Tracking biochemical changes induced by iron loading in AML12 cells with Synchrotron live cell, time-lapse infrared microscopy

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ABSTRACT

Hepatocytes are essential for maintaining homeostasis of iron and lipid metabolism in mammals. Dysregulation of either iron or lipids has been linked with serious health consequences, including non-alcoholic fatty liver disease (NAFLD). Considered the hepatic manifestation of metabolic syndrome, NAFLD is characterised by dysregulated lipid metabolism leading to a lipid storage phenotype. Mild to moderate increases in hepatic iron have been observed in approximately 30% of individuals with NAFLD; however, direct observation of the mechanism behind this increase has remained elusive. To address this issue, we sought to determine the metabolic consequences of iron loading on cellular metabolism using live cell, time-lapse Fourier transform infrared (FTIR) microscopy utilising a synchrotron radiation source to track biochemical changes. Use of Synchrotron FTIR is non-destructive and label-free, and allowed observation of spatially-resolved, sub-cellular biochemical changes over a period of 8 hours. Using this approach, we have demonstrated that iron loading in AML12 cells induced perturbation of lipid metabolism congruent with steatosis development. Iron loaded cells had approximately three times higher relative ester carbonyl concentration compared to controls, indicating accumulation of triglycerides. The methylene/methyl ratio qualitatively suggests the acyl chain length of fatty acids in iron loaded cells increased over the 8 hour period of monitoring compared to a reduction observed in the control cells. Our findings provide direct evidence that mild to moderate iron loading in hepatocytes drives de novo lipid synthesis, consistent with a role for iron in the initial hepatic lipid accumulation that leads to development of hepatic steatosis.

INTRODUCTION

Iron and lipids are essential for maintaining normal cellular function. The biological functions of iron are primarily dependent on its ability to mediate electron transfer, essential for mitochondrial respiration, DNA biosynthesis and to catalyse enzymatic reaction [1]. This capacity also makes iron a strong pro-oxidant, requiring cells and organisms to tightly regulate iron metabolism to prevent oxidative damage [2]. The role of lipids within cellular function is far more varied, being major constituents of membranes, and being involved in cell signalling and energy storage. In mammals, the liver is a focal point for regulation of both iron and lipid metabolism [2, 3]. However, despite multiple levels of regulation, dysregulation of iron and lipids can occur, and may result in either accumulation or deficiency [4-6].

NAFLD has emerged as a major global health problem of the 21st century [7]. Currently estimated to affect 20-30% of the world’s population, NAFLD has become one of the most prevalent chronic liver diseases. Considered the hepatic manifestation of the metabolic syndrome, NAFLD is associated with insulin resistance, obesity, type II diabetes and cardiovascular disease [8-10]. NAFLD ranges from
simple steatosis to non-alcoholic steatohepatitis (NASH), and represents a spectrum of chronic liver diseases [11]. A significant proportion (20-30%) of individuals with simple steatosis progress to NASH, potentially leading to end stage liver failure and hepatocellular carcinoma [11, 12]. Even without disease progression, individuals display increased morbidity and mortality related to cardiovascular disease and a number of different cancers [13].

Iron overload has been demonstrated to significantly alter hepatic lipid metabolism in mouse models of NASH development [14, 15]. In these models, iron overload down-regulates pathways involved in lipid catabolism and increases lipogenesis pathways. Furthermore, we have previously shown that iron overloaded mice exhibited an increase in hepatic cholesterol and an upregulation of many genes involved in the cholesterol biosynthesis pathway [16]. Disruptions in these pathways are known to be associated with hepatic accumulation of lipids, clearly demonstrating that iron has a role in dysregulation of lipid metabolism within NASH development.

Mild to moderate iron loading is observed in approximately 30% of individuals who present with NAFLD [17, 18]. However, the mechanism by which iron accumulates within these individuals remains unclear. The physiological impact of iron loading on NAFLD has focused primarily on disease progression, given that oxidative stress is a large component of NASH [19, 20]. It is well established that excess iron causes oxidative stress in NASH via direct damage to macromolecules, increased pro-inflammatory cytokines and reduced very low density lipoprotein secretion, leading to cell death [19]. Although the role iron plays in disease progression has been investigated, the effect of iron overload on initial disease development is not well understood. Excess iron is stored within hepatocytes, which are the main cell type responsible for regulating hepatic uptake, catabolism and lipogenesis of lipids. It is therefore unsurprising that iron-induced disruptions in hepatocyte function are associated with metabolic dysregulation [21].

Recently, the advantages of complementary, “multi-modal” imaging techniques have become increasingly recognised. This is especially true in the case of synchrotron-based techniques, where X-ray fluorescence microscopy (XFM) provides the capability to reveal elemental information that complements biochemical distributions revealed by FTIR. To date, FTIR and XFM have proved invaluable for associating alterations in cell metal homeostasis with biochemical markers of altered metabolism, oxidative stress or protein misfolding [22-24].

FTIR microscopy is an established vibrational spectroscopy technique frequently used to characterise the biochemical profiles of complex biological samples [25, 26]. In FTIR microscopy, molecular bonds representing different chemical functional groups absorb infrared radiation at different wavelengths resulting in unique absorbance fingerprints [27]. These absorbance fingerprints allow for label-free,
non-destructive determination of relative concentration and distribution of protein, lipids, carbohydrates and nucleic acids [25]. Investigation of biological samples using FTIR microscopy has primarily been limited to dried cells and tissues because of a strong interference by water [28]. However, coupling a synchrotron infrared light source to an FTIR microscope provides a substantial increase in beam throughput, allowing the intense IR beam to be focused on to individual live cells and to pass through the strongly absorbing aqueous medium, which together leads to an improvement of spectral quality in terms of signal-to-noise ratio [29, 30]. Additionally, reducing the absorption contribution from water by limiting the path length through which infrared radiation must travel within an aqueous solution by use of a custom FTIR-compatible microfluidic chamber, allows biologically relevant spectra to be acquired [31, 32].

The objective of this study was to investigate the extent to which iron overload would perturb lipid metabolism leading to a steatosis phenotype. To achieve this, we utilised synchrotron XFM and FTIR microscopy to study changes in iron and lipid concentration and distribution in single, live, iron loaded AML12 cells. We observed significant perturbations in lipid metabolism within iron loaded cells over an 8 hour period, consistent with steatosis development and indicating a potential role for iron in NAFLD development.

MATERIALS AND METHODS

Cell Culture

The mouse hepatocyte cell line, AML12 (CRL-2554TM), was maintained in Dulbecco’s Minimum Essential Medium / Ham’s F12 (DMEM/F-12; Sigma, Sydney, Australia) supplemented with 5% foetal calf serum (FCS), 10 µg/mL insulin, 5 µg/mL transferrin, 7 ng/mL selenium (ITS; Gibco) and 40 ng/mL dexamethasone (Sigma, Sydney, Australia), 1000 units/mL penicillin and 1000 µg/mL streptomycin (maintenance medium). Cells were incubated at 37 °C in an humidified atmosphere of 95% air / 5% CO₂.

Live cultures were transported to the Australian Synchrotron for live cell FTIR microscopic measurement and allowed to recover for 48 hours prior to passaging. Approximately 15,000 cells were seeded on to 1 mm thick CaF₂ windows (Crystran Ltd, Poole, Dorset, UK) and allowed to adhere for 24 hours. At the start of the experiment, maintenance medium was removed and replaced with experimental medium (maintenance medium containing 0.5% FCS instead of 5% FCS), with or without 30 µg/mL ferric ammonium citrate (FAC; Sigma, Sydney, Australia) and incubated at 37 °C in an humidified atmosphere. For XFM analysis, approximately 20,000 AML12 cells were seeded on to 1 µm thick silicon nitride membranes (MCN, Melbourne, Australia) and allowed to adhere for 24 hours. Following 12 or 24 hour incubations in experimental media detailed above, cells were fixed with 3% paraformaldehyde and 1.5% glutaraldehyde in PBS followed by three rinses in tris-glucose buffer and air dried.
FTIR flow cell

For live cell FTIR microscopy, a modified Bioptechs (Butler, PA, USA) FCS3 flow cell was utilised. This modified setup replaced the glass windows with FTIR-compatible CaF$_2$ windows (40 mm diameter, 1 mm thick). The bottom CaF$_2$ window had drilled inlets with T-channels milled to improve medium flow across the entire sample area (Crystran Ltd, Poole, Dorset, UK). A Kapton™ (DuPont, Wilmington DE, USA) sealing spacer of 7.6 µm thickness was placed between the top and bottom CaF$_2$ windows and was stamped out as previously described [31]. The spacer was used to provide an optical path length short enough to avoid complete absorbance by the OH vibration of water (1650 cm$^{-1}$), while simultaneously preventing compression of the cells being studied. A closed loop of silicone tubing filled with culture medium, connected between the flow cell inlet and outlet connections, provided a reservoir to sustain the cells during measurement.

Synchrotron FTIR microscopy

Synchrotron FTIR microscopy is a well-established method to study biochemical parameters such as protein and fatty acid content at cellular resolution [33-35]. Synchrotron FTIR measurements were undertaken at the Australian Synchrotron’s Infrared Microspectroscopy beamline. At 15.5 hours, cells were removed from the incubator, transferred to the modified FTIR flow cell and allowed to equilibrate to ambient temperature (25 °C) before being placed on the sample stage of a Bruker Hyperion 3000 microscope (Bruker Optik, Ettlingen, Germany) with a liquid nitrogen cooled narrow band mercury cadmium telluride (MCT) detector coupled to a Vertex V80v spectrometer. Spectra were collected in transmission mode at 4 cm$^{-1}$ resolution over the spectral range of 3900-750 cm$^{-1}$. A circular aperture defining an 8.3 µm diameter measurement area was used and spectra were collected with 32 co-added scans. A background spectrum of 128 co-added scans was collected adjacent to the cell under investigation following every 50$^{th}$ spectrum of the map. Each cell was mapped with a 3 µm step size in X and Y directions every hour from 16 to 23 hours with 4-5 biological replicates per condition at 8 consecutive timepoints.

FTIR spectral analysis

Spectra were collected and pre-processed using OPUS 8.0 (Bruker Optik, Ettlingen, Germany). Spectra were trimmed over the range of 3050 – 1350 cm$^{-1}$ and baseline corrected using a rubber-banding algorithm with 10 baseline points over 10 iterations. CytoSpec v2.00.03 (CytoSpec, Berlin, Germany) was then used to generate chemical images from integrated areas under different characteristic bands, including (i) amide II band from proteins (1590 – 1480 cm$^{-1}$), (ii) ν(C=O) stretching vibration of esters from lipids (1760 – 1705 cm$^{-1}$), and (iii) νas(C-H) stretching modes from methylene groups (2944 – 2907 cm$^{-1}$), methyl groups (2980 – 2946 cm$^{-1}$), as well as olefins (3020 – 3000 cm$^{-1}$). ImageJ v1.52i
[36] was used to calculate average and summed intensities from the chemical images. A threshold of 0.01 integrated area of the methylene spectral images was used to generate cellular outlines for each timepoint per cell.

**X-ray fluorescence microscopy**

Iron distribution maps of control and iron loaded AML12 cells were collected at the Australian Synchrotron’s XFM beamline. A 15.8 keV energy monochromatic X-ray beam was focused using a Kirkpatrick-Baez mirror pair to an approximately 2 µm FWHM spot size and X-ray emissions from samples were collected in event-mode using a low-latency, 384-channel Maia detector. Iron distribution maps were generated via raster scanning the cells through the beam with an effective 5 ms dwell time per 1 µm step size. The full emission spectra were used to reconstruct quantitative elemental maps of area density using GeoPIXE version 6.7 (CSIRO, Australia). Quantitative analysed cellular iron data were extracted as TIFF files and imported into ImageJ v1.52i. Cellular outlines obtained from phase contrast images were used to determine the average concentration of iron per cell. Three biological replicates per condition were analysed representing 50-100 single cells.

**Statistics**

Statistical analysis was conducted using GraphPad Prism v8.3.0 (San Diego, California, USA). Comparisons were conducted using Fisher’s least significant difference test for comparisons between each timepoint mean. Results are expressed as mean ± standard deviation (SD). Linear regression analysis was conducted, and differences between control and iron-loaded cells determined. Significance was inferred at the nominal alpha = 0.05 value. Differences between frequency distributions were calculated using a Kolmogorov-Smirnov test.

**RESULTS**

Control and iron loaded AML12 cells were investigated with XFM and live cell, time-lapse FTIR microscopic techniques at the Australian Synchrotron. The degree of cellular iron loading was first examined by XFM. Cells incubated with 30 ng/mL ferric ammonium citrate exhibited a significantly higher iron content than controls after 12 hours of iron loading (P < 0.0001) and this level of loading was still present at 24 hours (Fig. 1A & B). There was no significant difference in iron concentration in control cells between 12 and 24 hours. The result indicated that cells subjected to iron loading imported and retained the metal to a significantly higher concentration than controls over the entire 24 hour incubation period. Additionally, the intracellular iron signal was punctate (Fig. 1A), suggesting that iron was compartmentalised within the cells, consistent with normal cellular iron uptake mechanisms, probably within ferritin or subcellular organelles [37]. Thus, changes to macromolecules
observed in iron-loaded live cells by time-lapse FTIR microscopy will have been measured in the presence of significantly higher iron concentrations than controls.

Live cell time-lapse FTIR measurement was conducted on control and iron loaded AML12 cells following a 16 hour incubation in their respective experimental media. Four to five cells per condition were monitored individually from 16 to 23 hours. The average size of the cells was the same at all timepoints, indicating that (i) iron loading did not cause changes in cell size during the 8 hour period and, (ii) changes in relative concentration of functional groups between conditions were not caused by differences in cell volumes (Supplementary Fig. 1).

Figure 2 shows the average absorbance (Fig. 2A) and 2nd derivative (Fig. 2B) FTIR spectra from live iron loaded (n = 5) and control (n = 4) cells at 16 and 23 hours. The amide I peak at 1640 cm$^{-1}$ was excluded from analysis due to distortions in this region caused by differences in water absorption between the cell culture media and live cells as seen in the figures. Three main spectral regions, 3050-2800 cm$^{-1}$, 1770-1700 cm$^{-1}$ and 1590-1350 cm$^{-1}$, were used for the analysis. These regions, which contain bands of biological relevance, have been reported not to be subject to (i) the significant distortion of the amide I band caused by water absorption, and (ii) the refractive effect of the cumulative 2 mm thick CaF$_2$ windows used in this study [31, 38]. The band assignment for functional groups is detailed in the Materials and Methods. Figure 2C shows the average absorbance spectra of the ester carbonyl region. In biological systems, this carbonyl band arises due to $\nu$(C=O) stretching vibrational mode of esters from lipid triglycerides and fatty acids [31]. Iron accumulation in AML12 cells caused an increased absorbance of the ester carbonyl band compared to control cells and no difference was observed for the aldehyde carbonyl band in either group. This suggests that the proportion of triglycerides to free fatty acids (FFA) is higher in iron loaded cells compared to controls, indicating that although there is more lipid present, lipid metabolism of iron loaded cells may not have been disrupted sufficiently that it leads to excessive accumulation of potentially toxic FFA in the cytoplasm.

The $\nu$(C-H) stretching region was also examined to reveal information on lipid structure. A pronounced increase in the amount of total lipid unsaturation was observed, as indicated by the net increase in intensity of the $\nu$(C=CH-) band. In addition, analysis of the second-derivative spectrum revealed a shift of the $\nu$(C=CH-) band to higher wavenumbers, concomitant with the net increase in intensity (Figure 2D). The position of this band is commonly used for examining the relative degree of unsaturation within individual lipid molecules (ie, the number of double bonds per molecule) [31]. Therefore, the shift towards higher wavenumbers observed in this study may indicate a higher number of olefinic bonds (cis C=CH-) per lipid molecule, thus indicating a higher degree of lipid unsaturation in the iron loaded cells [38, 39].
Representative false-colour spectral maps of functional groups corresponding to proteins (amide II) and lipids (ester, methylene and methyl groups), along with brightfield images for control and iron loaded cells at 16 and 23 hours are shown in Figure 3. During the 8 hour time-lapse, the control cell developed two peripheral cellular processes containing optically dense regions (dark appearance) that can be seen in the upper right of the brightfield images (Fig. 3A & C). These optically dense regions corresponded with elevated concentrations of ester and methylene functional groups (Fig. 3I & K) suggesting they are regions high in lipid, possibly lipid droplets. Numerous optically dense regions were also observed centrally within the cell body of the individual iron loaded cell (Figure 3B & D) and co-localised with areas high in ester and methylene (Figure 3J, L, N, P). The distribution of protein represented by amide II is concentrated in the centre of the cells (Fig. 3E – H). The reason for this is probably two-fold; firstly, the centre of the cell contains many of the cellular organelles including the nucleus, increasing protein concentration and, secondly, this area is likely to be the thickest part of the cell. In both cells, a high concentration of methyl groups is present in the centre of the cell (Fig 3Q-T), corresponding with the location of the nucleus in brightfield images. Time lapse videos showing changes in cell morphology and distribution over 8 hours were generated from spectral maps (Supplementary video 1A-D). Supplementary video 1C shows the ester rich compartment separating into two smaller compartments of similar size, with the two ester-rich compartments appearing to have different methylene concentrations. The difference in the methylene/ester ratio suggests differences in lipid composition between the two compartments (Fig. 3O).

Semi-quantitative analysis of protein and lipid content within iron loaded and control cells was performed using average integrated areas under absorbance spectral bands to determine total functional group concentrations at each timepoint (Fig. 4). Iron loaded cells exhibited approximately 26% higher concentration of amide II compared to control cells at all timepoints (Fig. 4A), indicating that iron loaded cells have a higher concentration of protein compared to control cells. Both groups exhibited increases in the concentration of amide II between 16 and 23 hours. The significant increase in amide II during the 8 hours suggests that control and iron loaded cells are accumulating proteins possibly relating to cell growth or cell cycle progression. The control cells demonstrated a constant rate of increase in amide II concentration (Table 1) and there was no significant difference between the rate of increase for control and iron loaded cells. This suggests that control and iron loaded AML12 cells have similar protein metabolisms during the 8 hour time period within the flow cell and that the elevation in protein concentration in iron loaded cells occurred during the first 16 hours of incubation, prior to the start of imaging.

Iron accumulation resulted in significant alterations in overall cellular lipid metabolism (Fig 4B, C and D). Iron loaded cells exhibited higher concentrations of ester, methylene, methyl and olefinic
functional groups at 16 hours, indicating that increased cellular iron had already significantly perturbed lipid metabolism within the initial 16 hours of incubation. Ester concentration was 3.3 times higher in iron loaded cells compared to control cells at 16 hours, indicating an increase in lipid storage in the form of triglycerides. Significant increases in ester concentration were observed for both groups between 16 and 23 hours. A linear model was used to compare the rate of increase over the 8 hours of measurements (Fig. 4B). As indicated by the gradient over time, iron loaded cells had a higher rate of increase in ester concentration between 16 and 23 hours compared to control cells (Table 1). This suggests that excess iron induced alterations to lipid metabolism, causing cells to accumulate triglycerides at a higher rate than control cells.

Analysis of additional functional components of lipids provides further insights into differences in lipid composition between the control and iron loaded cells. Iron accumulation resulted in a 62% increase in methylene and a 78% increase in methyl concentration at 16 hours compared to control cells (Fig 4C & D). These differences were maintained for the remaining 8 hours, with iron loaded cells exhibiting higher methylene and methyl concentrations at all timepoints compared to control. During the 16 to 23 hour period, both groups demonstrated increases in methylene and methyl concentrations. The rate of change of methylene and methyl concentrations over time was assessed using a linear model. Iron loaded cells exhibited a higher constant rate of increase in methylene concentration compared to control cells (Table 1), leading to a total increase 4 times larger than controls between 16 and 23 hours. The difference in the rate of increase in methyl concentration between control and iron loaded cells was not statistically significant. The methylene to methyl ratio was used to indicate changes in hydrocarbon acyl chain length within cells (Fig. 4F), and these results indicate that chain length within iron loaded cells was shorter than control cells at 16 hours. However, over the ensuing 8 hours, the ratio of methylene to methyl increased in iron loaded cells, whilst it decreased in control cells (Fig 4F; Table 1). This suggests that iron accumulation resulted in alterations in lipid metabolism which caused an accumulation of longer chain fatty acids. Comparisons of the representative chemical maps of methylene/methyl ratio indicated that the increase in lipid chain length corresponded with high ester concentrations, suggesting that iron loading increases the chain length of triglycerides.

The olefinic band located at 3020 cm\(^{-1}\) was used as an indicator of the relative amount of unsaturated lipids. Iron loaded AML12 cells exhibited 60% higher olefinic concentration compared to controls, suggesting a greater concentration of unsaturated lipids (Fig. 4E). The olefinic/methylene ratio was used to qualitatively determine whether iron accumulation altered the proportion of double bonds present in lipid structure. Figure 4G indicates that iron loading did not alter the proportion of double bonds compared to control cells at any timepoint, despite the increase in chain length and overall lipid content. Further, during the 16 to 23 hour period, no changes in the olefinic/methylene ratio were
observed for either group. This suggests that although iron loading altered overall lipid metabolism, it did not cause significant disruption to the metabolism of unsaturated lipids.

To investigate whether the differences in lipid composition were the result of a global cellular change, or localised sub-cellular changes, frequency histograms were generated on a per pixel basis for lipid signals in each image, and compared between control and iron loaded cells, for ester and methylene concentrations (Supplementary Fig. 2). The results suggest that iron loaded cells had a higher proportion of lipids concentrated in fewer regions of the cells compared to control cells, in which distribution appeared to be more uniform.

**DISCUSSION**

Dysregulated iron metabolism has been associated with metabolic diseases of lipid, carbohydrate and protein metabolism [40, 41]. Globally, the occurrence of metabolic diseases such as obesity, cardiovascular disease and NAFLD is increasing [42], so further understanding of the effect of iron accumulation on disease development is critical. In this study, we demonstrate the application of time lapse synchrotron FTIR microscopy to investigate iron-induced changes in the metabolic processes of single, live AML12 cells at the Australian Synchrotron’s Infrared Microspectroscopy beamline. Cells were iron loaded for 16 hours then tracked for the ensuing 8 hours. XFM analysis of fixed cells confirmed that incubation of cells with ferric ammonium citrate provided stable iron loading during this period, demonstrating that this is an appropriate model for determining iron induced alterations in cellular metabolism.

This study focussed on iron-induced alterations in lipid and protein metabolism. The bands of interest for the investigation are within the three spectral regions 3050-2800, 1770-1700 and 1590-1350 cm$^{-1}$, which did not show evidence of water or CaF$_2$ interference. We demonstrated that changes in concentration and distribution within a single live mammalian cell can be monitored over a period of 8 hours using time lapse FTIR microscopy using a synchrotron radiation source in a non-destructive manner and without the use of chemical labelling. This approach provided 3 µm spatial resolution. The use of single cell analysis was successful at providing insight into protein and lipid metabolism within a single cell; however, analysis of multiple cells was required to provide information on the response of a population of cells. In our study, iron loaded AML12 cells exhibited changes in lipid composition and concentration which are consistent with steatosis development. Our observations indicated higher concentrations of triglycerides and cholesteryl esters, indications of increased lipid chain length, and no difference in the ratio of unsaturated to saturated lipids for iron loaded cells compared to controls.
Cellular iron accumulation led to significant increases in ester concentrations within cells, implying an increase in triglyceride and cholesteryl esters compared to control cells [43]. Elevated ester concentrations were evident in iron loaded cells at 16 hours, indicating iron had already significantly upregulated lipid metabolic processes prior to imaging. Over the 16 to 23 hour period, iron loaded cells had a proportionally larger accumulation of esters than controls. This suggests that increased iron concentrations may alter cellular uptake, lipogenesis and catabolism of lipids leading to an increase in generation of storage lipids such as triglycerides. Our study indicates that the increased triglyceride concentrations are not uniformly distributed across the cells and are occurring within small regions. This distribution is consistent with the increased lipid being stored within lipid droplets, which is a characteristic feature of hepatic steatosis [44]. Traditionally, the study of iron’s role in hepatic steatosis has focused on increased oxidative stress causing disease progression to steatohepatitis and cirrhosis [14, 45]; however, this study is consistent with our previous report [16] suggesting that iron plays a role in causing the initial development of steatosis via dysregulated lipid metabolism.

No differences in FFA concentration were identified between iron loaded and control cells suggesting iron loading did not result in an increase in FFA accumulation, which would be potentially toxic. This indicates that although iron accumulation results in alterations in lipid metabolism, cells are still able to store lipids in biologically safe formats.

Increases in the ratio of methylene/methyl are suggestive of an increase in acyl chain length [46]. At 16 hours, the methylene to methyl ratio indicated longer chain lipids in control compared to iron loaded cells. During the subsequent 8 hours, this ratio decreased in control cells but increased in iron-loaded cells, suggesting a reduction in chain length in control and an increase in chain length in iron loaded cells. These findings are consistent with decreased fatty acid oxidation and/or increased fatty acid synthesis and elongation. Mechanistically, it is possible that iron induces changes to mitochondrial and peroxisomal function, impacting the catabolism of longer chain fatty acids to a greater extent than short chain fatty acids. Previous studies have suggested that regions high in methylene to methyl are indicative of lipids within membranes [47]. Whilst we cannot rule that out, in the current study, regions of increased chain length co-located with higher concentrations of esters, suggesting that long chain fatty acids were present as triglycerides. Furthermore, the morphology of these regions is consistent with the morphology of lipid droplets seen in conventional histological images. Nevertheless, the increase in lipid chain length may have consequences for the integrity of lipid membranes [48], depending on the proportion of excess lipids that accumulate within the membranes. Functionally, the increase in chain length and esters may represent a mechanism to minimise cytoplasmic accumulation of FFAs, which may be toxic to the cell.
The proportion of unsaturated to saturated fatty acids is indicated by the olefinic to methylene ratio. The unsaturated/saturated ratio was similar in both conditions, but unsaturated fatty acids were present at higher concentrations in iron loaded cells. This may have implications for NAFLD patients presenting with associated iron loading because a higher concentration of unsaturated lipids may increase susceptibility to oxidative-associated progression of the disease. Changes in the ratio of unsaturated to saturated lipids affect the integrity and fluidity of membranes and have been reported to be one mechanism whereby iron may cause perturbations in lipid biosynthetic pathways [21]. However, in the current study, changes to the ratio were not observed, possibly due to the higher concentration of unsaturated lipids that appeared to be located within lipid droplets.

The cellular concentration of protein, as measured using the amide II peak, differed between the two groups. Elevated amide II concentrations were observed in iron loaded AML12 cells compared to control cells. During the 16 to 23 hour period, amide II concentrations increased at the same rate for both groups. A possible explanation is that excess lipid accumulation in the iron loaded cells caused an increase in mitochondrial content, increasing protein concentrations within the cell by 16 hours. It has previously been demonstrated that the mitochondrial content of steatotic cells is elevated compared to control due to an increase in mitochondrial biogenesis [49]. We propose the iron-induced alterations in lipid metabolism observed in the current study reduced the efficiency of mitochondrial metabolism, potentially causing an increase in mitochondrial biogenesis in response. This is consistent with the observed increase in acyl chain length due to a reduced capacity of mitochondria to oxidise fatty acids.

The results of this study demonstrate, in vitro, that iron-induced alterations in lipid metabolism lead to significant lipid accumulation within 16 hours of presentation of iron, which continues during the following 8 hours. In addition to the provision of biochemical insight in the context of NAFLD, this study further emphasises the growing importance of multi-modal microscopy techniques, in this case combined XFM and time-lapse FTIR, to reveal a more complete biochemical picture than either technique alone, and potentially offers a template for future studies to gain increased biochemical information in a wide range of cell types and disease processes. In the context of NAFLD, our approach used the opportunities provided by Synchrotron time-lapse FTIR microscopy to investigate alterations in single cell metabolic processes and measure differences in the distribution and relative concentration of macromolecules over time without the use of chemical labelling. Using this approach, we have demonstrated an overall accumulation of lipids in AML12 cells exposed to exogenous iron. Our imaging methodology has also revealed that more subtle changes to chain length and bond saturation are associated with this lipid accumulation and, most importantly, that the lipids appear to
be stored as triglycerides and not potentially toxic free fatty acids. Taken together, this study provides direct observation of a role for iron in the initial lipid accumulation that leads to hepatic steatosis.

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DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this paper.
REFERENCES

1. Zhang, D. L., Ghosh, M. C. and Rouault, T. A. (2014) The physiological functions of iron regulatory proteins in iron homeostasis - an update. Front Pharmacol. 5, 124
2. Wallace, D. F. (2016) The Regulation of Iron Absorption and Homeostasis. Clin Biochem Rev. 37, 51-62
3. Postic, C., Dentin, R. and Girard, J. (2004) Role of the liver in the control of carbohydrate and lipid homeostasis. Diabetes Metab. 30, 398-408
4. Swanson, C. A. (2003) Iron intake and regulation: implications for iron deficiency and iron overload. Alcohol. 30, 99-102
5. Marrades, M. P., Gonzalez-Muniesa, P., Martinez, J. A. and Moreno-Aliaga, M. J. (2010) A dysregulation in CES1, APOE and other lipid metabolism-related genes is associated to cardiovascular risk factors linked to obesity. Obes Facts. 3, 312-318
6. Park, J., Rho, H. K., Kim, K. H., Choe, S. S., Lee, Y. S. and Kim, J. B. (2005) Overexpression of Glucose-6-Phosphate Dehydrogenase Is Associated with Lipid Dysregulation and Insulin Resistance in Obesity. Mol. Cell. Biol. 25, 5146
7. Sayiner, M., Koenig, A., Henry, L. and Younossi, Z. M. (2016) Epidemiology of Nonalcoholic Fatty Liver Disease and Nonalcoholic Steatohepatitis in the United States and the Rest of the World. Clin Liver Dis. 20, 205-214
8. Zelber-Sagi, S., Nitzan-Kaluski, D., Halpern, Z. and Oren, R. (2006) Prevalence of primary non-alcoholic fatty liver disease in a population-based study and its association with biochemical and anthropometric measures. Liver Int. 26, 856-863
9. Firneisz, G. (2014) Non-alcoholic fatty liver disease and type 2 diabetes mellitus: the liver disease of our age? World J Gastroenterol. 20, 9072-9089
10. Younossi, Z. M., Golabi, P., de Avila, L., Paik, J. M., Srishord, M., Fukui, N., Qiu, Y., Burns, L., Afendy, A. and Nader, F. (2019) The global epidemiology of NAFLD and NASH in patients with type 2 diabetes: A systematic review and meta-analysis. J Hepatol. 71, 793-801
11. Milic, S., Lulic, D. and Stimac, D. (2014) Non-alcoholic fatty liver disease and obesity: biochemical, metabolic and clinical presentations. World J Gastroenterol. 20, 9330-9337
12. McPherson, S., Hardy, T., Henderson, E., Burt, A. D., Day, C. P. and Anstee, Q. M. (2015) Evidence of NAFLD progression from steatosis to fibrosing-steatohepatitis using paired biopsies: implications for prognosis and clinical management. J Hepatol. 62, 1148-1155
13. Adams, L. A., Lymp, J. F., St Sauver, J., Sanderson, S. O., Lindor, K. D., Feldstein, A. and Angulo, P. (2005) The natural history of nonalcoholic fatty liver disease: a population-based cohort study. Gastroenterology. 129, 113-121
14. Hanga, P., Morgan-Stevenson, V., Maliken, B. D., Nelson, J. E., Washington, S., Westerman, M., Yeh, M. M. and Kowdley, K. V. (2016) Iron overload results in hepatic oxidative stress, immune cell activation, and hepatocellular ballooning injury, leading to nonalcoholic steatohepatitis in genetically obese mice. Am J Physiol Gastrointest Liver Physiol. 310, G117-127
15. Tan, T. C., Crawford, D. H., Jaskowski, L. A., Murphy, T. M., Heritage, M. L., Subramaniam, V. N., Clouston, A. D., Anderson, G. J. and Fletcher, L. M. (2011) Altered lipid metabolism in Hfe-knockout mice promotes severe NAFLD and early fibrosis. Am J Physiol Gastrointest Liver Physiol. 301, G865-876
16. Graham, R. M., Chua, A. C., Carter, K. W., Delima, R. D., Johnstone, D., Herbison, C. E., Firth, M. J., O’Leary, R., Milward, E. A., Olynick, J. K. and Trinder, D. (2010) Hepatic iron loading in mice increases cholesterol biosynthesis. Hepatology. 52, 462-471
17. Valenti, L., Dongiovanni, P., Fracanzani, A. L., Santorelli, G., Fatta, E., Bertelli, C., Tauli, E., Fiorelli, G. and Fargion, S. (2003) Increased susceptibility to nonalcoholic fatty liver disease in heterozygotes for the mutation responsible for hereditary hemochromatosis. Dig Liver Dis. 35, 172-178
18. Nelson, J. E., Wilson, L., Brunt, E. M., Yeh, M. M., Kleiner, D. E., Unalp-Arida, A., Kowdley, K. V. and Nonalcoholic Steatohepatitis Clinical Research, N. (2011) Relationship between the pattern of
hepatic iron deposition and histological severity in nonalcoholic fatty liver disease. Hepatology. 53, 448-457
19 Rolo, A. P., Teodoro, J. S. and Palmeira, C. M. (2012) Role of oxidative stress in the pathogenesis of nonalcoholic steatohepatitis. Free Radic Biol Med. 52, 59-69
20 Videla, L. A., Rodrigo, R., Orellana, M., Fernandez, V., Tapia, G., Quinones, L., Varela, N., Contreras, J., Lazarte, R., Csendes, A., Rojas, J., Maluenda, F., Burdiles, P., Diaz, J. C., Smok, G., Thielemann, L. and Poniachik, J. (2004) Oxidative stress-related parameters in the liver of non-alcoholic fatty liver disease patients. Clin Sci (Lond). 106, 261-268
21 Dongiovanni, P., Fracanzani, A. L., Fargion, S. and Valenti, L. (2011) Iron in fatty liver and in the metabolic syndrome: a promising therapeutic target. J Hepatol. 55, 920-932
22 Caine, S., Hackett, M. J., Hou, H., Kumar, S., Maley, J., Ivanishvili, Z., Suen, B., Szmielinski, A., Jiang, Z., Sylvain, N. J., Nichol, H. and Kelly, M. E. (2016) A novel multi-modal platform to image molecular and elemental alterations in ischemic stroke. Neurobiol Dis. 91, 132-142
23 Hackett, M. J., Aitken, J. B., El-Assaad, F., McQuillan, J. A., Carter, E. A., Ball, H. J., Tobin, M. J., Paterson, D., de Jonge, M. D., Siegele, R., Cohen, D. D., Vogt, S., Grau, G. E., Hunt, N. H. and Lay, P. A. (2015) Mechanisms of murine cerebral malaria: Multimodal imaging of altered cerebral metabolism and protein oxidation at hemorrhage sites. Science Advances. 1, e1500911
24 Summers, K. L., Fimognari, N., Hollings, A., Kiernan, M., Lam, V., Tidy, R. J., Paterson, D., Tobin, M. J., Takechi, R., George, G. N., Pickering, I. J., Mamo, J. C., Harris, H. H. and Hackett, M. J. (2017) A Multimodal Spectroscopic Imaging Method To Characterize the Metal and Macromolecular Content of Proteinaceous Aggregates (“Amyloid Plaques”). Biochemistry. 56, 4107-4116
25 Petibois, C., Wehbe, K., Belbachir, K., Noreen, R. and Deleris, G. (2009) Current Trends in the Development of FTIR Imaging for the Quantitative Analysis of Biological Samples. Acta Physica Polonica - Series A General Physics. 115, 507-512
26 Baker, M. J., Trevisan, J., Bassan, P., Bhargava, R., Butler, H. J., Dorling, K. M., Fielden, P. R., Fogarty, S. W., Fullwood, N. J., Heys, K. A., Hughes, C., Lasch, P., Martin-Hirsch, P. L., Obinaju, B., Sockalingum, G. D., Sule-Suso, J., Strong, R. J., Walsh, M. J., Wood, B. R., Gardner, P. and Martin, F. L. (2014) Using Fourier transform IR spectroscopy to analyze biological samples. Nat Protoc. 9, 1771-1791
27 Petibois, C. and Deleris, G. (2006) Chemical mapping of tumor progression by FT-IR imaging: towards molecular histopathology. Trends Biotechnol. 24, 455-462
28 Whelan, D. R., Bambery, K. R., McNaughton, D. and Wood, B. R. (2013) Synchrotron Fourier transform infrared (FTIR) analysis of single living cells progressing through the cell cycle. Analyst. 138, 3891-3899
29 Carr, G. L. (2001) Resolution limits for infrared microspectroscopy explored with synchrotron radiation. Review of Scientific Instruments. 72, 1613-1619
30 Reffner, J. A., Martoglio, P. A. and Williams, G. P. (1995) Fourier transform infrared microscopical analysis with synchrotron radiation: The microscope optics and system performance (invited). Review of Scientific Instruments. 66, 1298-1302
31 Tobin, M. J., McNaughton, D. and Wood, B. R. (2013) FTIR spectroscopy of single live cells in aqueous media by synchrotron IR microscopy using microfabricated sample holders. Vibrational Spectroscopy. 53, 34-38
32 Doherty, J., Cinque, G. and Gardner, P. (2017) Single-cell analysis using Fourier transform infrared microspectroscopy. Applied Spectroscopy Reviews. 52, 560-587
33 Vongsivivut, J., Perez-Guaita, D., Wood, B. R., Heward, P., Khambatta, K., Hartnell, D., Hackett, M. J. and Tobin, M. J. (2019) Synchrotron macro ATR-FTIR microspectroscopy for high-resolution chemical mapping of single cells. Analyst. 144, 3226-3238
34 Hackett, M. J., Borondics, F., Brown, D., Hirschmugl, C., Smith, S. E., Paterson, P. G., Nichol, H., Pickering, I. J. and George, G. N. (2013) Subcellular biochemical investigation of purkinje neurons using synchrotron radiation fourier transform infrared spectroscopic imaging with a focal plane array detector. ACS Chem Neurosci. 4, 1071-1080
Kidman et al  Tracking biochemical changes with Synchrotron live cell FTIR

35  Hackett, M. J., Caine, S., Liu, X., May, T. E. and Borondics, F. (2015) Development of single-beam wide-field infrared imaging to study sub-cellular neuron biochemistry. Vibrational Spectroscopy. 77, 51-59
36  Schneider, C. A., Rasband Ws Fau - Eliceiri, K. W. and Eliceiri, K. W. (2012) NIH Image to ImageJ: 25 years of image analysis. Nature Methods. 9, 671-675
37  Graham, R. M., Chua, A. C. G. and Trinder, D. (2012) Plasma iron and iron delivery to the tissues. In Iron Physiology and Pathophysiology in Humans (Anderson, G. J. and McLaren, G. D., eds.). pp. 117-140, Humana Press, Totowa, NJ
38  Vongsivivut, J., Heraud, P., Gupta, A., Thyagarajan, T., Puri, M., McNaughton, D. and Barrow, C. J. (2015) Synchrotron-FTIR microspectroscopy enables the distinction of lipid accumulation in thraustochytrid strains through analysis of individual live cells. Protist. 166, 106-121
39  Vongsivivut, J., Heraud, P., Gupta, A., Puri, M., McNaughton, D. and Barrow, C. J. (2013) FTIR microspectroscopy for rapid screening and monitoring of polyunsaturated fatty acid production in commercially valuable marine yeasts and protists. Analyst. 138, 6016-6031
40  Choi, J. S., Koh, I. U., Lee, H. J., Kim, W. H. and Song, J. (2013) Effects of excess dietary iron and fat on glucose and lipid metabolism. J Nutr Biochem. 24, 1634-1644
41  Britton, R. S., O’Neill, R. and Bacon, B. R. (1990) Hepatic mitochondrial malondialdehyde metabolism in rats with chronic iron overload. Hepatology. 11, 93-97
42  James, P. T., Rigby, N. and Leach, R. (2004) The obesity epidemic, metabolic syndrome and future prevention strategies. Eur J Cardiovasc Prev Rehabil. 11, 3-8
43  Nara, M., Okazaki, M. and Kagi, H. (2002) Infrared study of human serum very-low-density and low-density lipoproteins. Implication of esterified lipid C=O stretching bands for characterizing lipoproteins. Chemistry and Physics of Lipids. 117, 1-6
44  Mashek, D. G., Khan, S. A., Sathyanarayan, A., Ploeger, J. M. and Franklin, M. P. (2015) Hepatic lipid droplet biology: Getting to the root of fatty liver. Hepatology. 62, 964-967
45  Liochev, S. I. and Fridovich, I. (1999) Superoxide and iron: partners in crime. IUBMB Life. 48, 157-161
46  Derenne, A., Claessens, T., Conus, C. and Goormaghtigh, E. (2013) Infrared Spectroscopy of Membrane Lipids. In Encyclopedia of Biophysics (Roberts, G. C. K., ed.). pp. 1074-1081, Berlin, Heidelberg
47  Kucuk Baloglu, F., Garip, S., Heise, S., Brockmann, G. and Severcan, F. (2015) FTIR imaging of structural changes in visceral and subcutaneous adiposity and brown to white adipocyte transdifferentiation. Analyst. 140, 2205-2214
48  Rawicz, W., Olbrich, K. C., McIntosh, T., Needham, D. and Evans, E. (2000) Effect of chain length and unsaturation on elasticity of lipid bilayers. Biophys J. 79, 328-339
49  Carabelli, J., Burgueto, A. L., Rosselli, M. S., Gianotti, T. F., Lago, N. R., Pirola, C. J. and Sookoian, S. (2011) High fat diet-induced liver steatosis promotes an increase in liver mitochondrial biogenesis in response to hypoxia. J Cell Mol Med. 15, 1329-1338
Table 1. Changes in functional groups in control and iron loaded AML12 cells over time.

|                | Amide II | Ester | Methylene | Methyl | Olefinic | Methylene/ Methyl | Olefinic/ Methylene |
|----------------|----------|-------|-----------|--------|----------|-------------------|---------------------|
| **Control**    |          |       |           |        |          |                   |                     |
| $R^2$          | 0.63     | 0.74  | 0.68      | 0.85   | 0.033    | 0.90              | 0.12                |
| $P$ value      | 0.057    | 0.006 | 0.011     | 0.001  | 0.67     | 0.0003            | 0.40                |
| Gradient ± SD  | 1.263 ± 0.39 x 10^{-2} | 2.35 ± 9.16 x 10^{-3} | 8.16 ± 2.27 x 10^{-4} | 8.33 ± 1.44 x 10^{-4} | NA | -3.98 ± 0.53 x 10^{-2} | NA |
| **Iron loaded**|          |       |           |        |          |                   |                     |
| $R^2$          | 0.36     | 0.91  | 0.68      | 0.70   | 0.19     | 0.51              | 0.03                |
| $P$ value      | 0.034    | 0.0003| 0.012     | 0.01   | 0.28     | 0.046             | 0.68                |
| Gradient ± SD  | 1.072 ± 0.58 x 10^{-2} | 0.004 ± 0.001 | 2.54 ± 0.71 x 10^{-3} | 6.24 ± 1.67 x 10^{-4} | NA | 1.58 ± 0.63 x 10^{-2} | NA |

Rates of change and linear regression parameters of functional groups shown in Figure 4. Where a regression was not significant, no gradient is shown (NA, Not applicable).
**Figure 1:**

**Fig. 1.** (A) Representative images of iron and chlorine distribution in control and iron-loaded AML12 cells at 12 and 24 hours. Chlorine is distributed throughout the cell and here indicates cellular boundaries. Scale bar is 10 µm. (B) Iron distribution in control and iron loaded AML12 cells after 12 and 24 hours of incubation. Significant differences between control and iron-loaded cells denoted by ***P < 0.0005. Minima and maxima of Cl and Fe are 1200-5000 µg/cm² and 75-150 µg/cm², respectively.

**Figure 2:**

**Fig. 2.** Representative average, baseline-corrected (A) absorbance and (B) 2nd derivative FTIR spectra of iron loaded AML12 cells and their corresponding controls observed at 16 and 23 hours. The broad spectral regions used for the analysis were 3050 - 2800 cm⁻¹, 1770 - 1700 cm⁻¹ and 1590 - 1350 cm⁻¹. The amide I peak (1640 cm⁻¹) was excluded from analysis due to interference caused by the absorption of water. (C) Comparison of carbonyl (1720 cm⁻¹) and ester (1745 cm⁻¹) bands present in average absorbance spectra of iron loaded AML12 cells and controls at 16 and 23 hours. (D) Comparison of olefinic peaks (unsaturated lipids) in 2nd derivative spectra of iron loaded AML12 cells and controls at 16 and 23 hours. The olefinic peak was found at 3014 and 3011 cm⁻¹ in iron loaded and control cells, respectively.

**Figure 3:**

**Fig. 3.** Comparison of brightfield (BF) images (A-D) and chemical images (E-T) observed for a representative control and iron loaded cell at 16 and 23 hours. Chemical images were compared based on the distribution of amide II (E-H), esters (I-L), methylene groups (M-P) and methyl groups (Q-T), respectively. Scale bar is 15 µm. Parameters for colour thresholds are the same for each functional group. Blue represents low concentration and red represents higher concentration. Minima and maxima of amide II, ester, methylene and methyl are 0-0.25, 0-0.2, 0-0.2 and 0-0.05, respectively.

**Figure 4:**

**Fig. 4.** Total cellular concentration of chemical groups in control (blue) and iron loaded (red) AML12 cells. (A) amide II, (B) esters, (C) methylene groups, (D) methyl groups, (E) olefinic groups, (F) methylene/methyl and (G) olefinic/methylene. Data were generated by integrated areas under specific absorption peaks. Dotted curves are 95% confidence intervals for the regression. Data are mean ± SD. The degree of significance is shown for differences between iron loaded and control cells at each timepoint *P < 0.05, **P < 0.005, ***P < 0.0005.
Fig 3
