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Deliverable Report D8.3:
**Data set describing the impact of drug treatment on the mitochondrial
function in tissue samples from patients**

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Work package 8

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PP	Restricted to other programme participants (including the Commission Services)	
RE	Restricted to a group specified by the consortium (including the Commission Services)	
CO	Confidential, only for members of the consortium (including the Commission Services)	

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PUBLISHABLE SUMMARY

Report describing work towards the generation of data on the mitochondrial function of liver and heart biopsies taken from patients treated with drugs on the project drug list.

OBJECTIVES

Using the methods developed through D8.1, to measure the mitochondrial function of liver and heart biopsies provided by WP06.

INTRODUCTION

The objective of the deliverable was to assess the mitochondrial capacity of biopsy samples taken from patients treated with drugs on the project drug list. Biopsy samples were to be supplied by WP06. This was to be assessed by measuring oxygen consumption under defined conditions using a fluorescence-based oxygen-sensitive phosphorescent probe, and ROS using ESR.

A method and SOP to facilitate the assessment of oxygen consumption in Biopsy material was developed as part of Deliverable report D8.1 using snap frozen mouse liver as a model looking at both quartz cuvette measurements of collagenase digested liver and crude liver homogenate measured on low volume 96 well plates. All measurements are plate reader based. Sample data measuring liver fragments in quartz cuvettes demonstrate the impact of the substrate provided on oxygen consumption, with glucose showing lower oxygen consumption than succinate. The fact the succinate, which is cell impermeable, demonstrates such high levels of oxygen consumption suggests considerable cell membrane damage allowing succinate access to the cellular mitochondrial network. This is supported by the observation that the addition of digitonin does not increase cell respiration (Fig. 1A). While this method was shown to be capable of assessing mitochondrial dysfunction, limited through-put and a requirement for biomaterial normalization presented difficulties in applying such measurement to the analysis of subtle changes between ex vivo samples. Measurements of crude liver homogenate were therefore assessed and optimized using lower volume hand homogenisation. While this approach does not allow the assessment of a pure mitochondrial fraction as available when using cell fractionation, it does facilitate the use of approximately ten-fold less material and, as the homogenate can be easily pipetted, it overcomes some of the throughput and normalisation issues associated with measurement of tissue pieces.

Data shows clear concentration dependence with higher homogenate concentrations showing higher levels of oxygen depletion (Fig. 1B). In addition, antimycin inhibition is observed illustrating that ETC activity is being measured specifically. It should also be noted that the addition of ADP has little effect on oxygen depletion (Fig. 1C) indicating that again, these mitochondria have been uncoupled by freezing and thawing process. The method of measuring snap frozen samples is therefore well suited to assessing ETC inhibition but not the assessment of uncoupling of mitochondria or membrane potential mediated changes in ETC activity. This can be overcome by performing the measurement on fresh samples.

NOTE: This approach is also compatible with the low volume high sensitivity method developed within Deliverable report D8.2 since completion of Deliverable report D8.1 to facilitate single spheroid measurement allowing measurements in volumes as low as 10µL.

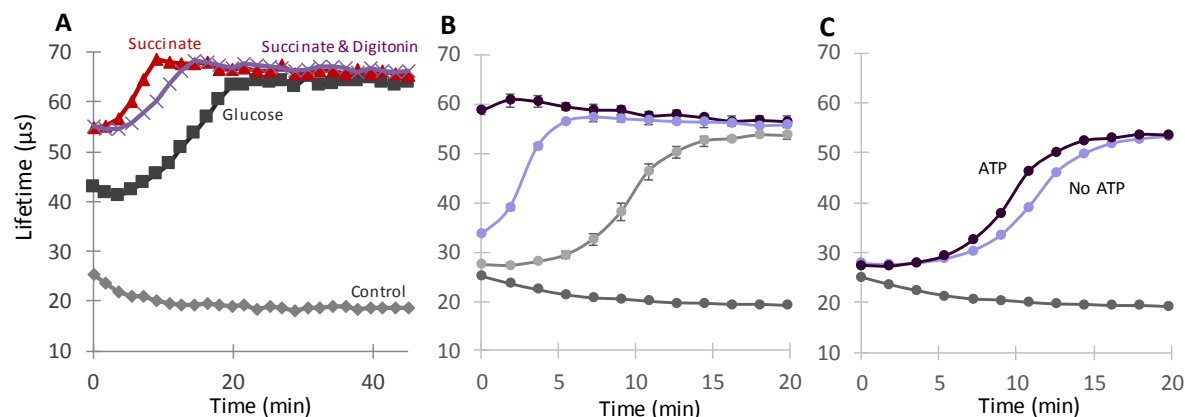


Figure 1: Measurements of collagen digested snap frozen mouse liver slimes in quartz cuvettes demonstrating the effect of substrate and cell permeabilisation on oxygen consumption. B: Concentration dependence of crude liver homogenate prepared from snap frozen liver samples measuring state 2 respiration on succinate. C: Effect of ADP addition to succinate-driven oxygen consumption of crude homogenate.

A method and SOP to facilitate the assessment of radical/ROS formation in Biopsy material was developed as part of Deliverable report D8.1. For mitochondrial ROS formation, mitochondria can be isolated and analysed by ESR while direct tissue ROS levels can be measured indirectly by detection of vitamin C/ascorbic acid radicals in the supernatant of homogenized tissue samples show (Fig. 2A) which can be interpreted as derived from ROS formation. Vitamin C reacts rapidly with superoxide, hydroxyl, alkyl, peroxy, and alkoxy radicals resulting in a one electron oxidized ascorbyl radical. Therefore the steady-state concentration of the resonance-stabilized ascorbyl radical serves as a marker for the degree of oxidative stress, this however requires a significant amount of material and as such can be applied to larger samples of material but is not applicable to small biopsies. Directly measuring (mitochondrial) radical formation at low temperature with ESR was therefore favored. As described in SOP 4 (D8.1), after placement into liquid nitrogen in a finger dewar. In human liver biopsies, radicals like thiyl radicals derived from GSH, and related to ROS formation can be directly observed (Fig 2B). Thiyl radical formation was further investigated in liver biopsies of 7 patients that were treated with acetaminophen during a pylorus preserving pancreaticoduodenectomy (modified Whipple's procedure). During the operation three liver biopsies were taken and were snap frozen in liquid nitrogen. These were further analysed by ESR as described in SOP 4. There was a significant increase in the level of thiyl radicals in liver tissue 1 h after acetaminophen challenge in these patients (Fig. 2C). These data demonstrate that methods are available to facilitate ESR measurements in human biopsy samples.

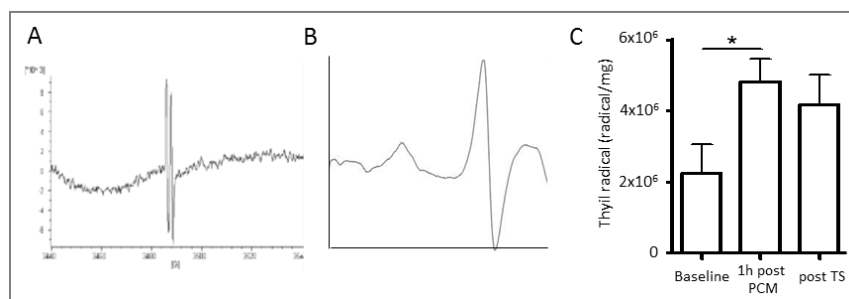


Figure 2: A: Vitamin C radical detected in homogenized human liver sample. B: Typical ESR spectrum of a thiyl radical observed as broad signals (45G) with $g = 2.02$ detected in a human liver biopsy at low temperature in liquid nitrogen. C: Level of thiyl radicals measured in liver biopsies of patients that underwent a Whipple procedure: there was a significant increase 1 hour after acetaminophen (PCM) compared to baseline (* $p=0.04$).

RESULTS

During the development of the project it became apparent that, despite significant methodological advancement in the sensitivity of functional (WP8) and 'omic (WP07) measurement methods, there would be insufficient material supplied from single needle biopsies to satisfy all measurements planned for patient biopsy samples. As the data density and resolution deliverable from genomic, proteomic and metabolomic analyses is significantly higher than that from functional analysis, and the transport of material less sensitive; these 'omics measurements were prioritised within the consortium for receipt of valuable patient material samples to ensure maximum data return to the project from the amount of patient material that could be sourced. Indeed, as reported through WP07, additional prioritisation within these analyses was also required to maximise data output. Because of this consortium sample prioritisation, it has not been possible to generate functional data on biopsy material.

DIFFICULTIES

As outlined above, significant difficulties arose around access to patient samples for *ex vivo* mitochondrial analysis (oxygen consumption & ESR). This emerged as an issue due the fact that the amount of material required to conduct all analyses across WP07 and WP08 exceeded the amount of material available via needle biopsy. While it was hoped that a combination of continued methodological improvement through WP07 and 08, and possible access to larger *ex vivo* samples would ameliorate this, the progress made in these areas during the project, although significant, was not sufficient to facilitate all of the *ex vivo* analysis planned. In this scenario, 'omics measurements were prioritised to ensure maximum data return to the project, meaning that no functional mitochondrial analysis (Oxygen consumption or ESR). Instead it was determined that information on mitochondrial function would best be attained, considering the limitation in biomaterial, through assessing mitochondrial protein expression and metabolite patterns through proteomic and metabolomic analysis respectively.