



Assessing the Effect of Preservation in Heart Transplant Protocol: Cold Ischemia Versus Normothermic Perfusion

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Abstract

Cold Ischemia (CI) preservation is the traditional heart storage method employed in heart transplants. However, its limitations are among the causes of present donor shortage. A new method relying on normothermic *ex vivo* heart perfusion has emerged thanks to the development of Organ Care System (OCS) by Trans Medics Inc. ¹H NMR was employed to analyze the metabolomic profile of serum samples taken before and after heart transplants in which the organ was preserved either with OCS method or with the traditional CI. Differences emerged between the sera collected just before the explant and the sera sampled at the implant, after de clamping the organ. These differences were ascribed to transient homeostasis fault. With the employed data collection protocol and the considered number of transplants, the CI and OCS preservation methods did not reveal any clear metabolite pattern signature. A decrease of nearly all of the measured metabolite levels was observed in the receiving patient sera with OCS preserved heart. This result should be ascribed also to the criteria that were adopted to select the preservation method, rather than to lower metabolic levels or different stress levels of the organ tissue. A modified sampling protocol should be tested to assess the effect of the preservation method on the hearth transplant.

Keywords: Blood Serum Metabolomics; Cold Ischemia; Heart Transplant; Normothermic Perfusion; Nuclear Magnetic Resonance; Organ Preservation

Introduction

Despite the progress in mechanical circulatory support and stem cell therapy, heart transplantation is still the best treatment of advanced heart failure [1]. Although universally adopted, cold ischemic preservation is not optimal because low levels of aerobic metabolism deplete ATP storage and increase acidosis and myocardial oedema [2,3]. An alternative to Cold Ischemia (CI) is the method of normothermic *ex vivo* heart perfusion with the portable perfusion device Organ Care System (OCS) developed by Trans Medics Inc. (Andover, Massachusetts) [4,5]. The first advantage of this preservation method is the decrease in cold

ischemic time which has been repeatedly demonstrated to be a fundamental factor in transplantation survival [6,7]. This implies that the donor pool can be expanded and that the time to perform the implant by the surgeon increases. Another benefit of OCS is the possibility to control the quality of the explanted heart during the transport and soon before the implant in the recipient [8]. Although a better preservation of the cardiac muscle using *ex vivo* perfusion has been demonstrated to improve follow up of survival length in animal models, the same evidence is still missing for human patients. In addition, a full characterization of the state and function of the transplanted hearts by employing the different preservation methodologies is still lacking. As the product of all the biochemical reactions occurring in cells, the metabolites, i.e. low molecular weight molecules such as amino acids, sugars, lipids, organic acids etc., are exquisite reporters of organ function. Several techniques have been employed to study the metabolic

profile of different bio-specimens (e.g. biofluids, cell extracts and tissues) like Liquid Chromatography with Mass Spectrometry (LC-MS), tandem mass spectrometry, Gas Chromatography with Mass Spectrometry (GC-MS), High-Performance Liquid Chromatography (HPLC) and Nuclear Magnetic Resonance (NMR) spectroscopy [9]. As commonly recognized, when extreme sensitivity is not an issue, NMR is the most powerful and versatile technique among the different approaches available for metabolomics identification and quantification [10]. By exploiting the magnetic properties of some nuclei present in biomolecules, mainly ^1H , ^{13}C and ^{31}P , NMR is able to provide structural information and absolute quantification of metabolites through a nondestructive experiment requiring relatively small samples, inexpensive preparation, reasonable acquisition times and often single-shot automated sessions. On the other hand, contrary to mass spectra or chromatographic profiles, NMR analysis is inherently complex because of the nature of the spectral data that often consist of very crowded patterns with multiple signals for single metabolites. Once assignment is performed, however, this complication may offer an internal consistency control or an advantageous feature enabling quantification, despite signal overlap. Metabolite profiling was used to monitor transplant organ physiology in order to assess organ reperfusion injury and possible organ dysfunction [11]. Usually, these analyses are performed *ex vivo* on biofluids like blood and urine. Concerning heart transplant, the concentrations of metabolites related to inflammatory process, i.e. nitrates, thromboxane A2 or B2 and neopterin, were found to be significantly increased in the urine of patients showing heart rejection [12,13]. Furthermore, a higher concentration of lipids and lipoproteins in serum was discovered to be a prognostic marker of rejection [14]. To assess cardiac function and rejection, *in vivo* ^1H and ^{31}P NMR measurements were exploited [15-17].

Increased N-acetyl aspartate, myo-inositol and creatine levels were observed in brain tissues of patients exhibiting congestive heart failure [15], whereas decreased phosphocreatine/phosphate, ATP/phosphate and ADP/phosphate ratios in heart tissues were associated with acute rejection and ischemia [16,17].

In this work, we analyzed by ^1H NMR the metabolomic profile of serum samples taken before and after heart transplants in which the organ was preserved either with OCS method or with the traditional CI. Through statistical analysis of NMR data, we found that there are differences between the sera sampled at the explant and those collected at the implant, after de clamping the transplanted heart, in line with a temporary loss of homeostasis in the receiving patient. With the adopted protocol, no significant concentration difference of specific metabolites was found when either cold ischemia or organ care system were employed for heart preservation. The systematic decrease of nearly all of the quantified metabolite levels, that was observed in the transplanted patient

sera when normothermic perfusion preservation had been adopted, should most likely arise also from the criteria for the selection of the preservation protocol, that concern the donor organ conditions, rather than from the metabolic levels or the stress levels of the recipient patients.

Materials and Methods

Materials

All materials used to prepare serum solutions (phosphate salts, D_2O), were from Sigma (St. Louis, MO, USA).

Preservation procedures

Preservation procedures for graft transportation included standard cold storage (Cold Ischemia, CI group) and *ex-vivo* normothermic perfusion (Organ Care System, OCS group). In CI group, St Thomas cardioplegia infusion and cold storage at 3-4 °C were employed. In the OCS group, after graft retrieval employing St Thomas cardioplegia, the aorta and the pulmonary arteries were connected to the OCS cannulae and the heart was continuously perfused with a solution composed donor blood mixed with 1.2-1.5 litres priming solution. Blood returning from the coronary sinus through the right heart and from the left ventricle was thus flowing in an oxygenator and delivered into the aortic root by a pulsatile pump. The heart was continuously assessed by the aortic pressure, coronary flow and differential lactate profile.

Blood Sampling

Twenty-five heart transplantations were considered and serum samples of donors and recipients were collected before organ removal from the donor patients and after organ de clamping in the transplanted patients, respectively. For most of the patients, blood samples from both coronary sinus (20 samples from donors and 25 from recipients) and peripheral vein (25 samples from donors and 25 from recipients) were collected. Donor and recipient demographics are reported in Table 4. A worldwide growing trend to use marginal donors established over the last years. Marginal donors are aged donors, sometime with a history of drug abuse or cardiac arrest episodes. From these donors most often an organ with suboptimal characteristics is obtained. Therefore, the donor/recipient selection to randomize the conservation protocol cannot be properly applied as the choice of that protocol depends on the surgeon. Given the limited size of the considered transplantation cohort and the above mentioned general issues, no randomization was performed. The considered transplantation cohort, albeit limited in absolute terms, is surely a demanding sample from the viewpoint of the required clinical effort. Out of the considered transplantations, 13 were performed with organs from *ex-vivo* normothermic perfusion using OCS from Trans Medics Inc., whereas the traditional CI organ preservation was employed for the

remaining 12 cases. The particular gravity of the donor conditions was determinant to submit the explanted hearts to OCS rather than CI preservation. Sample collection was authorized by the CEUR (Comitato Etico Unico Regionale) of Friuli Venezia Giulia region (Approval No. CEUR-2016-Os-030-ASIUD; Protocol No. 18386, Aug. 1st 2016). The whole work here described has been carried out in accordance with the code of ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans.

Serum sample preparation

Blood was collected in tubes before heart retrieval (t_0) and at declamping time (t_2) from coronary sinus and peripheral vein. Plasma and serum samples were obtained by blood centrifugation at 2400 rpm for 15 minutes at 4 °C and the aliquots collected were stored at -80 °C until analysis.

NMR spectroscopy

Sera were diluted (1:1 or 1:0.2) with PBS buffer, pH 6.5, and 5 % of D₂O was added for lock purposes. The experiments were collected at 298 K with the 11.7 T Bruker Avance NMR spectrometer of the Udine University Biophysics Laboratory operating at 500 MHz (¹H frequency). 1D ¹H NMR spectra were acquired with Carr-Purcell-Meiboom-Gill (CPMG) [18,19] spin-echo sequences using (τ -180°- τ) train of 96 ms and τ = 0.5 ms. During the relaxation delay (4s) a low-power selective presaturation pulse was applied to the solvent resonance that was completely suppressed prior to acquisition by detecting the signal after two consecutive WATERGATE modules [20,21]. A synthetic electronic reference signal (ERETIC, Electronic Reference to access *In vivo* Concentrations) [22] was introduced as a chemical shift and intensity reference. Experiments were recorded with 512 scans over a spectral width of 16 ppm. Two-dimensional ¹H-¹H-TOCSY [23] and ¹H¹³C-HMQC [24] spectra of some representative samples were acquired with 64 scans, 256 increments, 80 ms mixing time and 1 s relaxation delay, and 128 scans, 128 increments and 1 s relaxation delay, respectively. Spectra were processed with Topspin 4.0 and phased, baseline-corrected and integrated with Mestrenova.

Statistical Analysis

All statistical analyses have been performed within the statistical software R [25], using the standard manipulation

and statistical analysis functions provided by the package. Due to the wide spread of the signal intensities for the assessed metabolites, either within single samples and among different samples, the experimental data matrix was treated by Singular Value Decomposition (SVD) analysis. The first singular vectors and singular values were used to rebuild a matrix which was the best fit of rank 1 of the original data matrix where each value was simultaneously proportional to a sample scaling coefficient and a metabolite scaling coefficient. Analysis of variance (ANOVA) and Wilcoxon tests were also performed using the corresponding routines of the R software package.

Results

Spectroscopic Assessment

When blood is centrifuged, after the addition of anticoagulants, it is possible to separate the plasma by collecting the supernatant and discarding the sedimented cellular part. By avoiding the introduction of anticoagulants, the centrifugation removes also the clotting proteins such as fibrinogen and prothrombin leading to a less viscous solution called serum [26]. Although the protein content is reduced in serum, samples invariably contain also high molecular weight components, i.e. proteins and lipids, along with small metabolites. Furthermore, several serum samples considered in this work showed an intense orange colour suggesting that partial haemolysis had occurred during the centrifugation steps. The red blood cell lysis actually causes massive release of macromolecules, especially proteins. Besides the onset of possible affinity interactions, the very presence of broad NMR signals from macromolecules hinders the observation of the sharp resonances from small metabolites. To remove this broad signal interference, all NMR spectra were edited by exploiting the differences in transverse relaxation times (T_2) between high and low molecular-weight components. With the application of a T_2 filter through a Carr-Purcell-Meiboom-Gill (CPMG) pulse-train prior to acquisition [19,20], the signals from high molecular-weight species were largely removed (Figure 1A). Since in the aromatic region some peaks arising from coupled spins were found to exhibit J-modulation artefacts that show up as intensity oscillations, we found that correction was possible, in spite of the filter length required to suppress the broad components, by fine-tuning the CPMG train duration (96 ms) while keeping very short refocusing intervals (Figure 1B) (27,28).

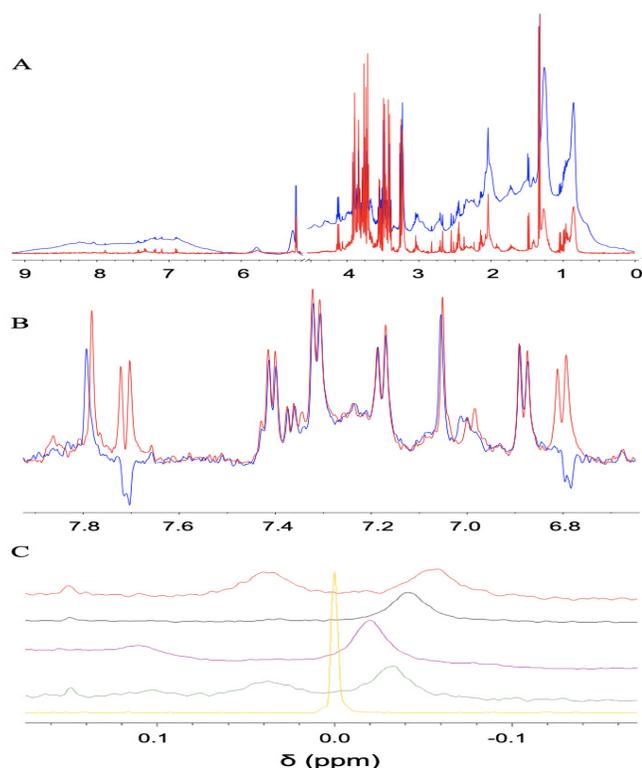


Figure 1: A) Overlay of ^1H NMR spectra of blood serum samples using 1D NOESY unedited sequence (blue) and CPMG T_2 edited sequence (red). B) Aromatic region overlay of CPMG ^1H NMR spectra of blood serum samples recorded with 80 ms filter (blue) and 96 ms filter (red). C) Example of four DSS signals in CPMG ^1H NMR spectra of blood serum samples (red, black, purple and green) superimposed with a DSS reference sample (yellow). All spectra were acquired at 500 MHz.

As chemical shift and concentration reference we first added to the samples a measured amount of internal standard, namely 4,4-Dimethyl-4-Silapentane-1-Sulfonate Sodium Salt (DSS), but we found that a variable interaction degree of this standard with the macromolecules present in the samples prevented any possibility of relying on internal referencing (Figure 1C). To circumvent the problem, we resorted to ERETIC [22], an approach that introduces an electronically-synthesized signal during acquisition. The ERETIC signal was calibrated for chemical shift and absolute concentration with respect to a 20 mM DSS reference solution. For the absolute concentration measurement, the following equation was used [29]:

$$\frac{[\text{unknown}]}{[\text{standard}]} = \frac{I_{\text{unknown}} N_{\text{standard}}}{I_{\text{standard}} N_{\text{unknown}}}$$

Where $[\text{unknown}]$ and $[\text{standard}]$ are the concentrations

of the metabolite species to be determined and of the standard, respectively, I_{unknown} and I_{standard} are their intensities and N_{unknown} and N_{standard} are the number of nuclei contributing to the respective signals.

After optimizing the acquisition of spectra, the different signals were assigned to the metabolites on the basis of previously published assignments [30-32], available databases [33,34] and spin-system recognition controls through 2D NMR spectra (Figure S1). Seventeen metabolites were identified. Three highly crowded regions could not be univocally assigned due to overlap of many different signals and the Branched-Chain Amino Acids (BCAAs) valine, isoleucine and leucine were considered all together. The list of the assigned metabolites and of the overlapping unidentified signals that were considered, along with the corresponding chemical shift intervals (the so-called sampling buckets in metabolomics jargon), is reported in Table 1. It can be noted immediately that ATP is absent. This important biomarker that exhibits very characteristic signals in the NMR spectrum [33,34] was not present in any of the analysed sera because no extraction from the cellular fraction was performed in order to quickly freeze the samples and therefore to prevent the typical catabolic losses that occur during the manipulations. A typical ^1H NMR spectrum is shown in Figure 2.

Metabolite	Bucket (ppm) and assignment
Acetate	1.92-1.90 (s, CH_3)
Alanine	1.50-1.43 (d, CH_3)
Aspartate	2.94-2.90 (dd, βCH); 3.01-2.98 (dd, βCH)
Citrate	2.57-2.49 (d, 2CH); 2.70-2.64 (d, 2CH)
Creatinine	4.07-4.03 (s, CH_2)
Formate	8.46-8.43 (s, CH)
α -Glucose	5.25-5.20 (d, C1H)
Glutamate	2.34-2.30 (t, γCH_2)
Glutamine	2.47-2.42 (m, γCH_2); 2.15-2.10 (m, βCH_2)
Glycine	3.59-3.57 (s, αCH_2)
Histidine	7.81-7.76 (s, C2H ring); 7.03-6.96 (s, C4H ring)
Lactate	4.16-4.06 (q, CH); 1.34-1.30 (d, CH_3)
Pyruvate	2.38-2.36 (s, CH_3)
Phenylalanine	7.45-7.39 (d, C3H, C5H ring); 7.34-7.28 (d, C2H, C6H ring)
Threonine	4.34-4.19 (m, βCH)

Tyrosine	6.93-6.84 (d, C3H, C5H ring); 7.20-7.13 (d, C2H, C6H ring)
Urea	5.95-5.63 (br, 2NH ₂)
Valine, Isoleucine, Leucine	1.05-0.92 (series of CH ₃)
Unassigned 1	3.94-3.62
Unassigned	3.58-3.32
Unassigned 3	3.30-3.14
Symbols in parenthesis indicate signal multiplicity (s, singlet; d, doublet; dd, doublet of doublets; t, triplet; q, quartet; m, multiplet; br, broad).	

Table 1: Metabolite assignments and corresponding occurrence intervals over which integrals were measured.

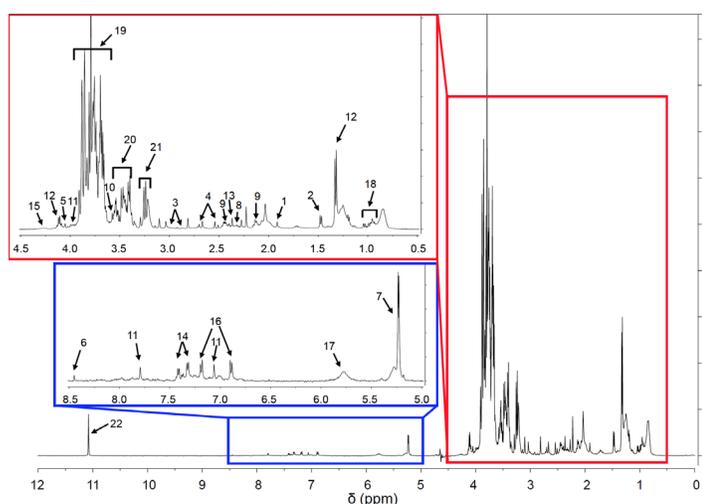


Figure 2: Representative ¹H NMR spectrum of a serum sample recorded at 500 MHz, in PBS pH 6.5 at 298 K. Numbers correspond to the following assignments: 1 acetate, 2 alanine, 3 aspartates, 4 citrate, 5 creatinine, 6 Formate, 7 glucoses, 8 glutamates, 9 glutamines, 10 glycine, 11 histidine, 12 lactate, 13 pyruvates, 14 phenylalanine, 15 threonine, 16 tyrosine, 17 urea, 18 branched-chain amino acids, 19 unassigned 1, 20 unassigned 2, 21 unassigned 3, 22 ERETIC signal.

The unassigned region extending from 3.94 to 3.62 ppm contains α protons of some amino acids (leucine, glutamine, glutamate, aspartate, lysine and alanine) along with β protons of choline, C3H, C5H, C6H of α -glucose and C6H of β -glucose. The region covering the interval from 3.58 to 3.32 ppm is mainly

dominated by glucose signals: C2H, C4H of α -glucose and C3H, C4H, C5H of β -glucose. In addition to glucose protons, also α protons of choline, *myo*-inositol and taurine (if present) fall within the same spectral interval. The signal envelope of this second region, indeed, showed a high correlation (0.971) with C1H α -glucose signal. The third unassigned region (3.30-3.14 ppm) includes C2H of β -glucose and trimethyl ammonium protons of choline, phosphocholine and glycerol-phosphocholine. From the concentration measurement of α -glucose based on its C1H signal (5.20-5.25 ppm), it was possible to calculate the amount of β -glucose considering the equilibrium ratio between the two anomers [35] (C1H of β -glucose resonates near the water signal, so it is unobservable due to solvent suppression). Since the third unassigned region consists of β -glucose C2H and cholines, by subtracting the β -glucose contribution, the intensity of this region can be used to estimate the quantity of cholines.

The urea exchangeable proton signal is affected by saturation transfer from water presaturation leading to unreliable absolute quantitation. The urea signal intensity was therefore corrected by a factor obtained from a preliminary calibration of the signal intensity variation as a function of the power level of solvent presaturation (Figure S2).

From a qualitative point of view, while the majority of recorded spectra presented a similar pattern of peaks, some spectra showed additional signals of uncertain attribution and consistent broadening of a few regions independently of the organ preservation protocol (Figure 3).

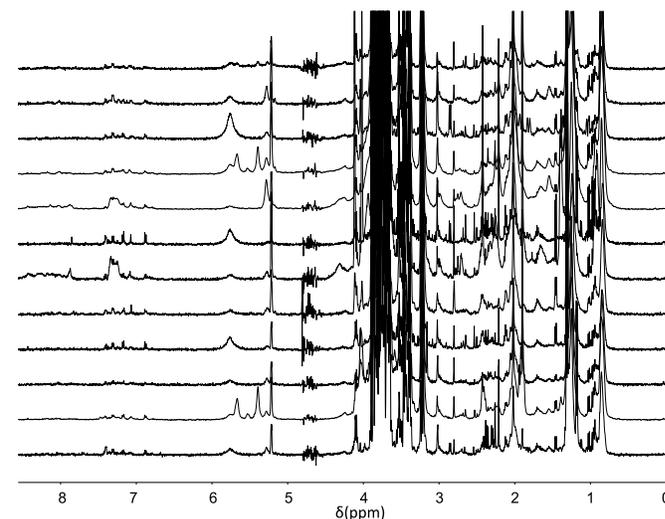


Figure 3: Stacked ¹H NMR spectra of representative profiles of serum samples from different patients. It can be seen that some spectra present additional or broadened peaks.

This is clearly related to inter-individual variability. Actually, many different exogenous factors are known to

contribute substantially to the metabolic profile like diet, gut bacteria, medications, renal function and comorbidities [36]. The large spread of the calculated metabolite concentrations (Table S1) confirms this observation.

For almost each transplant, independently of the preservation method, four serum samples were collected. Blood from coronary sinus and peripheral vein was collected both at the explant, from the donor patient, and at the implant, just after declamping the organ in the receiving patient. Therefore, each sample is characterized by the following attributes: location of blood sampling (S for coronary sinus, P for peripheral vein); phase of blood sampling (EX for sampling at organ explant, PD for post-declamping sampling, i.e. at organ implant); preservation type of the organ (CI or OCS).

After purging spurious signals, concentrations were estimated for 19 individual metabolites or undifferentiated metabolite classes: tyrosine, phenylalanine, urea, glucose, threonine, lactate, creatinine, citrate, alanine, aspartate, Formate, glutamate, glutamine, pyruvate, glycine, acetate, histidine, cholines and a compound signal for valine, isoleucine and leucine.

Statistical Inference

The simultaneous scaling coefficients that were derived by SVD analysis agree almost quantitatively with those obtained by scaling alternatively by sample or by metabolite (Figure 4). We have chosen this normalization method because it is genuinely two-dimensional, i.e. with simultaneous normalization by columns (metabolites) and by rows (samples).

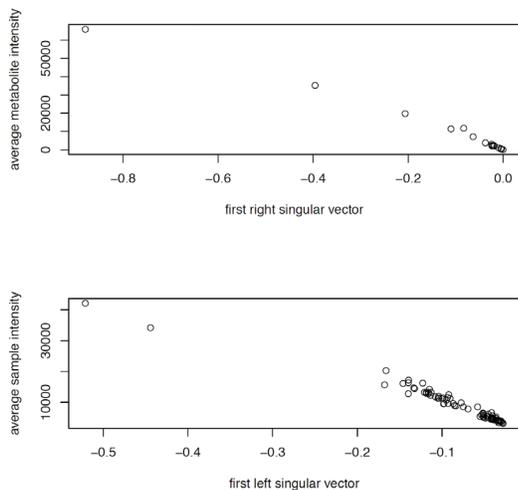


Figure 4: Plots of the average metabolite (sample) intensity versus the first right (left) singular vector, showing the linear relation between the two. The first singular vectors (right and left, the sign is unimportant as long as it is the same for both) are used simultaneously to normalize the data in SVD based normalization.

A first clustering of the samples to check for emerging features of the data set was performed using a Euclidean-distance-based average clustering analysis. The only apparent feature is the close pairing of samples from the same phase (EX or PD) with different sampling location (P or S) of blood specimens (Figure 5). This again highlights that the Individual Patient Pattern (IPP) is predominant. Figure 5: Dendrogram of analysed samples (average linkage, euclidean distance). The specific heart is indicated by GR followed by a number. The following characters separated by an underscore indicate the location of blood sampling (P or S, peripheral vein or sinus coronarius, respectively), the phase of blood sampling (EX or PD, at explant or post-unclamping, respectively) and heart preservation (CI or OCS, cold ischemia of Organ Care System, respectively).

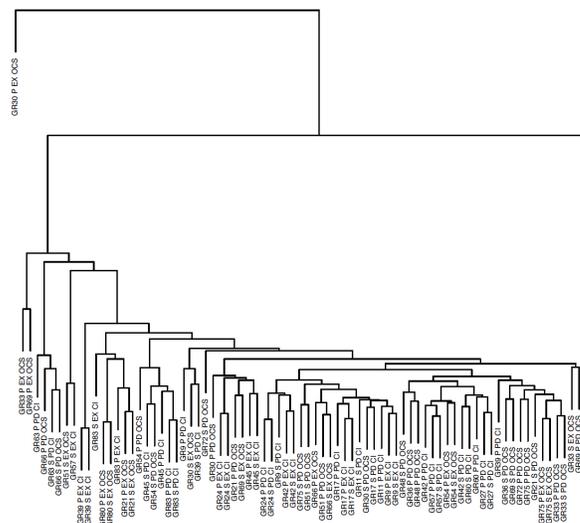


Figure 5: Dendrogram of analysed samples (average linkage, euclidean distance). The specific heart is indicated by GR followed by a number. The following characters separated by an underscore indicate the location of blood sampling (P or S, peripheral vein or sinus coronarius, respectively), the phase of blood sampling (EX or PD, at explant or post-unclamping, respectively) and heart preservation (CI or OCS, cold ischemia of Organ Care System, respectively).

We subsequently inspected if there is an effect of the location of blood sampling, i.e. coronary sinus (S) and peripheral vein (P), on the metabolite profile. Since the ratios of the metabolite concentrations at the two sampling locations were found different throughout each transplant depending apparently on the moment of the specimen collection, i.e. before the Explant (EX) and after the implant (Post-Declamping) (PD) (but essentially reflecting again the IPP), we decided to proceed by separating the EX samples from

the PD ones, to end up with 23 paired samples (S and P) for PD and 17 analogous pairs for EX. On the data from these specimens, we performed the paired *t*-test to check the effect of the location of blood sampling on each metabolite. Since no significant difference in variance after the multiple test correction was found (except for acetate which showed a large variance in EX samples), the equal-variance paired *t*-test was carried out, with post-refinement of the *p*-values for multiple testing by the false discovery rate correction. For the EX samples, there are no metabolites with significantly different occurrence in S and P specimens (*p*-value < 0.05). This is consistent with a homeostatic condition of the donor patients. The same analysis on PD samples showed a few metabolites with significantly different abundance in S and P samples. These are aspartate, glucose, lactate, tyrosine and urea (Table 2).

Metabolite	Average S/P ratio	p-value
Aspartate	1.06	0.022
Glucose	0.95	0.033
Lactate	1.42	0.0052
Tyrosine	0.87	0.022
Urea	0.75	0.0052

Table 2: Paired *t*-test results: metabolites significantly differently abundant in S and P sera considering only PD specimens.

The presence of significant differences between S and P samples snapshots the transient non-equilibrium condition of the receiving organism, just after the implant when the transplanted heart is unclamped. Furthermore, the increase in lactate and the decrease in urea in the coronary sinus with respect to the peripheral vein are clear signs of a stressed and uncompensated state of the just transplanted heart.

On attempting the same statistical tests to assess the effect of organ preservation (CI vs. OCS), which means splitting further the PD pool into two subsets, no significant metabolite difference could be found to separate P and S sera. If, on the other hand, the same P and S data were joined into single pools and considered only according to the CI or OCS preservation protocol, only phenylalanine and branched-chain amino acids (BCAAs) showed a meaningful difference between OCS and CI (Table 3).

Metabolite	Average OCS/CI ratio	p-value
Phenylalanine	1.50	0.016
BCAAs	1.27	0.021

Table 3: Paired *t*-test results: metabolites with significantly different concentration ratios in OCS and CI subsets of the whole PD pool, with joined P and S sampling.

Donor data		
	CI (n = 13)	OCS (n = 12)
Age (year) mean ± SD	47.7±13.4	45.9±11.3
Age > 55 years, % (N)	15 (2)	33 (4)
Male, % (N)	54 (8)	75 (9)
Ischemic time (min) mean ± SD	217.8±62.6	148±34.3
Infection, % (N)	31 (4)	8 (1)
Cardiac arrest, % (N)	15 (2)	33 (4)
Recipient data		
Age (year) mean ± SD	57±10.3	56±9.6
Male, % (N)	85 (11)	100 (12)
Body mass index > 30, % (N)	15 (2)	8 (1)

Table 4: Donor and recipient demographics.

Apparently, by showing an increase in phenylalanine and BCAAs, OCS-preserved hearts seem to exhibit a higher level of stress. This result, however, could not be entirely ascribed to the impact of OCS preservation on heart transplant, as suggested by the negative controls on EX data. The same CI vs. OCS analysis without separating P and S sampling was conducted, in fact, on the EX data pool. The donor patients whose heart would have been preserved with OCS showed a meaningfully higher concentration of urea (*p*-value = 0.008), i.e. a typical indicator of kidney function failure or recent heart attack. This is consistent with the explicit bias of surgeons who submit to OCS preservation the worse explanted hearts, in terms of donor conditions.

If S/P ratios were considered in relation to OCS and CI preservation, no significant difference was found between the two groups.

Linear models are not able to significantly discriminate by ANOVA test between OCS and CI considering either all PD samples or only PD subgroups, i.e. only S or only P sera. In the ANOVA tests, the effects of metabolite, preservation method and their joint effect have been included in the linear model.

The results obtained with normalized concentration values, i.e. considering the whole measured signal pool of every single blood specimen as normalization constant for the corresponding dataset vector (19 species), should be reproduced also when absolute concentration values are used instead. SVD analysis is constitutively independent of the actual concentration values and provides the same outcome with either absolute and normalized concentration matrices. Accordingly, the average values of the absolute concentrations for the individual metabolite species

should not exhibit any statistically significant trend when grouped according to the organ preservation protocol or/and the sample draw origin. This can be appreciated by inspection of Tables S2 and S3. In Table S2 the mean absolute concentrations are reported for each metabolite in any samples (explant and implant) drawn at coronary sinus, along with the corresponding standard deviations. In spite of the large individual variability, the concentration values are in line with the typical ones given in literature. The large standard deviations are however responsible for the lack of statistical significance of metabolite absolute concentration ratios, whatever the chosen combination, i.e. either OCS/CI ratio at explant or implant, and PD/EX ratio for OCS- or CI-preserved hearts (Table S3).

From Table S3, however, it is apparent that the metabolite levels after preservation by OCS are consistently smaller than those after preservation by CI, i.e. 18 out of 19 ratios are less than 1.0, whereas the same ratios are mostly (12 out of 19) larger than 1.0 at explant, i.e. before preservation. Moreover, a nearly systematic decrease in absolute metabolite levels is observed after preservation by OCS, whereas no clear trend emerges for CI preservation (for glucose the decrease after OCS preservation is even statistically significant with a p -value of 0.009). A Wilcoxon test performed on the ratios of Table S3, treated as numbers whose *a-priori* expected mean is 1.0, resulted in highly significant p -value (3.81×10^{-5}) for the OCS/CI ratios determined at implant, whereas the same ratio at explant did not give a significant p -value (0.07). The same statistical test performed on the decrease in metabolite concentrations (PD/EX ratios) after preservation by OCS gave a p -value of 0.0033, whereas no significance was found for preservation by CI (p -value = 0.156).

Discussion

In spite of the effort required to perform and manage the number of heart transplants that were here considered, the results of the metabolomics analysis so far obtained indicate that the size of the examined data set is still insufficient to draw statistically sound conclusions. This effect of the data set size is stressed by the protocol of analysis, as discussed in the following. Therefore, besides the acknowledged merits of the OCS over the traditional CI preservation method, in terms of the cold ischemic time limitation and quality control of the explanted heart, the available data do not allow us to answer the basic questions on the further advantages and convenience of the OCS for the general protocol of the heart transplant intervention.

Nonetheless, the collected data give some interesting clues and therefore are valuable from the viewpoint of a methodological discussion. Indeed, while highlighting the relevance of the inter-individual variability, the IPP factor, which certainly determines, to a large extent, the necessity of extending the data set dimension,

the statistical analysis also shows that our limited experimental data pool cannot be safely considered unaffected by the bias which the donor patient conditions introduce on the selection of the heart preservation method. This further calls for an extension of the experimental data set.

The results we have reported show very clearly the occurrence of lower absolute concentration levels for all but one of the identified metabolites in PD coronary sinus sera from OCS-preserved hearts with respect to CI-preserved organs (Table S3). Coupled with the statistically significant decrease of absolute metabolite concentrations following OCS preservation (inferred from PD/EX ratio, Table S3), the result appears in contrast with previously published data based on the comparison between hypothermic perfusive preservation and static cold storage of porcine kidneys [37,38]. The concentrations of the metabolites in the storage fluid of the perfusion apparatus were systematically higher when hypothermic perfusion rather than static cold storage had been adopted. The finding was interpreted to represent a higher metabolic level of perfusion-preserved kidneys over statically-stored ones, and therefore to support a beneficial role of perfusion-based protocols. Apart from the differences between heart and kidney and between hypothermic and the normothermic conditions, the results we describe here are obtained in a totally different context. Our analysis concerns the blood sera collected in the donor and receiving patient organisms, whereas the mentioned literature data [37,38] were essentially relative to the metabolites that diffuse from the organ tissue to the storage fluid in the perfusion machine (in the kidney tissue, the concentration differences between perfusive and static cold storage were much less pronounced). Much more interesting would be relating the general decrease of the measured absolute metabolite levels to functional inference. Although it is tempting to infer lower tissue stress from the concentration reduction of pyruvate and lactate, or absence of urea cycle dysfunction, or activation of an amino-acid-based anaplerotic pathway [39], the bias influencing the choice of the OCS-based protocol for functionally compromised organs can sensibly alter those conclusions.

The whole result of our statistical analysis, however, unequivocally picks up an important characteristic of the data, namely that the metabolic pattern we inspect by close and quick blood sampling at coronary sinus and peripheral vein upon explant or upon implant of the heart is dominated by the presence or absence, respectively, of homeostatic equilibrium. Hence the study only partially achieved the aim to which it was designed, namely to interrogate the metabolic pattern for the occurrence of diagnostic, transient differences in proximity of the transplanted organ, i.e. at the coronary sinus, in order to correlate such differences with the organ preservation method, after correcting for any possible donor variability effect. While successfully highlighting the transient

imbalance of the metabolic pattern at the implant, our experimental set proves too limited to discern possible metabolic effects of the organ preservation method.

Given the limited number of metabolites that can be reliably quantified through our NMR approach and in consideration of the IPP factor, a substantial increase of the data set size to overcome the IPP factor variability, and to remove possible bias in the selection of OCS preservation rather than CI, may not necessarily lead, by reasonable time and effort investments, to appreciate effects of the organ preservation protocol.

Therefore, if a metabolomics study such as that here described must provide prognostic conclusions concerning the organ preservation method before long-term monitoring of the heart transplant survival score will reach a proper statistical reliability, a modification of the adopted experimental protocol should be implemented. Blood sampling should be carried out only from the receiving patient and essentially at peripheral vein locations, one time prior to transplant and two or three times after transplant (say after 6, 12 and 24 hours), along with coronary sinus and peripheral blood draws at the implant. Thus, with a minor increase of work load with respect to the previous protocol, it would be possible to collect more homogeneous data, without disregarding those transient features that occur at the very moment of heart transplant. The collected data set should provide information on the short-term metabolic pattern evolution of the receiving patient. With statistically adequate dimensions (not necessarily much larger than the size here considered), the effect of the organ preservation method on the outcome of the transplant could be assessed more directly.

In conclusion our preliminary study has suggested valuable methodological refinements to improve the design of a metabolomics investigation on the effect of the heart preservation method.

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Data Availability

The data used to support the findings of this study are included in the present article and in the related Supplementary Material.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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References

1. Kirklin JK, Naftel DC, Kormos RL, Stevenson LW, Pagani FD, et al. (2013) Fifth INTERMACS Annual Report: Risk Factor Analysis from More than 6,000 Mechanical Circulatory Support Patients. *J Heart Lung Transplant* off Publ Int Soc Heart Transplant 32: 141-156.
2. Banner NR, Thomas HL, Curnow E, Hussey JC, Rogers CA, et al. (2008) The Importance of Cold and Warm Cardiac Ischemia for Survival after Heart Transplantation. *Transplantation* 86: 542-547.
3. Russo MJ, Iribarne A, Hong KN, Ramlawi B, Chen JM, et al. (2010) Factors Associated with Primary Graft Failure after Heart Transplantation. *Transplantation* 90: 444-450.
4. Hassanein WH, Zellos L, Tyrrell TA, Healey NA, Crittenden MD, et al. (1998) Continuous Perfusion of Donor Hearts in the Beating State Extends Preservation Time and Improves Recovery of Function. *J Thorac Cardiovasc Surg* 116: 821-830.
5. Messer S, Ardehali A, Tsui S (2015) Normothermic Donor Heart Perfusion: Current Clinical Experience and the Future. *Transpl Int* 28: 634-642.
6. Goldsmith KA, Demiris N, Gooi JH, Sharples LD, Jenkins DP, et al. (2009) Life-Years Gained by Reducing Donor Heart Ischemic Times. *Transplantation* 87: 243.
7. Anonymous (2006) World Transplant Congress 2006 Oral Abstracts. *Am J Transplant* 6: 65-472.
8. Hamed A, Tsui S, Huber J, Lin R, Poggio EC, et al. (2009) 19: Serum Lactate Is a Highly Sensitive and Specific Predictor of Post Cardiac Transplant Outcomes Using the Organ Care System. *J Heart Lung Transplant* 28: S71.
9. Zhang A, Sun H, Wang P, Han Y, Wang X (2011) Modern Analytical Techniques in Metabolomics Analysis. *Analyst* 137: 293-300.
10. Beckonert O, Keun HC, Ebbels TMD, Bundy J, Holmes E, et al. (2007) Metabolic Profiling, Metabolomic and Metabonomic Procedures for NMR Spectroscopy of Urine, Plasma, Serum and Tissue Extracts. *Nature Protoc* 2: 2692-2703.
11. Wishart DS (2005) Metabolomics: The Principles and Potential Applications to Transplantation. *Am J Transplant* 5: 2814-2820.
12. Zhao Y, Katz NM, Lefrak EA, Foegh ML (1997) Urinary Thromboxane B2 in Cardiac Transplant Patients as a Screening Method of Rejection. *Prostaglandins* 54: 881-889.
13. Mügge A, Kurucay S, Böger RH, Bode-Böger SM, Schäfers HJ, et al. (1996) Urinary Nitrate Excretion Is Increased in Cardiac Transplanted Patients with Acute Graft Rejection. *Clin Transplant* 10: 298-305.
14. Eugene M, Le Moyec L, de Certaines J, Desruennes M, Le Rumeur E, et al. (1991) Lipoproteins in Heart Transplantation: Proton Magnetic Resonance Spectroscopy of Plasma. *Magn Reson Med* 18: 93-101.

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15. Lee CW, Lee JH, Kim JJ, Park SW, Hong MK, et al. (1999) Cerebral Metabolic Abnormalities in Congestive Heart Failure Detected by Proton Magnetic Resonance Spectroscopy. *J Am Coll Cardiol* 33:1196-1202.
16. Kober F, Caus T, Riberi A, Confort-Gouny S, LeFur Y, et al. (2002) Objective and Noninvasive Metabolic Characterization of Donor Hearts by Phosphorous-31 Magnetic Resonance Spectroscopy. *Transplantation* 74:1752-1756.
17. Lukes DJ, Madhu B, Kjellström C, Gustavsson ML, Mjörnstedt L, et al. (2001) Decreasing Ratios of Phosphocreatine to Beta-ATP Correlates to Progressive Acute Rejection in a Concordant Mouse Heart to Rat Xenotransplantation Model. *Scand J Immunol* 53: 171-175.
18. Meiboom S, Gill D (1958) Modified spin-echo method for measuring nuclear relaxation times. *Rev Sci Instrum* 29: 688-691.
19. Carr HY, Purcell EM (1954) Effects of diffusion on free precession in nuclear magnetic resonance experiments. *Phys Rev* 94: 630-638.
20. Piotto M, Saudek V, Sklenár V (1992) Gradient-tailored excitation for single-quantum NMR spectroscopy of aqueous solutions. *J Biomol NMR* 2: 661-665.
21. Hwang TL, Shaka AJ (1995) Water Suppression That Works. Excitation Sculpting Using Arbitrary Wave-Forms and Pulsed-Field Gradients. *J Magn Reson* 112: 275-279.
22. Akoka S, Barantin L, Trierweiler M (1999) Concentration Measurement by Proton NMR Using the ERETIC Method. *Anal Chem* 71: 2554-2557.
23. Braunschweiler L, Ernst RR (1983) Coherence transfer by isotropic mixing: Application to proton correlation spectroscopy. *J Magn Reson* 53: 521-528.
24. Müller L (1979) Sensitivity enhanced detection of weak nuclei using heteronuclear multiple quantum coherence. *J Am Chem Soc* 101: 4481- 4484.
25. R Core Team (2018) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. <https://www.R-project.org/>.
26. Best and Taylor's Physiological basis of medical practice.
27. Hahn EL (1950) Spin Echoes *Phys Rev* 80: 580-594.
28. Allerhand A, Thiele E (1966) Analysis of Carr-Purcell spin-echo experiments on multiple-spin systems. II The effect of chemical exchange. *J Chem Phys* 45: 902-916.
29. Holzgrabe U, Deubner R, Schollmayer C, Waibel B (2005) Quantitative NMR Spectroscopy-Applications in Drug Analysis. *J Pharm Biomed Anal* 38: 806-812.
30. Psychogios N, Hau DD, Peng J, Guo AC, Mandal R, et al. (2011) The Human Serum Metabolome. *PLOS ONE* 6: e16957.
31. Sitter B, Sonnewald U, Spraul M, Fjösne HE, Gribbestad IS (2002) High-Resolution Magic Angle Spinning MRS of Breast Cancer Tissue. *NMR Biomed* 15: 327-337.
32. Roslund MU, Tähtinen P, Niemitz M, Sjöholm R (2008) Complete Assignments of the ¹H and ¹³C Chemical Shifts and JH, H Coupling Constants in NMR Spectra of d-Glucopyranose and All d-Glucopyranosyl-d-Glucopyranosides. *Carbohydr Res* 343:101-112.
33. Wishart DS, Jewison T, Guo AC, Wilson M, Knox C, et al. (2013) HMDB 3.0-The Human Metabolome Database in 2013. *Nucleic Acids Res (Database issue)* 41: D801-807.
34. Ulrich EL, Akutsu H, Doreleijers JF, Harano Y, Ioannidis YE, et al. (2008) Bio Mag Res Bank. *Nucleic Acids Res (Database issue)* 36: D402-D408.
35. Maebayashi M, Ohba M, Takeuchi T (2017) Anomeric Proportions of D-Glucopyranose at the Equilibrium Determined from ¹H-NMR Spectra I. Investigation of Experimental Conditions and Concentration Dependence at 25.0 °C. *J Mol Liq* 232: 408-415.
36. Rhee EP, Gerszten RE (2012) Metabolomics and Cardiovascular Biomarker Discovery. *Clin Chem* 58: 139-147.
37. Guy AJ, Nath J, Cobbold M, Ludwig C, Tennant DA, et al. (2014) Metabolic analysis of perfused state during hypothermic machine perfusion of human cadaveric kidneys. *Transplantation* 99: 754-759.
38. Nath J, Smith TB, Kamlesh P, Ebbs SR, Hollis A, et al. (2017) Metabolic differences between cold stored and machine perfused porcine kidneys: A ¹H NMR based study. *Cryobiology* 74: 115-120.
39. Ikegami R, Shimuzu I, Yoshida Y, Minamino T (2018) Metabolomic Analysis in Heart Failure. *Circulation* J 82: 10-16.