Supplemental Material for
Altered Vitamin A Metabolism in Human Liver Slices Corresponds to Fibrogenesis
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Liver Preparation for Cell Sorting and Flow-cytometry
Liver tissue wedges were sequentially perfused with Washing buffer (1 × HBSS and 10 mM HEPES, pH 7.2-7.5), Perfusion buffer, Washing buffer, and the recirculating Collagenase buffer at a flow rate of 12 mL/min (Gilson’s MINIPULS 3, Middleton, WI, USA). Perfusion buffer contained 1 × Hank’s Balanced Salt Solution (HBSS, without Ca2+, Mg2+, or phenol red, from Gibco), 10 mM HEPES (pH 7.2-7.5) and 0.5 mM EDTA (pH 8.0). Collagenase buffer contained 1 × HBSS (Gibco), 5 mM MgCl2, 5 mM CaCl2, 5 mM HEPES (pH 7.2-7.5), 0.5% w/v Collagenase IV (Sigma-Aldrich, St. Louis, MO, USA), 0.25% w/v Protease (Sigma), 0.125% w/v Hyaluronidase (Sigma), 0.05% w/v DNase I (Sigma). Fresh aliquots of enzymes were added to the buffer on the day of the perfusion. 40 mL of Perfusion buffer and 20 mL of Collagenase buffer were used for each 10 g of liver tissue. Buffers were pre-warmed to 37°C prior to the perfusion step.

The perfused liver tissue samples were gently mashed with a sterile syringe plunger through a sterile mesh strainer in ice-cold DMEM medium. The cell suspensions were filtered through a 100μm sterile strainer and centrifuged at 50g at 4°C for 3 minutes to separate hepatocytes in the pellets from non-parenchymal cells in the supernatant. The supernatants were transferred to a new tube and kept on ice. The pellets went through three cycles of washing with ice-cold DMEM and being pelleted at 50g at 4°C for 3 minutes. To further improve the purity of isolated hepatocytes, the cell pellets were resuspended in 5mL of ice-cold PBS, overlaid with 10mL of 25% Percoll gradient solution. This was centrifuged at 1,400g (no brake) at 4°C for 20 minutes. The pellet, containing purified live hepatocytes, was stored for RNA extraction if the viability was greater than 50%. Viability was determined using the trypan blue exclusion assay (Thermo Fisher Scientific, Waltham, MA, USA). The non-parenchymal cells (NPC) in the supernatants were centrifuged at 500g at 4°C for 7 minutes. The pellets were resuspended in 5mL of ice-cold PBS, overlaid with 10mL of 50% and 25% Percoll gradients, and centrifuged at 1400g (no brake) at 4°C for 20 minutes. Cell layers were collected into 20mL PBS each, and centrifuged again at 500g at 4°C for 7 minutes. A fraction of the pellets, containing total NPC, were saved for RNA extraction. The rest of the pellets were resuspended with ice-cold Flow buffer containing 1X PBS, 2% FBS, and 1 mM EDTA.

The isolated non-parenchymal cells were stained with an antibody mixture that included eBioscience (San Diego, CA, USA): anti-CD45 (Cat. No. 15-0459-42); BioLegend (San Diego, CA, USA): anti-CD3 (Cat. No. 317330), anti-CD11b (Cat. No. 553310), anti-CD14 (Cat. No. 301834), anti-CD31 (Cat. No. 303120), anti-CD32 (Cat. No. 303206), anti-CD68 (Cat. No. 333814), and anti-CD271 (Cat. No. 345110). In addition, cells were also stained with LIVE/DEAD Fixable Far Red Dead Cell Stain Kit (Cat. No. L10120, Life Technologies, Carlsbad, CA, USA). The incubation mixture was kept at 4°C for 30 min in the dark on a rocking platform. The mixture was centrifuged at 500g at 4°C for 7 min. Cells were washed once with Flow buffer. The antibody-
labeled cells were sorted with a BD Aria III (BD Biosciences, San Jose, CA, USA). Analysis of cell populations was performed using FlowJo software, version 9.8.5 (FlowJo, LLC, Ashland, OR, USA). Kupffer cells were selected as the CD45+, CD3−, CD14+, CD68+, CD32+ populations.1,2 LSECs were selected as CD45−, CD31+, CD11b−.3 HSCs were selected as CD45−, CD271+, autofluorescence positive with the emission wavelength at 460 nm, SSC-H > 150 units.4 Cells were pelleted at 500 × g at 4°C for 7 min and were stored in -80°C before RNA extraction.

**Liver slice preparation, histology and cultures**

Liver cores were cut into 250 μm-thick slices using a vibrating microtome, Leica VT1200 S (Nussloch, Germany) with Dulbecco’s Modified Eagles Medium (DMEM) as the cutting medium, as previously described.5 Day 0 slice samples were immediately removed from the DMEM to either be fixed or stored at -80°C. The remaining liver slices were cultured individually on 0.4 μm millicell organotypic inserts in 24 well plates (Millipore Corporation, Billerica, MA, USA). Slices were cultured in 1X advanced-DMEM medium, 5% Fetal Bovine Serum (FBS), 1X GlutaMAX, 0.5X Penicillin-Streptomycin, 1X Insulin-Transferrin-Selenium supplement, and 15 mM HEPES (pH 7.2 - 7.5) (Gibco, Grand Island, NY, USA) and maintained on a rocking platform at 17 rpm in a humidified incubator at 37°C and 5% CO2. The medium was changed every two to three days.

For liver slice histological staining, slices were fixed using 10% neutral-buffered formalin at room temperature for 24 hours. The fixed liver slices were embedded in paraffin and were sliced into 4 μm-thick sections. Trichrome stain, picrosirius red stain, and alpha-smooth muscle actin stain followed the standard protocol in the Pathology Research Services Laboratory at Department of Pathology at University of Washington. The images were recorded with Nanozoomer Whole Slide Scanner (Hamamatsu City, Shizuoka Pref., Japan) and visualized with NDP.view2 software (Hamamatsu).

**RNA extraction and multiplex qPCR from human liver slices**

The RNA of liver slices or the sorted liver cells was isolated with TRIzol and the Direct-zol RNA MiniPrep Kit (Zymo Research, Irvine, CA, USA) and converted to cDNA using the QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany). Next, a pre-amplification was performed using cDNA as templates, 0.2 fold-diluted primer mixture of TaqMan assays of interest (Thermo Fisher Scientific), and BIO-X-ACT Short Mix reagents (Bioline USA Inc., Taunton, MA, USA). The pre-amplified samples were diluted five-fold with RNase- and DNase-free H2O. Samples were analyzed with a 48 × 48 dynamic array and a BioMark HD microfluidics system (Fluidigm, San Francisco, CA, USA). The Fluidigm Real-Time PCR Analysis software was used to calculate Ct thresholds, using the settings of quality threshold 0.65, baseline correction linear, Ct threshold method auto detection. Typically, three replicates of liver slices from each patient were analyzed for each time point. CT values were normalized to the average of three house-keeping genes ACTB, GAPDH and HPRT1 and expressed as ‘Relative Abundance’, 2^-ΔCT.

**Kinetic modeling of vitamin A metabolic flux and signaling**

A kinetic model of the Vitamin A metabolic flux in the liver slices (normalized to 1 mg liver tissue) was constructed using MATLAB and Simulink platform (R2018a; MathWorks, Natick, MA). The flux model was established based on known metabolic pathways for vitamin A in human liver and included retinyl palmitate, retinol, retinaldehyde, and arRA as shown in Figure S1. All model parameters are summarized in Table S1, and all governing equations are described below. Specifically, the retinyl ester and retinol were connected via hydrolysis and LRAT-
dependent retinyl esterification; retinol and retinal were connected via oxidation and reduction (e.g. unspecified RDH and Dhrs enzymes); retinol also has additional elimination pathway incorporated in the model that represents previously identified retinol elimination\(^6\) and secretion as RBP4-retinol bound complex from the liver\(^7,8\); retinal is metabolized to form atRA via ALDH1A-dependent oxidation and atRA is eliminated via CYP26. The developed model was first optimized and used to conduct steady state simulations of retinoid concentrations in liver tissue after continuous infusion of retinol. The baseline rate constants of the kinetic model were verified by comparing the simulated concentration ratios to observed concentration ratios\(^9\) of ester/retinol and retinol/atRA as shown in Figure S2.

To define the potential changes in enzyme activities that could explain the observed time-dependent changes in retinoid concentrations observed in human liver slices over 96-hour culture, the individual enzyme expressions/activities were altered in a stepwise manner based on known regulation and alterations in primary retinoid metabolizing enzymes. Specifically, five scenarios with enzymatic changes as function of time were simulated: 1) LRAT expression/activity was decreased; 2) CYP26 expression was induced in addition to scenario 1; 3) retinal reductase expression/activity was decreased in addition to scenario 2; 4) retinol oxidase expression/activity was decreased in addition to scenario 3; and 5) retinol additional elimination pathway was induced in addition to scenario 4. To initiate the time course simulations for endogenous retinoids, experimentally determined baseline retinoid concentrations in liver slices (Table S2) were used as initial conditions at \(t=0\) and the simulations were conducted for 96 hours. The MATLAB model file and a representative code script are included as Supplementary Material.

To define which one of the kinetic scenarios 1-5 described above is most plausible to explain the observed kinetic changes in vitamin A metabolism in the liver slice model, the tissue concentrations of retinyl palmitate-\(d_6\), retinol-\(d_6\), and atRA-\(d_6\) in human liver slices were simulated for 4 hours after dosing retinol-\(d_6\) at 0-hour, 12-hours, and 44-hours post-slicing. In addition, a scenario similar to scenario 5 described above but including a low baseline retinal reductase expression/activity and non-decreasing retinal reductase and retinol oxidase with time to reflect potential donor specific differences was included. The simulations were initiated with dosing 20 pmol retinol-\(d_6\) and allowed to proceed for 4 hours, using the simulated midpoint enzyme expression/activity value at 2-hour, 14-hour, and 46-hour post-slicing from above as constant parameters.

**Supplementary Figure 1.** The schematic diagram of the kinetic model describing the metabolic flux of vitamin A in human liver which includes retinyl palmitate, retinol, retinaldehyde, and atRA. The \(k_{\text{esterification}}, k_{\text{hydrolysis}}, k_{\text{oxi}}, k_{\text{other}}, k_{\text{red}}, k_{\text{ALDH}}, \text{and } k_{\text{CYP26}}\) represent the rate constant of retinol esterification, retinyl ester hydrolysis, retinol oxidation, retinol additional elimination, retinaldehyde reduction, ALDH-mediated retinal metabolism and CYP26-mediated atRA metabolism in the retinoid flux model system, respectively. The detailed parameter values are summarized in Table S1.
Retinoid Flux Model Equations

The differential equations for the retinoid flux model are listed below:

\[
\frac{dA_{\text{ester}}}{dt} = k_{\text{esterification}}A_{\text{retinol}} - k_{\text{hydrolysis}}A_{\text{ester}}
\]

\[
\frac{dA_{\text{retinol}}}{dt} = k_{\text{hydrolysis}}A_{\text{ester}} + k_{\text{red}}A_{\text{retinal}} - A_{\text{retinol}}(k_{\text{esterification}} + k_{\text{oxi}} + k_{\text{other}})
\]

\[
\frac{dA_{\text{retinal}}}{dt} = k_{\text{oxi}}A_{\text{retinol}} - k_{\text{red}}A_{\text{retinal}} - k_{\text{ALDH}}A_{\text{retinal}}
\]

\[
\frac{dA_{\text{atRA}}}{dt} = k_{\text{ALDH}}A_{\text{retinal}} - k_{\text{CYP26}}A_{\text{atRA}}
\]

where, the \(A_{\text{ester}}, A_{\text{retinol}}, A_{\text{retinal}}, \) and \(A_{\text{atRA}}\) represent the amount of retinyl ester, retinol, retinal, and \(atRA\) in the retinoid flux model system, respectively. The \(k_{\text{esterification}}, k_{\text{hydrolysis}}, k_{\text{oxi}}, k_{\text{other}}, k_{\text{red}}, k_{\text{ALDH}}, \) and \(k_{\text{CYP26}}\) represent the rate constant of retinol esterification, retinyl ester hydrolysis, retinol oxidation, retinol additional elimination, retinal reduction, ALDH-mediated retinal metabolism and CYP26-mediated \(atRA\) metabolism in the retinoid flux model system, respectively. The details of all rate constant values are summarized in Table S1.

The retinoid flux model developed here is normalized to 1 mg liver tissue, therefore:

\[
C_{\text{ester}} = \frac{A_{\text{ester}}}{1 \text{ mg liver tissue}}
\]

\[
C_{\text{retinol}} = \frac{A_{\text{retinol}}}{1 \text{ mg liver tissue}}
\]

\[
C_{\text{retinal}} = \frac{A_{\text{retinal}}}{1 \text{ mg liver tissue}}
\]

\[
C_{\text{atRA}} = \frac{A_{\text{atRA}}}{1 \text{ mg liver tissue}}
\]

To explore the plausibility of mechanistic hypotheses that may explain the time-dependent changes of endogenous and \(d_6\)-labeled retinoids observed in human liver slices, multiple important pathways or enzyme expression/activity levels were altered in the simulations as shown below:

\[
\frac{dE_{\text{LRAT}}}{dt} = -k_{\text{LRAT}}E_{\text{LRAT}}
\]

\[
\frac{dE_{\text{RDH,oxi}}}{dt} = -k_{\text{RDH,oxi}}E_{\text{RDH,oxi}}
\]

\[
\frac{dE_{\text{RDH,red}}}{dt} = -k_{\text{RDH,red}}E_{\text{RDH,red}}
\]

\[
\frac{dE_{\text{other}}}{dt} = -k_{\text{syn,other}} \left( 1 + \frac{E_{\text{max,other}} \cdot C_{\text{atRA}}}{EC_{50,\text{other}} \cdot C_{\text{atRA}}} \right) - k_{\text{deg,other}}E_{\text{other}}
\]
\[
\frac{dE_{\text{CYP26}}}{dt} = -k_{\text{syn,CYP26}} \left( 1 + \frac{E_{\text{max,CYP26}}c_{\text{atRA}}}{E_{\text{C50,CYP26}} + c_{\text{atRA}}} \right) - k_{\text{deg,CYP26}} E_{\text{CYP26}}
\]

where \( E_{\text{LRAT}} \), \( E_{\text{RDH,oxi}} \), \( E_{\text{RDH,red}} \), \( E_{\text{other}} \), and \( E_{\text{CYP26}} \) represent the amount of enzyme responsible for retinyl esterification, retinol oxidation, retinal reduction, retinol additional elimination, and CYP26-mediated \( \alpha \text{tRA} \) metabolism in the retinoid flux model system, respectively. The \( k_{\text{LRAT}} \), \( k_{\text{RDH,oxi}} \), and \( k_{\text{RDH,red}} \) represent the declining rate constant to empirically describe the reduction in expression/activity of LRAT and enzymes responsible for retinol oxidation and retinal reduction, respectively. For induction, both retinol additional elimination pathway enzyme(s) and CYP26 enzyme were hypothesized to be induced by hepatic \( \alpha \text{tRA} \) concentration. The \( k_{\text{syn,other}} \) and \( k_{\text{syn,CYP26}} \) represent the synthesis rate of retinol additional elimination enzyme(s) and CYP26, respectively; the \( k_{\text{deg,other}} \) and \( k_{\text{deg,CYP26}} \) represent the degradation rate of retinol additional elimination enzyme(s) and CYP26, respectively; the \( E_{\text{max,other}} \) and \( E_{\text{max,CYP26}} \) represent the maximum fold induction for retinol additional elimination enzyme and CYP26, respectively; the \( EC_{50,\text{other}} \) and \( EC_{50,\text{CYP26}} \) represent the concentration of \( \alpha \text{tRA} \) that gives half-maximal response of induction for retinol additional elimination enzyme and CYP26, respectively. The detailed values of all relevant parameters governing enzyme changes are summarized in Table S1.

Assuming the level of retinol additional elimination enzyme(s) and CYP26 reached steady state at time\( =0 \), then:

\[
k_{\text{syn,other}} = k_{\text{deg,other}} E_{\text{other},t=0}
\]

\[
k_{\text{syn,CYP26}} = k_{\text{deg,CYP26}} E_{\text{CYP26},t=0}
\]

Therefore, if

\[
E'_{\text{other}} = \frac{E_{\text{other}}(t)}{E_{\text{other},t=0}}
\]

\[
E'_{\text{CYP26}} = \frac{E_{\text{CYP26}}(t)}{E_{\text{CYP26},t=0}}
\]

by substitution of \( k_{\text{syn}} \):

\[
\frac{dE'_{\text{other}}}{dt} = k_{\text{deg,other}} \cdot \left( 1 + \frac{E_{\text{max,other}}c_{\text{atRA}}}{EC_{50,\text{other}} + c_{\text{atRA}}} - E'_{\text{other}} \right)
\]

\[
\frac{dE'_{\text{CYP26}}}{dt} = k_{\text{deg,CYP26}} \cdot \left( 1 + \frac{E_{\text{max,CYP26}}c_{\text{atRA}}}{EC_{50,CYP26} + c_{\text{atRA}}} - E'_{\text{CYP26}} \right)
\]

For all enzymes, the enzyme amount was assumed to be directly and proportional (linearly) associated with the rate constant of the respective enzyme-mediated biotransformation, therefore:
\[ k_{\text{esterification}}(t) = k_{\text{esterification},t=0} \cdot \frac{E_{\text{LRAT}}(t)}{E_{\text{LRAT},t=0}} \]

\[ k_{\text{oxi}}(t) = k_{\text{oxi},t=0} \cdot \frac{E_{\text{RDH},\text{oxi}}(t)}{E_{\text{RDH},\text{oxi},t=0}} \]

\[ k_{\text{red}}(t) = k_{\text{red},t=0} \cdot \frac{E_{\text{RDH},\text{red}}(t)}{E_{\text{RDH},\text{red},t=0}} \]

\[ k_{\text{other}}(t) = k_{\text{other},t=0} \cdot \frac{E_{\text{other}}(t)}{E_{\text{other},t=0}} \]

\[ k_{\text{CYP26}}(t) = k_{\text{CYP26},t=0} \cdot \frac{E_{\text{CYP26}}(t)}{E_{\text{CYP26},t=0}} \]
**Supplementary Table 1.** The summary of model parameter values used in the kinetic model describing the metabolic flux of vitamin A in human liver. The definition of each parameter was described in detail in the Supplementary Methods. The values of all kinetic rate constants were verified using the steady state simulations (Figure S4) which agreed with observed retinoids ratios. The induction and degradation parameters of CYP26 have been previously published and the induction parameters of k\(_{\text{other}}\) (retinol additional elimination pathway) were assumed to be the same as the induction parameters of CYP26 except for the maximum fold induction.

| Parameter   | Unit   | Value  |
|-------------|--------|--------|
| k\(_{\text{hydrolysis}}\) | hr\(^{-1}\) | 0.01 |
| k\(_{\text{esterification}}\) | hr\(^{-1}\) | 0.2 |
| k\(_{\text{oxi}}\) | hr\(^{-1}\) | 0.2 |
| k\(_{\text{red}}\) | hr\(^{-1}\) | 4 |
| k\(_{\text{other}}\) | hr\(^{-1}\) | 0.1 |
| k\(_{\text{ALDH}}\) | hr\(^{-1}\) | 0.1 |
| k\(_{\text{CYP26}}\) | hr\(^{-1}\) | 1 |
| k\(_{\text{LRAT}}\) | hr\(^{-1}\) | 0.05 |
| k\(_{\text{RDH, oxi}}\) | hr\(^{-1}\) | 0.02 |
| k\(_{\text{RDH, red}}\) | hr\(^{-1}\) | 0.3 |
| k\(_{\text{deg, other}}\) | hr\(^{-1}\) | 0.014 |
| E\(_{\text{max, other}}\) | - | 3 |
| EC\(_{\text{50, other}}\) | umol/L | 0.09 |
| k\(_{\text{deg, CYP26}}\) | hr\(^{-1}\) | 0.014 |
| E\(_{\text{max, CYP26}}\) | - | 33 |
| EC\(_{\text{50, CYP26}}\) | umol/L | 0.09 |

**Supplementary Figure 2.** Steady state simulations of retinyl ester, retinol, retinaldehyde, and atRA tissue concentrations using the developed vitamin A metabolic flux model after continuous infusion of retinol. The simulated steady state ratio of ester/retinol is approximately 20 and the simulated steady state ratio of retinol/atRA is approximately 200, which are consistent with the observed tissue concentration ratios reported previously.
Supplemental Figure 3: Retinyl ester (palmitate) and retinol concentrations as a function of time in culture during liver slice culturing. Human liver slices were cultured on Transwell plates. Slices and media samples were taken on t=0, 12, 24, 48, 96, and 168 hours (168h for two subjects only) and the vitamin A metabolites were quantified using LC-MS/MS. Concentrations in slices were normalized to the slice weight in mg. The time course is shown for retinyl ester palmitate in slices (A) and media (C), and. Retinol in slices (B) and media (D) for up to four days in tissue (n=4-5 donors, duplicates) and in media (n=3-4 donors). The inset of panel A shows 2 donors on different scale. Statistical outliers (blue symbols) were excluded for retinol tissue measurements in panel B. All data are shown as mean ± SD within replicates in a given donor.
Supplemental Figure 4: Temporal changes to the expression of genes involved in Vitamin A homeostasis (A-D). Human liver slices were prepared from a single donor and cultured on Transwell plates for up to one week prior to RNA extraction and gene expression analysis. Assays were run on samples from 1-3 slices per time point depending on sample quality, normalized to the mean of three housekeeping genes and basal expression at t=0. Data is shown as mean or mean ± SD where appropriate.
**Supplementary Table 2: Summary of basal retinoid concentrations in the liver slices from individual donors for time course (donors 1-5) and activity (donor 6) assays.** Values indicated with a > sign were above the highest concentration (upper limit of quantification) of the standard curve during LC-MS/MS quantification.

| Donor | Retinyl Ester (Palmitate) (pmol/mg) | Retinol (pmol/mg) | Retinoic Acid (pmol/mg) | Fold Change RA at t=12h |
|-------|----------------------------------|------------------|------------------------|------------------------|
| 1     | 557.2                            | 13.2             | 0.044                  | 6.0                    |
| 2     | >1000                            | 22.7             | 0.045                  | 57.8                   |
| 3     | 167.5                            | 2.1              | 0.011                  | 2.3                    |
| 4     | 508.6                            | 16.1             | 0.040                  | 94.6                   |
| 5     | 27.2                             | 1.1              | 0.018                  | 2.1                    |
| 6     | >1250                            |                  |                        |                        |
Supplementary Figure 5. Simulations of \textit{d}_6-labeled retinoids (retinyl ester, retinol, and \textit{at}RA) concentrations in human liver slices over 4 hours in culture after dosing retinol-d6 at 0-hour (shown in black), 12-hour (shown in red), and 44-hour (shown in blue) post-slicing using the developed retinoid flux model in 3 additional scenarios that complement the 4 scenarios in Figure 7: 1) LRAT expression/activity was decreased (A-C); 2) LRAT expression/activity was decreased and CYP26 expression was induced (D-F); and 3) LRAT and oxidase expression/activity were decreased, CYP26 and retinol additional elimination pathway was induced, the baseline retinal reductase expression/activity was assumed to be low and does not decrease over time (G-I). The left, middle, and right columns show \textit{d}_6-labeled retinyl ester, retinol, and \textit{at}RA respectively.
Supplemental References

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