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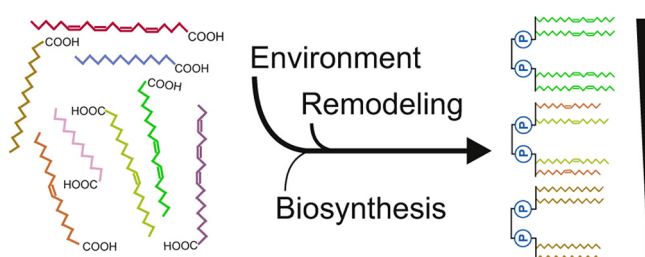
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**Abstract** The molecular assembly of cells depends not only on the balance between anabolism and catabolism but to a large degree on the building blocks available in the environment. For cultured mammalian cells, this is largely determined by the composition of the applied growth medium. Here, we study the impact of lipids in the medium on mitochondrial membrane architecture and function by combining LC-MS/MS lipidomics and functional tests with lipid supplementation experiments in an otherwise serum-free and lipid-free cell culture model. We demonstrate that the composition of mitochondrial cardiolipins strongly depends on the lipid environment in cultured cells and favors the incorporation of essential linoleic acid over other fatty acids. Simultaneously, the mitochondrial respiratory complex I activity was altered, whereas the matrix-localized enzyme citrate synthase was unaffected. This raises the question on a link between membrane composition and respiratory control. In summary, we found a strong dependency of central mitochondrial features on the type of lipids contained in the growth medium. This underlines the importance of considering these factors when using and establishing cell culture models in biomedical research. In summary, we found a strong dependency of central mitochondrial features on the type of lipids contained in the growth medium.

**Supplementary key words** lipids • FAs • MS • cardiolipin • mitochondria

Immortalized human cell lines are widely used models for biochemistry, cell biology, and human diseases and used in many research laboratories worldwide. They have evaded natural senescence by accumulation of proliferating mutations and can therefore be grown for prolonged periods of time in vitro (1). However, they require a variety of essential nutrients that are supplied with the cell culture medium. Different cell lines require



different medium compositions to be viable and proliferate. For HeLa cells, the first published immortalized cell line derived from cervical cancer cells of Henrietta Lacks in 1951 (2), Harry Eagle developed his MEM (3), which was modified by Renato Dulbecco in 1959 (DMEM) (4) consisting of inorganic salts, amino acids, vitamins, D-glucose, sodium pyruvate, and phenol red and is now one of the most used cell culture media (5). In addition, also nonsynthetic components are added, often derived from FBS, which contains a complex mixture of proteins, growth factors, as well as lipids and is added to the medium before usage.

To avoid batch-to-batch variation, to promote animal-free cell culture, but also to reduce costs, serum-free and chemically defined media have been developed (6). Nevertheless, serum-containing growth conditions are still heavily used in many research laboratories. Obviously, the composition of cell culture media affects cellular composition, metabolism, proliferation, and other functions. The impact of different media components on cellular functions has been studied with variable intensity. In particular, the lipid content of the medium has received little attention in the development of recent decades. For example, most cell culture media, such as MEM, DMEM, and RPMI, do not contain any lipids, and the only external source is nonchemically defined FBS, even though essential fatty acids cannot be synthesized de novo by human cells,

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scavengers, a process that leads to CL externalization to the outer mitochondrial membrane, where they recruit proapoptotic proteins resulting in the release of cytochrome *c* (cyt *c*) into the cytosol and consequently apoptosis (19, 20) or mitophagy (21, 22). Importantly, the acyl side chain composition of CL is not controlled by substrate specificity of their biosynthesis but requires additional enzymatic remodeling processes (Fig. 1A) (23, 24). In total, three enzymes have the ability to remodel CL, but only one—tafazzin—is so far reported to be pathogenic when impaired. Notably, CL compositions vary strongly not only across species (8) but also between tissues within one organism (10). Furthermore, a clear dependency of the CL composition and nutritionally available lipids is known (25). In addition to the aforementioned stated observations, such CL compositions have been shown to affect certain enzymatic specificities such as the cyt *c* peroxidase function, which has a higher affinity toward polyunsaturated CLs (26).

Here, we address the question to which extent externally added fatty acids impact on CL compositions in an otherwise lipid-free environment. We investigate the speed by which the mitochondrial membrane lipid compositions adapt to changes in the fatty acyl environment and how these changes influence mitochondrial functions. To tackle this, we use a completely controlled serum-free and lipid-free cell culture model for HeLa, which can be readily supplemented with a set of fatty acids and heart lipid extract.

## MATERIALS AND METHODS

### Cell culture

If not stated otherwise, all cells were grown at 37°C, 100% humidity, and in an atmosphere of 5% CO<sub>2</sub>. HeLa cells were maintained in 25 cm<sup>2</sup> flasks in DMEM, 1 g/l D-glucose, and sodium bicarbonate (D5546; Sigma-Aldrich, St. Louis, MA) supplemented with 10% (v/v) heat-inactivated fetal calf serum (10500-064; Gibco, Invitrogen, Carlsbad, CA), 2 mM L-glutamine (17-605E; Lonza Group, Basel, Switzerland), and 100 U/ml penicillin-streptomycin (17-602E; Lonza Group, Basel, Switzerland). For serum- and lipid-free cell culture, medium was exchanged to customized panserin 401 without lipids (Pan-Biotech, Aidenbach, Germany) for at least 2–3 days before further treatment.

### Fatty acid-BSA conjugates

For lipid supplementation, fatty acid-BSA complexes were generated as described (8). Briefly, fatty acids were weighed and dissolved as a 100 mM stock in 100 mM NaOH. The mixture was incubated at 70°C (for stearic and palmitic acid [PA], 85°C) in a water bath until complete solubilization. Fatty acid-free BSA (A7030; Sigma-Aldrich, St. Louis, MA) was dissolved in cell culture medium (2.8 mM, panserin 401 without lipids), incubated at 37°C, and thoroughly vortexed until complete solvation. While still warm, the 100 mM fatty acid (FA) solution was diluted 1:5 in the 2.8 mM BSA solution (final concentration: 20 mM FA in 2.28 mM BSA) shaken for 20 min at 55°C at 1,000 rpm. This BSA-complexed fatty acid stock

solution was added to the cell culture medium for later supplementation (1:800 for 25 μM FA in 2.8 μM BSA solution). Medium was prepared freshly and placed at 4°C for short-term storage. If not stated otherwise, the medium was exchanged every second day. PUFAs were stored at –20°C in aliquots under an argon atmosphere.

### Fatty acid supplementation of HeLa cells

For fatty acid supplementation experiments, 0.25 million cells, grown under standard conditions, were split in six wells with panserin 401 without lipids and incubated for 2 days. Then, the medium was exchanged to panserin 401 containing 25 μM of the respective supplemented fatty acid for 72 h. Cells were harvested by trypsinization, washed with PBS, and pelleted by centrifugation. Cell pellets were stored at –20°C for subsequent HPLC-MS/MS analysis. For fatty acid exchange experiments, 0.2 million cells were seeded in a 6-well plate for 48 h in panserin 401 without lipids before exchanging the medium to the new target condition. Cells were pelleted as described previously at the respective time points. Analogue, for respirometry, 0.5 million cells were seeded in 75 cm<sup>2</sup> flasks for 48 h in panserin 401 without lipids before exchanging the medium to the new target condition.

### Heart lipid supplementation of HeLa cells

About 20 g of fresh pig heart (purchased at the local butchery) were homogenized in 500 ml 1× PBS using a hand blender (Bosch, Germany). The lipids were extracted following the Folch method (27) using 500 ml 2:1 chloroform/methanol mixture in a 1 liter separating funnel. Organic phases containing the lipid extract were evaporated in a Rotavapor (Büchi, Flawil, Schweiz) at 40°C and 750 mbar. The dried lipid extracts were weighed, to 25 mg/ml with 2:1 chloroform/methanol mixture and stored in 2 ml aliquots in glass vials at –80°C. For heart lipid supplementation, 40 mg heart lipid extract was added to 200 ml lipid-free panserin 401 and shaken in water bath at 50°C for 24 h (200 μg/ml final concentration). The supplemented medium was sterile filtered through 0.2 μm pore filter. HeLa cells were grown in 25 cm<sup>2</sup> flasks with serum-free panserin 401 without lipids supplemented with pig heart lipids for 3 days and were then harvested.

### HPLC-MS/MS analysis of CLs

CL analysis was performed as described (8). Briefly, sample material was homogenized in PBS, and lipids were extracted following the Folch method (27) with internal standard (CL(14:0)<sub>4</sub>, 0.5 μM). Lipid extracts were dissolved in HPLC starting condition and subjected to HPLC-MS/MS analysis. Separation was achieved by reversed-phase HPLC with an Agilent Poroshell 120 EC-C8 2.7 μm 2.1 × 100 mm column (Agilent Technologies, Santa Clara, CA) on a Dionex Ultimate 3000 HPLC (Thermo Fisher Scientific, Inc, Waltham, MA; 50°C column oven, 0.4 μl/min flow) with running solvent A (60/40 acetonitrile/H<sub>2</sub>O, 10 mM ammonium formate, and 0.2% FA) and running solvent B (90/10 isopropanol/acetonitrile, 10 mM ammonium formate, and 0.2% FA). Analytes were measured using a LTQ Velos MS (Thermo Fisher Scientific, Inc, Waltham, MA) operated in negative ESI mode (3.8 kV, 275°C capillary temperature, 460–1,650 *m/z*) and data-dependent MS<sup>2</sup> acquisition. Thermo raw data were converted to open-source MZML format, and peaks were integrated in MZmine2 (28). Identification was based on a



combination of accurate mass, (relative) retention times, and fragmentation spectra, compared with a library of standards. Normalization, quantification, and data analysis were performed by an in-house pipeline in R software (R Core Team, <https://www.r-project.org/>) (29). CL acyl composition was modeled by a two-step calculation: A matrix of all possible CL fragments was aligned to measured MS<sup>2</sup> spectra by “Broyden-Fletcher-Goldfarb-Shanno” minimization. These CL fragments were aligned to a matrix of possible FAs and minimized using the “Broyden-Fletcher-Goldfarb-Shanno” algorithm. Prediction of CL profile with modeled FA and CL fragments was used as quality control. Phospholipids were measured as described (30).

## Cell viability

Cell viability was determined using the Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Japan) in which, the reagent WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] was reduced by dehydrogenases active only in viable cells to formazan, an orange soluble dye measurable by a UV/visible system. Via a dilution series of each supplemented fatty acid, the LD<sub>50</sub> values were calculated for each fatty acid in HeLa. Briefly, 5,000 cells suspended in 50 µl panserin 401 were added to each well in a 96-well plate. About 50 µl of the same medium containing the supplemented fatty acids in different concentrations were then added and incubated for 3 days. Then 10 µl of the CCK-8 solution were added to each well, and the plate was incubated for 2 h at 37°C and 5% CO<sub>2</sub>. Finally, the absorption was measured at 450 nm by a plate reader (PHERAstar, BMG Labtech, Ortenberg, Germany).

## Enzyme activity assays

For measuring the enzymatic activity of complex I and citrate synthase (CS), mitochondrial-enriched fractions were prepared. Therefore, cell pellets were suspended in 150 µl saccharose buffer (250 mM saccharose, 20 mM Tris, 2 mM EDTA, 1 mg/ml BSA; pH = 7.2). After one freezing cycle in liquid nitrogen and thawing cycle at 37°C, the cells were centrifuged at 12,000 rcf for 90 min. The mitochondrial cell pellet was resuspended in 150 µl hypotonic buffer (25 mmol/l KH<sub>2</sub>PO<sub>4</sub>, 5 mmol/l MgCl<sub>2</sub>, pH = 7.2) and further split into two aliquots: One for complex I measurement and the other for CS and protein analysis.

The CS activity was quantified by coupling the release of CoA from acetyl-CoA with the oxidation of DTNB and the following appearance of TNB at 412 nm in a colorimetric reaction. Briefly, a reaction mix containing 0.15 mM DTNB, 0.5 mM oxaloacetic acid, and 0.1% Triton X-100 was prepared and incubated for 2 min at 30°C. For the measurement, 5,000 cells were used, the reaction was started by the addition of 0.3 mM acetyl-CoA, and the absorbance was recorded kinetically for 2 min.

The activity of CI is directly proportional to ubiquinone reduction, and the co-occurring NADH consumption was measured at 340 nm. To ensure that complex I activity was not limited by other complexes of the mitochondrial respiratory chain, specific inhibitors of complex III (antimycin A) and IV (KCN) were used as well as 2 µg/ml rotenone and 10 µg/ml antimycin A, respectively, to obtain the specific NADH oxidation from complex I. For complex I measurement, three additional freeze/thaw cycles of the sample were performed. The reaction mix (50 mM KH<sub>2</sub>PO<sub>4</sub>, pH = 7.5, 2.5 mg/ml BSA, 130 µM NADH, and 2 mM KCN) was

preincubated for 2 min at 30°C, the reaction was started by addition of 65 µg/ml ubiquinone, and the absorbance was measured kinetically for 5 min at 340 nm.

## High-resolution respirometry in permeabilized HeLa cells

Respiration of fatty acid-supplemented HeLa cells was measured with the Oroboros O2k-FluoRespirometer (Oroboros Instruments, Innsbruck, Austria) and recorded and analyzed using DatLab 7.4 (Oroboros Instruments, Innsbruck, Austria). Two O2ks, with two independent chambers per O2k, were operated in parallel. While one chamber contained cells treated with a fatty acid, the parallel one contained control cells. Instrumental quality control was performed routinely, including daily polarographic oxygen sensor calibrations in mitochondrial respiration medium MiR05 (MiR05-Kit; Oroboros Instruments, Innsbruck, Austria) at 37°C and instrumental O<sub>2</sub> backgrounds prior to each experimental set. After fatty acid supplementation and cell harvesting (for details, see [Materials and methods](#) section), cells were resuspended in MiR05 and added into the O2k chambers via partial volume replacement. During experiments, the oxygen range was kept between air saturation and 70 µM. Oxygen flux (pmol O<sub>2</sub>/(s·mL)) was automatically corrected for instrumental background in DatLab 7.4, and results were expressed as O<sub>2</sub> flow per cell (amol O<sub>2</sub>/(s·x)) (31–33).

The substrate-uncoupler-inhibition-titration (SUIT-008) protocol (34) was used to analyze the oxidative phosphorylation (OXPHOS) capacity of HeLa cells suspended in MiR05. Different pathway control states were induced by the addition of substrates, uncouplers, and inhibitors. ROUTINE respiration was measured in living cells before permeabilization of the plasma membrane with digitonin to enable full access to mitochondria of subsequently titrated substrates and inhibitors. The optimum concentration of digitonin was determined applying the SUIT-010 protocol, yielding an optimum concentration of 0.15 µg/ml (1.35 µM) (35). When cells were fully permeabilized, pyruvate (5 mM) and malate (2 mM) were added to support N-linked LEAK respiration followed by the addition of ADP (2.5 mM) to evaluate N-OXPHOS capacity. Cyt *c* (10 µM) was added after the titration of ADP to test the intactness of the mitochondrial outer membrane, followed by addition of glutamate (10 mM). To assess the NS-OXPHOS capacity, succinate (10 mM) was injected to the permeabilized cells. The maximum respiration was experimentally induced by subsequent titrations of the uncoupler CCCP (0.5 µM steps) to reach NS-ET capacity. To inhibit complex I, 0.5 µM rotenone was added, reducing respiration to S-ET capacity. Antimycin A (2.5 µM) was injected to obtain residual oxygen consumption (*Rox*).

## RESULTS

For establishing serum-free and lipid-free cell culture, we ordered a customized panserin 401 without lipids (Pan Biotech, Aidenbach, Germany) and used fatty acid supplementation mediated via fatty acid-free BSA (8). To obtain the optimal concentration for lipid supplementation, we determined the cytotoxicity of each supplemented fatty acid using a cell viability assay (CCK-8; Dojindo EU GmbH, Munich, Germany). The LD<sub>50</sub> values of the 10 tested fatty acids ranged between 36.6 µM for arachidonic acid (AA) and 472.3 µM for

TABLE 1. Viability of HeLa cells in the presence of supplemented fatty acid-BSA conjugates (n = 3)

Fatty Acid	Abbreviation	Name	Mean <sub>(LD50)</sub> /μM	SD/μM
Oleic acid	OA	18:1	472.3	3.4
Palmitoleic acid	PoA	16:1	285.5	5.8
Lauric acid	LaA	12:0	225.2	3.5
Linoleic acid	LA	18:2	182.8	0.6
Myristic acid	MA	14:0	137.5	14.3
Stearic acid	SA	18:0	83.6	4.4
α-Linolenic acid	ALA	18:3	66.0	2.6
Palmitic acid	PA	16:0	61.2	6.2
Arachidonic acid	AA	20:4	36.6	6.8
Docosahexaenoic acid	DHA	22:6	NA	NA

NA, not available.

oleic acid (OA; **Table 1**). No effect on the viability was observed with DHA in the accessible concentration range of 0–200 μM, which is in line with other observations (36, 37), but contrasts others (38, 39), underlining the need to determine viability for each respective model system. Because of the low LD<sub>50</sub> for AA, we used 25 μM fatty acid as a generally applicable concentration for subsequent supplementation experiments. Interestingly, these relatively low levels of supplemented fatty acids had a positive impact on cell proliferation (**supplemental Fig. S1**).

To characterize potential lipidomic alterations caused by fatty acid supplementation, HeLa cells were first grown in panserin 401 without lipids for 48 h followed by 72 h in the presence of the respective fatty acid-BSA conjugate, and the CL composition was quantified (**Fig. 1B**) as described (8). Strikingly, each cell culture medium had a unique effect on CL composition (**Fig. 1C**). This falls in line with our earlier observation that the exact CL composition is not transcriptionally regulated but rather dependent on the availability of fatty acyls (9). In the same samples, we could observe that the species compositions of phosphatidylethanolamine and phosphatidylcholine were altered in response to the different fatty acid-enriched conditions tested (**supplemental Fig. S4**). Also in these other phospholipid classes, PUFA supplementation had the strongest influence on the observed lipid profiles.

HeLa cultivated in panserin 401 without lipids can only utilize fatty acids derived from their own de novo biosynthesis, which deprives them of PUFAs, and indeed, the measured CL species were dominated by acyl side chains with a total of only four double bonds (**Fig. 1C, left panel**). Our fatty acid deconvolution revealed that palmitoleic acid (16:1) and OA (18:1) made up 80% of the total CL composition, showing an apparent preference of monounsaturated long-chain fatty acids over saturated ones (**Fig. 1C, right panel**).

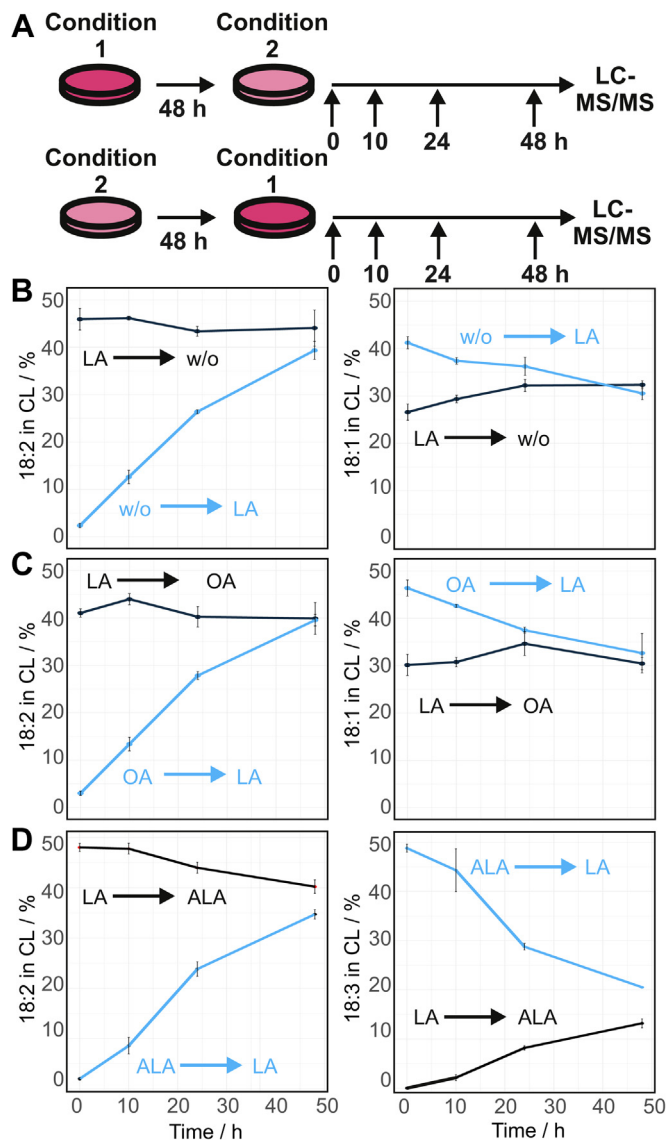
A principal component analysis of the CL profiles demonstrates that the effects of supplementation are fatty acid specific with varying intensity (**Fig. 1D**). The highest degree of incorporation was achieved with linoleic acid (LA; 18:2) to over 70% of all side chains in favor of 16:1 and 18:1 (**Fig. 1C, right panel**). Stearic acid (SA;

18:0) supplementation led to an equally strong increase of 18:1 incorporation as treatment with OA suggesting a quick desaturation process of this saturated fatty acid once taken up into the cell. Notably, PA (16:0) was also not incorporated as 16:0 itself but led to a slight increase of 16:1. This likely reflects the fact that PA is the major product of fatty acid biosynthesis and is subsequently elongated and/or desaturated into other nonessential fatty acids such as palmitoleic acid, SA, and OA (40). Myristic acid and AA were both incorporated to a level of 11% of CL side chains and α-linolenic acid (ALA) with 27.5%. DHA supplementation led to minor changes in CL profile with levels lower than the fatty acyl resolution of the mathematical side-chain modeling approach used (8). In absolute terms, upon LA treatment, a total of 76% of all CL side chains were altered, followed by ALA (34.2%), AA (31.4%), and OA (25.2%), whereas PA and DHA had the least impact with 8.9% and 8.3%, respectively (**supplemental Fig. S5**). Notably, the fatty acyl incorporation was concentration dependent. For example, when PA was supplied in excess at 200 μM—a concentration at which cell viability is already reduced to 10% within 72 h—the content of PA in CL increases from 4.6% to 40.4%, when compared with the 25 μM PA treatment (**supplemental Fig. S6**).

LA was the most influential of the 10 supplemented fatty acids, resulting to focus the CL profile around tetralinoleoyl-CL, rather than leading to multiple CL compositions as it was the case for all other supplementations (**Fig. 1B**). The fraction of LA content in CL side chains however plateaus at 75% after 4 days of treatment with 25 μM (**Fig. 1E**). This is in good agreement with tetralinoleoyl-CL being the most abundant molecular CL species in most mammalian tissues including heart and muscle (9). In contrast, ALA only incorporated half as frequently when being supplemented in the same concentration, highlighting a clear selectivity for LA. To check how strong the influence of a competing fatty acid was in context of dual mixtures, LA was supplemented with increasing amounts of OA (**Fig. 1F**). The result was a quenching of LA incorporation, but a significant molar surplus of OA (more than 3-fold) was needed to achieve a 50% reduction.

As our cell culture model is a proliferating system, we tested whether LA remains accumulated in CLs after its removal in the medium or if it is readily exchanged with other fatty acids in three different lipid exchange experiments (**Fig. 2A**). Therefore, cells were preconditioned with lipid-free (without; **Fig. 2B, upper panels**), OA (**Fig. 2B, center panels**), and ALA containing medium (**Fig. 2B, bottom panels**), which was then exchanged for LA containing medium and vice versa. The content of the respective fatty acyls in CL was then tracked over time by means of LC-MS/MS.

We observed a rapid LA incorporation in all three preconditioned scenarios, which was accompanied by a replacement of other fatty acyls including 18:1 and, when present, 18:3 (**Fig. 2B**). Quantitatively, these effects



**Fig. 2.** LA incorporation is preferred even if other FAs are available. **A:** Schematic depiction of lipid exchange cell culture experiments. **B:** Panserin 401 without and LA lipid exchange: While the amount of 18:2 increased from 2.5% to 38%, 18:1 decreased from 42% to 31% within 48 h upon adding 25  $\mu$ M LA. In reversed conditions, the amount of 18:2 dropped from 46% to 44% and 18:1 increased from 27% to 32%. Thus, 18:2 is relatively slowly exchanged in LA-depleted conditions. **C:** LA and OA exchange: In LA-pretreated conditions, neither 18:2 nor 18:1 content was affected after replacing LA with OA within 48 h. In contrast, 18:1 content decreased from 46% to 33% after OA to LA replacement, whereas LA content increased from 3% to 40% in the same time. Thus, 18:2 has a lower turnover rate in CL than 18:1. **D:** LA and ALA exchange: LA content decreased from 47% to 40% after ALA replacement, whereas ALA increased from 0% to 13% in 48 h. However, in reverse experiment conditions, LA increased from 3% to 34%, whereas ALA dropped from 48% to 21%.

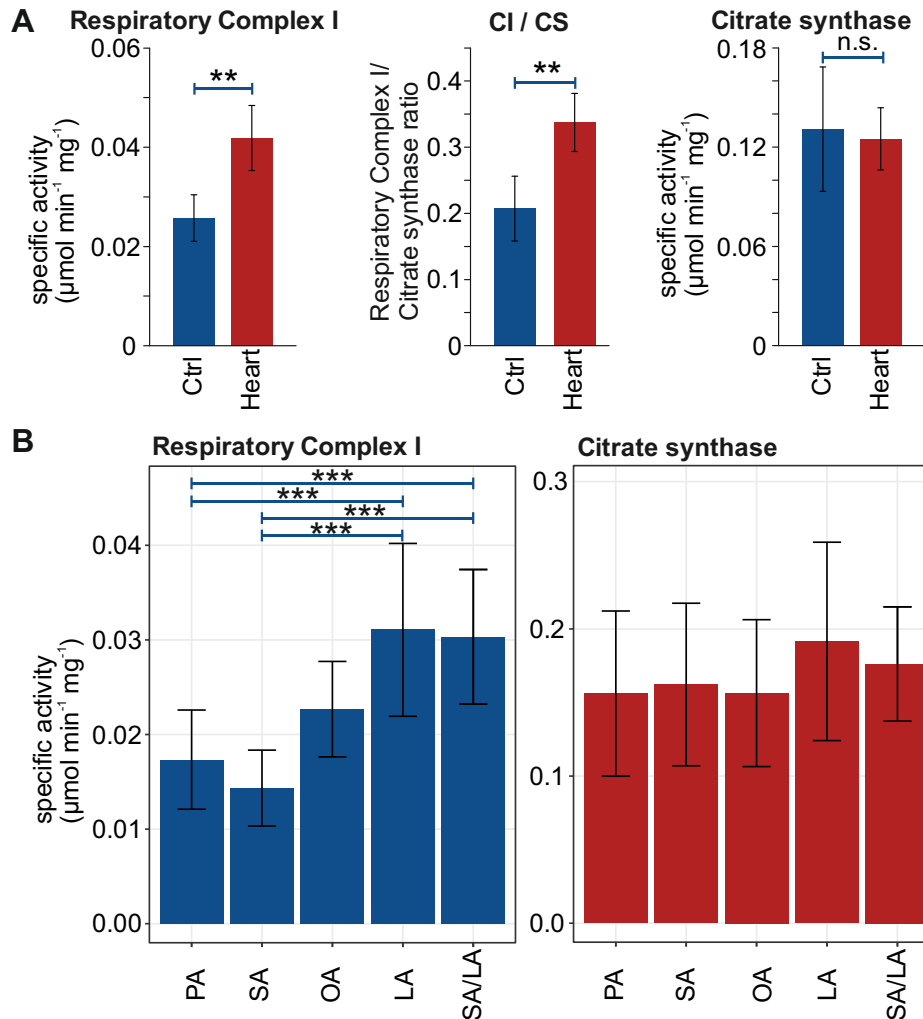
were slightly less pronounced when exchanging LA and ALA with each other. Upon LA withdrawal, however, the fraction of linoleoyl side chains in CLs remained largely stable within the period of 48 h. This effect was again least pronounced in the context of ALA. This was surprising for this proliferating system,

since at least a dilution factor of two would in theory be expected. This leads to the assumption that other cellular LA reservoirs are first depleted before significant changes occur in the mitochondrial CL composition. Together, this demonstrates that the CL side-chain composition is highly dependent on the availability of fatty acids. Thereby, the CL profile does not directly mirror the available fatty acyls, but the profile is modulated and altered by intrinsic specificities and preferences, especially for LA and to a lesser degree also for ALA.

Next, we used this lipid-free and serum-free model system to address the question how the changes in CL composition affect mitochondrial function. Previously, we demonstrated that supplementation of cultured cells with heart lipid extracts led to a more heart-like CL profile as well as an increase of CI activity (8). Although the heart lipid extract used was composed to a considerable extent of bound and free fatty acids, and an increased proportion of this can be assumed to be LA, it could not be specified whether both effects were caused by exactly the same compound contained in the mixture. Analyzing the impact of single fatty acid-BSA treatments allowed us to determine the individual effect of changing the lipid environment on mitochondria. The enzyme activity of the inner mitochondrial membrane-localized CI was measured after 4 days of fatty acid treatment and reaching a stable CL composition. The enzymatic activity of the matrix-localized CS served as control.

We observed that the CI activity followed a fatty acid supplement-dependent behavior. Heart lipid supplemented cells were characterized by an increased CI activity compared with controls, whereas CS was unaltered, leading to an increase in CI/CS ratio (Fig. 3A). When supplementing individual fatty acids, the highest specific activity was found in cells supplemented with 25  $\mu$ M LA. Equally high rates were also observed for the combined treatment of 25  $\mu$ M LA with 25  $\mu$ M SA. The latter, when supplemented alone, caused the lowest CI activity, whereas the effect caused by the combinatorial treatment appeared to be driven by LA alone. Both LA containing conditions resulted in specific activities significantly higher compared with treatment with the saturated fatty acids, PA and SA. In contrast, no significant changes between any of the measured conditions were detected regarding CS activity (Fig. 3B), which was determined from the same cell homogenates. Thus, LA stimulated CI activity in permeabilized HeLa cells irrespective of it being supplemented alone or in combination. This effect is in line with earlier observations made for the treatment of HeLa cells with whole heart lipid extracts (8).

This was followed up by testing whether the observed alteration of biochemical properties also affects mitochondrial respiration in HeLa cells. Thus,  $O_2$  consumption of differentially supplemented cells



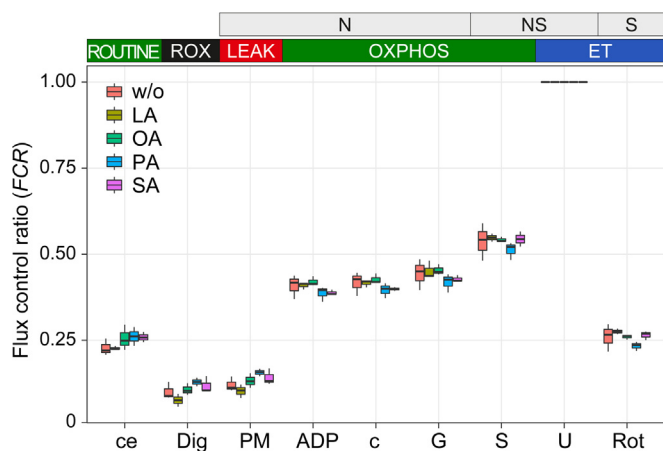
**Fig. 3.** LA increases the CI activity but not of CS. A: CI and CS activities in heart-lipid supplemented HeLa cells. (3 days, 200  $\mu\text{l/ml}$  heart lipid extract,  $n = 6$ , mean  $\pm$  SD). B: CI activity was significantly increased after 4 days in LA supplemented media, including a 1:1 mix of LA and SA. ( $n = 10$ , 25  $\mu\text{M}$  FA, mean  $\pm$  SD, significance was tested by ANOVA and Tukey post hoc correction,  $P = 7.59\text{e-}07$ ). CS activity was not affected by lipid supplementation in HeLa ( $n = 12$ ,  $P = 0.454$ ). Significance: \*\*\* $P < 0.001$ .

was measured in a high-resolution respirometer, following a specific SUIT-008 protocol (34). This allowed us to characterize the behavior of N- and S-linked pathways (NADH-dependent via CI and succinate-dependent via respiratory complex II) of the mitochondrial electron transfer system in a substrate-dependent manner (Fig. 4). Interestingly, we observed no significant differences of the flux control ratio (normalized for noncoupled electron transfer capacity) in living (ce) and permeabilized cells after digitonin treatment (Dig). The increased biochemical activity of CI upon supplementation of unsaturated fatty acids (Fig. 3) was apparently not rate limiting for fueling OXPHOS in cells when stimulated with NADH-linked substrates and succinate. Of note, we detected a trend toward reduced flux control ratios of N-linked OXPHOS in cells treated with saturated fatty acids (Fig. 3, ADP, c: cyt c, G: glutamate). The same behavior was also observed on the level of specific fluxes (supplemental Fig. S7).

## DISCUSSION

The acyl side-chain composition of CL is highly diverse not only in different organisms but also within tissues of the same organism (8, 9, 30). The postbiosynthetic enzymatic remodeling process of CL plays an important physiological role for mitochondrial membrane constitution, which can cause severe mitochondrial dysfunction, for example, when impaired as in the case of Barth syndrome (41). It is postulated that the diversity of CL is largely driven by two processes, *i*) the mitochondrial inner membrane's intrinsic strive to minimize free energy, which is strongly shaped by the protein-crowded environment in the membrane (42) and *ii*) the set of fatty acyls available for CL remodeling that determines the local free energy minimum that can be achieved (9, 43, 44). Taken together, these factors predict a strong dependence of mitochondrial membrane properties on the growth environment, which is with





**Fig. 4.** Fatty acid supplementation does not affect mitochondrial respiration ex vivo. Flux control ratios (*FCRs*) of living and permeabilized HeLa cultivated in different supplemented media (BSA mock control, LA, OA, PA, and SA) were normally distributed (Shapiro-Wilk's test,  $P > 0.05$ ) and not significantly altered (ANOVA,  $P = 0.054$ ,  $n = 3$ ). *FCRs* were baseline corrected for residual oxygen consumption (*Rox*) after inhibiting respiratory complex III with antimycin A. Corresponding  $O_2$  flow per cell can be found in supplemental Fig. S7. The header shows the different respiratory states according to the following sequential additions. ADP, ADP to activate OXPHOS; c, cytochrome *c* for control of intact mitochondrial outer membranes; ce, ROUTINE respiration of living cells; Dig, digitonin for cell permeabilization; G, glutamate as NADH-linked substrate; PM, pyruvate and malate as substrate for NADH-dependent OXPHOS; Rot, rotenone to inhibit complex I for determining succinate-dependent respiration; S, succinate to measure NADH and succinate-dependent OXPHOS; U, CCCP to dissipate the membrane potential to reach full electron transfer capacity.

regard to cultured cells defined by the medium and culturing conditions chosen.

As demonstrated in this study, by testing the impact of a range of fatty acid supplements in cell culture, the CL compositions as well as the CI activities respond to changes in the lipid content and composition in the medium. While the content of LA in human plasma is approximately 27% of free fatty acids, with total PUFA levels of 35–40% (45, 46), the typical lipid content of FBS mainly includes saturated and monounsaturated fatty acids with an LA fraction of as little as 5–8% (47, 48). This results in the general LA-deprived “CL phenotype” found across many different cultured cell lines (8). This does however not reflect the natural diversity of this lipid class. As a result, cells grown under these conditions tend to quickly incorporate large amounts of LA into CLs once this fatty acid is made available (Fig. 1E). Thereby, the degree of change that can be caused by the lipid fraction of the medium depends on the existing composition of the cells (Figs. 1F and 2). In this regard, a LA-saturated CL profile is noticeably more robust than other CL states. Not only CL but also other phospholipids respond in a similar way, but following a different specificity pattern

(supplemental Fig. S4), which is consistent with findings such as the PUFA-specific response of the phospholipid composition in rat muscles (49).

These observations are in agreement with the finding that the turnover of CL is slower than the turnover of other phospholipids (50–52). We made the observation that the CL state in cells can be highly stable as long as the lipid environment is not altered. However, when switching from LA-depleted to linoleic-rich growth conditions, we find a rapid incorporation of LA into CL (Fig. 2B), reflecting the preference of CL for linoleate incorporation (53). This is reflected in the CL compositions of most tissues, especially heart, where tetralinoleoyl-CL accounts for ~80% of CL and is depleted in Barth syndrome (41, 54).

The functional importance of a high LA content can be seen in the enzymatic activity assays. While matrix-localized CS was not affected, the membrane bound CI had a significantly higher enzymatic activity (Fig. 3B) highlighting the importance of lipid availability for respiratory complex function. The same result was observed using heart lipid extract for supplementation, which is in line with previous results (8), suggesting that LA could be a major determining factor for this increase. However, when analyzed in the context of living and permeabilized cells, these biochemical differences are compensated for and lead to a balanced respiratory behavior (Fig. 4). If respiratory complex I activity presents a threshold effect with a low flux control coefficient, an increased complex I activity is not expected to be captured by an increased NADH-linked pathway flux (55). Alternatively, this effect could be caused by antagonistic additivities of the fluxes in the convergent pathways of OXPHOS (32). An increased penetrance of the biochemical effects on cellular respiration could be expected under genuine physiological conditions such as in energy demanding tissues, indicating the limitations of the presently used cell culture model. In addition, fatty acids exert a distinct impact on the regulation of gene expression, thus providing another route to modulate the metabolic network and energy metabolism (56).

Our observations open up a multitude of possibilities by which the medium can influence the mitochondrial CLs, which are in line with the recognized unnatural lipid profile in cultured cells (57). These include, for example, batch effects caused by changes in the serum lipid composition. Also differences in the fatty acid depletion rates from the medium could lead to altered CL profiles (58). This would, for example, be the case if cells with differing growth rates are compared and LA in the medium becomes limiting for the faster growing cell line. The same also applies to chemically defined media. Often precise information about their



composition is not made public, especially with regard to the lipids used (6, 54, 59, 60). Of course, this renders it noticeably more difficult to assess to what extent the medium used is suitable for the desired cellular model system. In an ideal situation, cultivated cells could utilize the same sources and compositions of lipids as in their organism of origin. For CL, one of the main factors is the LA content, whereas other lipid classes might be more responsive to different sets of fatty acids (9).

Another important factor is the concentration with which lipids are supplied to cultured cells. Here, we chose 25  $\mu\text{M}$  for all supplementation experiments as this was a concentration that generated completely viable cells, even for AA (Table 1). Already this relatively low concentration strongly affected the side-chain composition of CLs (Fig. 1C). In contrast, other studies use higher concentrations ranging up to 500  $\mu\text{M}$  (61, 62). Such levels can cause increased apoptosis, cell death, and the induction of endoplasmic reticulum stress (63). Not every cell culture system easily tolerates such high loads of fatty acids. Concentrations of fatty acids that impair cell viability should be avoided unless this is the exact aim of the experiment. In any case, it is advisable to evaluate viability ranges not only for a given cell line in general but also in combination with its respective growth medium. Importantly, it should be noted that the observed behavior could not be transferred directly from one fatty acid to another (compare Table 1).


In a similar manner, also the solubility of fatty acids is variable, which affects the applicability for complex formation with BSA. This manifests itself by the fact that the formation of different fatty acid BSA conjugates (63) correlates with their respective melting points. Unsaturated fatty acids can be solubilized with BSA in higher concentrations than their saturated counterparts (64). Attempting to reach particularly high fatty acid concentrations could lead to increased levels of free fatty acids, which in turn can induce different physiological effects in the cell (65).

Lipids added to the cell culture medium clearly also contribute to cellular energy metabolism and anabolic requirements. We observed slightly increased growth rates of cells in the presence of low concentrations of fatty acid-BSA conjugates, irrespective of the type of fatty acid supplied (supplemental Fig. S1). Such a behavior was absent in fatty acid-free BSA mock controls. It can be assumed that these effects are particularly relevant if the medium used is otherwise lipid free, as is the case in this study. However, the variable impact of different fatty acid supplements on core mitochondrial functions as shown in Fig. 3 is independent of this general behavior with regard to cell proliferation and viability.

In conclusion, the (lipid) composition of cell culture media strongly affects not only mitochondrial phospholipid composition but also mitochondrial functions.

Supplementation, particularly with the essential LA, has a significant effect on mitochondrial activity and could be used to more realistically mimic mammalian tissue in cell culture.

### Data availability

Mass spectrometric raw data and extracted lipid profiles are deposited as Mendeley Dataset (<https://doi.org/10.17632/6pdcrm5p6k1>). Scripts for data analysis can be shared upon request (Markus A. Keller, [markus.keller@i-med.ac.at](mailto:markus.keller@i-med.ac.at)). 

### Supplemental data

This article contains [supplemental data](#).

### Author contributions

J. Z. and M. A. K. conceptualization; J. K. and M. A. K. methodology; J. K. and M. A. K. software; G. O., M.-L. E., Y. W., G. L., and J. K. formal analysis; G. O., M.-L. E., Y. W., K. L., G. L., and L. H. D. C. investigation; K. L., H. H. L., E. G., and S. D. resources; M.-L. E. and J. K. data curation; G. O. and M. A. K. writing-original draft; G. O., M.-L. E., Y. W., K. L., G. L., J. K., L. H. D. C., H. H. L., E. G., S. D., J. Z., and M. A. K. writing-review and editing; G. O., M.-L. E., Y. W., and M. A. K. visualization; E. G., S. D., J. Z., and M. A. K. supervision; E. G. and M. A. K. funding acquisition.

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### Conflict of interest

The authors declare that they have no conflicts of interest with the contents of this article.

### Abbreviations

AA, arachidonic acid; ALA,  $\alpha$ -linolenic acid; CCK-8, Cell Counting Kit-8; CL, cardiolipin; CS, citrate synthase; cyt  $c$ , cytochrome  $c$ ; FA, fatty acid; LA, linoleic acid; OA, oleic acid; OXPHOS, oxidative phosphorylation; PA, palmitic acid; CL,

respiratory complex I; SA, stearic acid; SUIT, substrate-uncoupler-inhibition-titration.

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