the cell antigens, allowing the exact quantification of antigen expression per cell.

Validation parameters

All validation procedures, unless specified otherwise, were performed on PBMCs (1x106 cells/ml) from subjects with obesity and NAFLD (N=3), following the standard operating procedures (SOP). Conventional sample acceptance criteria include non-hemolyzed and unclotted conditions. Assay reproducibility and stability as well as limit of detention and quantification are reported in online supplemental table 7.

Intra-assay precision (repeatability)

Each sample was processed in triplicate and run one time. Samples were processed and assayed on the same day to avoid the influence of sample instability. For the statistical evaluation, the mean of %CV was calculated for all samples (Mean %CV) and the intra-assay precision described as the Mean %CV.

Inter-assay precision (reproducibility)

Each sample was processed in triplicate and run two times. Samples were processed and assayed on the same day to avoid the influence of sample instability. Since the inter-assay samples were run on the same day, every effort was made to guarantee that the runs were independent of each other. Indeed, we have performed, for each analytical run, separate sample processing and acquisition. For the statistical evaluation, the mean of the %CV was calculated for each sample (Mean %CV) and the inter-assay precision was reported as the Mean %CV.

Lower limit of blank/detection

For determine the lower limit of blank/detection we have used a sample partially stained (CD14 staining) by omitting antibodies (Anti-ADFP antibody [EPR3713] (Alexa Fluor® 488) and RAB14 (D-5)). Through this strategy, the antigen of interest will not be detected.

Limit of Quantification

For determine the limit of quantification healthy volunteer (N=3) that minimally express the antigen of interest were used.

Antibody Tritation Curve

Antibody tritation was performed to identifying the correct concentration of antibody, to ensure the antibody performs within acceptable parameters and to eliminate nonspecific antibody binding. Serial concentrations (0.0625 µg/µl, 0.125 µg/µl, 0.25 µg/µl, 0.5 µg/µl and 1 µg/µl) of Anti-ADFP antibody [EPR3713] (Alexa Fluor® 488) were used to stain the samples. Serial concentrations (0.04 µg/µl, 0.1 µg/µl, 0.2 µg/µl, 0.4 µg/µl, and 0.6 µg/µl) of RAB14 (D-5), conjugated with Alexa Fluor® 488 secondary antibody, were used to stain the samples. Antibody tritation curves are reported in supplemental figure 8 C,D.

Cryopreservation of PBMCs

PBMCs were fixed for 45 min and then immediately stored at -80°C. Cells were thawed rapidly in a water bath at 37°C, washes and resuspended in PBS 1X. Staining for PLIN2, RAB14 CD14 and CD16 are reported in supplemental figure 9 A,B.

Reagents

We have provided a detailed list of reagents in online supplemental Table 8.

STATISTICS

We aimed to verify whether our biomarker predicted SAF-A levels. The whole dataset was split into a discovery and a validation sample with an equal number of subjects with NASH according to the SAF-A values; absence of NASH if SAF-A=0; presence of NASH if SAF-A≥1.

First, we tested those variables in addition to PLIN2 that were representative of metabolic, lipidic, and hepatic profiles, and were predictive of SAF-A, using a univariate analysis (Supplemental Table 1). We then assessed in a multivariate logistic model whether PLIN2 MFI, waist circumference, age, plasma glucose, triglycerides and ALT were accurate in predicting the presence (SAF-A≥1) or absence (SAF-A=0) of histologic activity. A stepwise elimination procedure was applied to find the subset of predictors, resulting in the best performing model. The variables entering the final model were PLIN2, waist circumference, glycemia and age (Supplemental Table 2).

Then, we ascertained whether our algorithm was able to predict the severity of SAF-A. For this purpose, the SAF-A score was recoded as a 3-level variable: SAF-A_level=0 if SAF-A=0; SAF-A_level=1 if SAF-A=1; and SAF-A_level=2 if SAF-A≥2 and a multinomial model, including only significant predictors in a stepwise regression, used.

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Supplemental Table 1. Results from the univariable logistic model for SAF-A prediction (presence/absence)

Variable	Beta coefficient	P value
PLIN2 (MFI)	2.774	3.25E-05
Waist circumference (cm)	0.120	3.03E-08
AST/Platelet index	3.690	0.100
Gender (male = 1)	0.020	0.963
Diabetes (presence = 1)	3.020	0.004
Age (years)	0.210	4.95E-08
BMI (kg/m^2)	0.312	1.61E-08
Hips Circumference (cm)	0.164	7.87E-07
HDL (mg/dl)	-0.038	0.001
LDL (mg/dl)	0.011	0.083
Triglycerides (mg/dl)	0.0278	0.0001
Cholesterol (mg/dl)	0.016	0.026
Glycemia (mg/dl)	0.101	7.13E-06
Insulinemia (IU/ml)	0.539	5.29E-05
HOMA-IR ((μIU/mL)(mmol/L)/22.5)	2.573	1.86E-05
AST (IU/l)	0.168	0.0003
ALT (IU/l)	0.145	4.84E-05
GGT (IU/l)	0.085	0.0017

In bold the variables that were tested in a multivariable model and that were chosen as representative of the metabolic (glycemia), lipidic (triglycerides) and hepatic profiles (ALT) along with PLIN2 (MFI), waist circumference and age

Supplemental Table 2. Final multivariable logistic model after a step regression procedure for SAF-A prediction (presence/absence)

Variable	Beta coefficient	P value
Intercept	-75.040	0.0015
PLIN2 (MFI)	2.971	0.0152
Waist circumference (cm)	9.028	0.0063
Age (years)	8.708	0.0034
ALT (IU/l)	0.145	4.84E-05

All variables were log-transformed

Supplemental Table 3. Classes of drugs used for diabetes, hypertension and hyperlipidemia treatment in the discovery and validation cohorts

	Discovery Cohort	Validation Cohort
Anti-Diabetes Medications		
SGLT2 inhibitors	60%	60%
GLP1 RAs	30%	35%
Pioglitazone	40%	45%
DPP IV inhibitors	10%	5%
Metformin	100%	100%
Anti-Hypertension Medications		
Beta-blockers	65%	60%
ACE inhibitors	70%	70%
Angiotensin II receptor blockers	30%	40%
Calcium channel blockers	15%	20%
Anti-Hyperlipidemic Medications		
Statins (Atorvastatin)	80%	75%
Cholesterol Absorption Inhibitors (Ezetimibe)	20%	25%

Percentage of patients using a specific class of drugs among those with T2D, hypertension and hyperlipidemia.

Supplemental Table 4. Final multinomial logistic model after a step regression procedure for SAF-A level prediction (SAF-A_level=0 if SAF-A=0; SAF-A_level=1 if SAF-A=1; and SAF-A_level=2 if SAF-A≥2)

Variable		Beta coefficient	P value
Intercept	level 1	-59.83898	0.015
	level 2	-71.00549	0.003
PLIN2 (MFI)	level 1	0.03320633	0.984
	level 2	4.24301361	0.005
Waist circumference (cm)	level 1	11.904624	0.012
	level 2	9.314543	0.032
Age (years)	level 1	8.215290	0.007
	level 2	7.477506	0.009
Glycemia (mg/dl)	level 1	-5.9964274	0.194
	level 2	-0.5078786	0.907

Supplemental Table 5. Results from the univariable logistic model for SAF-F prediction (presence/absence)

Variable	Beta coefficient	P value
RAB14 (ng/ml)	-0.09796	0.001042
Elastography	2.38349	4.88E-06
Waist circumference (cm)	0.153981	1.36E-06
Gender (male = 1)	0.212175	0.653057
Diabetes (presence = 1)	17.53943	0.98749
Age (years)	0.170324	4.62E-07
BMI (kg/m^2)	0.405609	1.14E-06
Hips Circumference (cm)	0.514379	0.010899
HDL (mg/dl)	-0.05248	0.000168
LDL (mg/dl)	0.014881	0.045473
Triglycerides (mg/dl)	0.024294	0.000229
Cholesterol (mg/dl)	0.011876	0.102235
Glycemia (mg/dl)	0.112788	1.10E-05
Insulinemia (IU/ml)	0.230373	0.000296
HOMA-IR ((μIU/mL)(mmol/L)/22.5)	1.041393	0.00011
AST (IU/l)	0.119634	0.005087
ALT (IU/l)	0.117491	0.000433
GGT (IU/l)	0.019475	0.130297

In bold the variables that were tested in a multivariable model and that were chosen as representative of the metabolic (glycemia), lipidic (HDL) and hepatic profiles (ALT) along with Rab14 or Elastography, waist circumference and age

Supplemental Table 6. Multinomial logistic model for SAF-F level prediction (SAF-F_level=0 if SAF-F=0; SAF-F_level=1 if SAF-F=1; and SAF-F_level=2 if SAF-F≥2) for individuals who underwent surgery procedure

Variable		Beta coefficient	P value
Intercept	level 1	60.61844	0.274
	level 2	30.86940	0.608
RAB14 (ng/ml)	level 1	-12.17215	0.110
	level 2	-15.95249	0.040
Waist circumference (cm)	level 1	-12.98499	0.273
	level 2	-12.31895	0.314
Age (years)	level 1	8.537065	0.211
	level 2	12.042225	0.114
Glycemia (mg/dl)	level 1	-0.2664244	0.959
	level 2	0.9475479	0.878
HDL (mgdl)	level 1	1.489295	0.426
	level 2	4.846866	0.085
ALT (IU/l)	level 1	-0.4477087	0.811
	level 2	0.3593118	0.867

Supplemental Table 7. Assay Reproducibility and Sensitivity

	PLIN2	**RAB14**
Intra-Assay Precision (%)	9.2	5.1
Inter-Assay Precision (%)	9.5	2.3
Limit of Detection (MFI)	0.43±0.02	0.55±0.01
Lower limit of blank (MFI)	0.7±0.05	0.8±0.02

Supplemental Table 8. List of reagents

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Human Glut2 Alexa Fluor® 488-conjugated Antibody	R&D Systems, Minneapolis, NE	FAB1414G
Human alpha-Smooth Muscle Actin PE-conjugated Antibody	R&D Systems, Minneapolis, NE	IC1420P
CD14-Pacific Blue	Beckman Coulter, Brea, CA	B00846
CD16-Phycoerythrin/Cyanin 7	Beckman Coulter, Brea, CA	6607118
Anti-ADFP antibody [EPR3713] (Alexa Fluor® 488)	Abcam, Cambridge, UK	ab201535
Goat Anti-Mouse IgG, Alexa Fluor® 488	Merk, Darmstadt, DE	SAB4600387
RAB14 (D-5)	Santa Cruz Biotechnology, Dallas, TX	sc-271401
Goat anti-Mouse IgG, Alexa Fluor® 568	Thermo Scientific, Waltham, MA	A-11031
Tris-HCl	Sigma-Aldrich, St. Louis MO	10812846001
Triton X-100	Merk, Darmstadt, DE	11754599001
Halt™ Protease Inhibitor Cocktail (100X)	Thermo Scientific, Waltham, MA	78429
Ammonium formate	Sigma-Aldrich, St. Louis MO	70221
Formic acid	Sigma-Aldrich, St. Louis MO	F0507
Hanks' Balanced Salt Solution (HBSS)	Merk, Darmstadt, DE	1.00496
EGTA	Merk, Darmstadt, DE	E3889
Fetal Bovine Serum	Thermo Scientific, Waltham, MA	16000044
Collagenase IV	Thermo Scientific, Waltham, MA	17104019
Pronase	Merk, Darmstadt, DE	10165921001
Lympholyte®-h cell separation media	Cedarlane Lab, Burlington, CA	CL5010
Phosphate-buffered saline	Thermo Scientific, Waltham, MA	10010023
4% paraformaldehyde solution	Merk, Darmstadt, DE	HT501128
Nile Red	Thermo Scientific, Waltham, MA	N1142
DAPI	Thermo Scientific, Waltham, MA	D1306
ProLong™ Gold Antifade	Life Technologies, Carlsbad, CA	P36931
ARCHITECT Insulin assay	Abbott Laboratories, Chicago, IL	8K41
Pierce™ BCA Protein Assay Kit	Thermo Fisher Scientific, Waltham,MA	23225
iTRAQ® Reagent - 8PLEX Multiplex Kit	Sigma-Aldrich, St. Louis MO	4381663
Classical Monocyte Isolation Kit, human	Miltenyi Biotec, Bergisch Gladbach,DE	130-117-337
CytoFLEX Daily QC beads	Beckman Coulter, Brea, CA	B53230
VersaComp Antibody Capture Kit	Beckman Coulter, Brea, CA	B22804