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Overlap in serum metabolic profiles between non-related diseases: Implications for LC-MS metabolomics biomarker discovery

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ABSTRACT

Untargeted metabolic profiling has generated large activity in the field of clinical biomarker discovery. Yet, no clinically approved metabolite biomarkers have emerged with failure in validation phases often being a reason. To investigate why, we have applied untargeted metabolic profiling in a retrospective cohort of serum samples representing non-related diseases. Age and gender matched samples from patients diagnosed with pneumonia, congestive heart failure, lymphoma and healthy controls were subject to comprehensive metabolic profiling using ultra-performance liquid chromatography-mass spectrometry (UPLC-MS). The metabolic profile of each diagnosis was compared to the healthy control group and significant metabolites were filtered out using *t*-test with FDR correction. Metabolites found to be significant between each disease and healthy controls were compared and analyzed for overlap. Results show that despite differences in etiology and clinical disease presentation, the fraction of metabolites with an overlap between two or more diseases was 61%. A majority of these metabolites can be associated with immune responses thus representing non-disease specific events. We show that metabolic serum profiles from patients representing non-related diseases display very similar metabolic differences when compared to healthy controls. Many of the metabolites discovered as disease specific in this study have further been associated with other diseases in the literature. Based on our findings we suggest non-related disease controls in metabolomics biomarker discovery studies to increase the chances of a successful validation and future clinical applications.

1. Introduction

Metabolic profiling has become an active field of clinical research in several areas of diagnostic medicine [1], but has so far failed to produce clinically approved novel small molecular biomarkers. Yet, it is evident from neonatal screening programs with up to 55 inborn errors of metabolism on the list of screenable diseases [2], that a disease can be very tightly linked to a metabolic signature. Likewise, it is a well-established fact that diseases like cancer have a dramatically altered metabolism to that of normal proliferating cells [3], providing a biochemical rationale for biomarker discovery efforts. So why are no novel disease metabolite biomarkers emerging?

A major difference between most inborn errors of metabolism and a disease like cancer or cardiovascular disease is that while the first most often consist of a point mutation resulting in a single malfunctioning protein, the latter is a multifactorial disease developed over longer periods of time often involving several errors occurring in a sequential fashion [3]. Further, the patient being screened for inborn errors of metabolism is most often only a few days old, has no established microbiota and rarely has any co-morbidities whereas the prospective patient being screened for cancer typically is in the age of 40–70, has a complex microbiota and is very likely to have some kind of co-morbidity even if undiagnosed, increasing the risk of identifying non-specific biomarkers.

Conceptually the biomarker process can be divided into a discovery- and a clinical validation phase. The discovery phase should include confirmation of findings in an external test set and a disease mechanism hypothesis in order to minimize the risk of costly validations of unspecific markers [4,5]. So far, no novel metabolite biomarkers have passed an independent clinical validation phase

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since the emergence of modern metabolomics. For example, sarcosine was associated with prostate cancer progression both in urine and tissue [6]. However subsequent studies using comparable patient samples failed to validate this association [7,8].

In the present study, we have performed metabolic profiling, using LC-MS, of serum from patients representing three completely unrelated diseases with respect to clinical disease presentation and etiology; non-Hodgkin lymphoma, community acquired pneumonia and congestive heart failure. Resulting profiles were compared separately to healthy controls. Our results indicate that the number of disease specific serum metabolites is surprisingly small. We propose a strategy for clinical discovery projects to increase chances of finding disease specific metabolic serum biomarkers.

2. Materials and methods

2.1. Clinical samples

Serum samples were acquired from the commercial vendor BioServe and had been collected in accordance with the Declaration of Helsinki [9]. Healthy controls ($n = 40$) and the following three diseases were included: Congestive heart failure ($n = 40$), lymphoma ($n = 40$) and community acquired pneumonia ($n = 25$). Samples were selected to match for gender and age between groups. Lymphoma samples were non-Hodgkin lymphomas stage I–IV. Congestive heart failure samples were stage III–IV (Table 1). Samples were collected at different hospitals according to a standardized protocol [10] following FDA guidelines and then stored at a central location. All samples were extracted and prepared at the same time and analyzed, in a randomized order, over a period of three days on the same instrument.

2.2. Serum sample preparation

Samples were stored at −80 °C. Prior to metabolite extraction, samples were thawed on ice. 50 μL serum per sample was mixed with 150 μL MeOH and then centrifuged at 15800*g* for 15 min. The supernatant was transferred to a new vial, dried down in a speed vacuum concentrator and stored at −80 °C. Prior to analysis, samples were re-dissolved in 50 μL 1:1 MeOH:H_2O. An aliquot of each sample was pooled for quality control (QC) samples.

2.3. UPLC-MS analysis

Metabolites were separated by reversed phase liquid chromatography and detected by electrospray ionization mass spectrometry operated in positive and negative mode. The platform used was an Agilent 1290 UPLC-system connected online to an Agilent 6550 Q-ToF mass spectrometer equipped with a JetStream source. 5 μL per sample of the re-dissolved supernatant were injected onto a C18 column (Kinetex 100 × 2.1 mm 2.6 um 100 Å, Phenomenex) using the following mobile phases: H_2O with 0.1% formic acid (A) and 3:1 acetonitrile:isopropanol with 0.1% formic acid (B). All solvents were of HPLC grade and the water was Milli-Q. The following gradient was used at a flow rate of 0.5 ml/min: Min 0, 5% B; min 8, 95% B; min 10, 95% B; min 10.2, 5% B; min 12, 5% B. MS data was collected between m/z 70–1700 with the following electrospray ionization settings: Gas temperature 300 °C; gas flow 8 L/min; nebulizer pressure 40 psi; sheet gas temperature 350 °C; sheet gas flow 11; Vcap 4000; fragmentor 100; skimmer1 45; OctapoleRFPeak 750.

Samples were run in randomized order with a QC-sample injection interspersed between every 5 samples. The QC-injections were used *i)* to calculate the repeatability of the analytical method and *ii)* to evaluate the effect of sample normalization. Additional QC-sample injections were also made to acquire data dependent MS/MS spectra for metabolite identification purposes. Blank samples were injected throughout the sample series to monitor carry over.

2.4. Raw data preprocessing

Raw data was processed using the “Find by Molecular Feature”-function in the software MassHunter Qual version B.06.00 (Agilent) for peak detection and removal of isotopes and adducts. Generated CEF files were then imported into the software Mass Profiler Professional version B.12.05 (Agilent) for normalization, alignment, peak filtering and statistical analysis. Samples were normalized to have equal median intensity. The normalization was based on the assumption that all serum samples have a similar metabolic profile. The intention of a sample-wise signal correction method like median normalization is hence to correct for technical variation in e.g. sample preparation and MS signal intensity [11]. A two-step filtering approach was used for peak quality control: firstly, only peaks present in at least 75% of samples in at least one group were retained; secondly, only peaks above a specified intensity level in the raw data were used for further analysis.

2.5. Univariate statistical analysis

To identify single metabolite markers discriminating disease from healthy controls, univariate statistical analysis was applied. For each disease, Student's *t*-test with Benjamini Hochberg FDR correction for multiple testing was performed. Missing values were excluded and the comparison of pneumonia with healthy controls was done by *t*-test not assuming equal variances due to differences in sample size.

Metabolite features with FDR <0.05 were examined in the raw data using the “Find by Formula”-function in MassHunter Qual in combination with manual curation. Low quality features such as PEG contaminations and erroneous peak detection were removed from the data set. In the case of poor automatic peak area integration, features were re-integrated manually. The significance of

Table 1
Clinical samples.

Characteristic		Healthy controls (C)	Congestive heart failure (H)	Community acquired pneumonia (P)	Lymphoma (L)
n		40	40	25	40
Age, years[a]		52 (41–59)	54 (44–60)	54 (41–60)	51 (41–60)
Male/female ratio		20/20	20/20	12/13	20/20
Disease stage (n)	I	N/A	–	N/A	8
	II		–		14
	III		32		9
	IV		8		9

[a] Data is presented as median (range).

the re-integrated curated data was then confirmed with a Student's *t*-test using the software Prism version 6.07 (GraphPad). Prism was further used to perform Pearson correlation analyses and to create box plots. Overlap analysis of metabolites differing between disease and healthy controls was visualized using the online tool Venny [12].

2.6. Metabolite identification

Accurate mass measurements were subjected to database searches in the public databases METLIN [13] and Human Metabolome Database [14] as well as an in-house library comprising 384 synthetic standards. Database hits were then confirmed by retention time match (in-house library only) and MS/MS spectral match from the QC-samples. When data dependent MS/MS spectra were missing, samples were re-analyzed using targeted MS/MS. In a few cases, samples were also fractionated by LC and relevant fractions were then analyzed by direct infusion on a Thermo Scientific Orbitrap MS instrument to acquire MS^n structural information. In the case of multiple possible database hits, e.g. for structural isomers, metabolites were annotated with compound class only.

Phospholipid species such as phosphatidylcholine (PC) and lysophosphatidylcholines (LPC) were annotated using the formula X:Y, where X = number of carbon atoms and Y = number of double bonds. A phospholipid "O" annotation (e.g. LPC(O-X:Y)) represents an alkyl ether bond; a "P" annotation (e.g. LPC(P-X:Y)) represents an alkenyl ether bond. The suffix "ox." denotes oxidation.

3. Results

3.1. Quality control

The analytical repeatability of each of the selected metabolite features was calculated as ≤8.6% based on the QC-samples, which is considered an acceptable coefficient of variation according to FDA guidelines [15]. Carry over was below the limit of detection as determined by comparing the peak areas of lipophilic metabolites (such as LPCs) in patient samples to blank sample injections. The data were further visualized by principal components analysis and box plot to verify sample homogeneity.

The clinical sample table (Table 1) shows disease and control samples with equal gender ratios and matched age span. BMI was significantly different (Student's *t*-test $p < 0.05$) in the control group compared to the three disease groups (control vs. heart failure $p = 0.03$; control vs. lymphoma $p = 0.03$; control vs. pneumonia $p = 0.01$). Therefore BMI was considered a potential confounding factor. Pearson correlation between BMI and all selected metabolites respectively showed however no significant correlation (Supplementary Table 1).

3.2. 178 metabolites differed in disease compared to healthy controls

Following MeOH extraction, serum samples were analyzed by ultra-performance liquid chromatography-mass spectrometry (UPLC-MS), detecting approximately 1200 metabolites per sample (Fig. 1). After signal correction by sample-wise median intensity normalization and frequency/abundance filtering, 590 and 506 metabolites remained from the UPLC-MS positive and negative mode analysis, respectively. Of these, 354 metabolites significantly differed in disease compared to healthy controls in positive mode, while the corresponding number for negative mode was 205, determined by Student's *t*-test with Benjamini Hochberg multiple testing correction FDR limit of 5%. Removal of low quality metabolic features and features overlapping between ionization modes resulted in a total of 178 differential metabolites, 106 and 72 in positive and negative mode, respectively. Differential metabolites were subsequently analyzed for overlap between diseases compared to healthy controls. 66% of the differential metabolites were identified.

3.3. A majority of differential metabolites overlap in disease

The number of metabolites differing between disease and control was largest in pneumonia ($n = 134$), while the corresponding number was 91 in lymphoma and 109 in heart failure (Fig. 2). Likewise, pneumonia was the disease with the largest number of unique metabolites ($n = 51$). Only 12 and 6 metabolites were unique to heart failure and lymphoma, respectively. Examples of unique metabolites are displayed in Supplementary Fig. 1.

61% of the differential metabolites overlapped between two or all three diseases compared to healthy controls. 26% of metabolites overlapped between all three diseases, and a majority of these were down-regulated in comparison to controls. Metabolites unique to a certain disease and those overlapping between only two diseases were to a larger extent up-regulated or regulated in opposite directions in different diseases (Fig. 2).

3.4. 43% of metabolites overlapping between all three diseases are lysophosphatidylcholines (LPCs) which may be connected to immune responses

Of the metabolites overlapping between all three diseases (Supplementary Table 2), the dominating metabolite class was LPCs, of which all were down-regulated in disease compared to healthy controls. A literature search for associations of this metabolite class with disease showed a strong association between serum LPC levels and various diseases (Fig. 3).

3.5. Implications for the design of biomarker discovery studies

We used our dataset to conceptually investigate the impact of study design on results interpretation. One form of biomarker discovery study design is to compare the disease of interest with healthy controls [1,16]. In the present dataset this was for example illustrated by the comparison of lymphoma patients with healthy control subjects. One example of a metabolite differing between these two groups was LPC(18:3) (Fig. 4A). Another possible study design is to compare progression states of a disease such as stages of a cancer [17]. Observing the relative concentration of LPC(18:3) from stage I to stage IV lymphoma, a significant trend emerged (Fig. 4B). When however congestive heart failure and pneumonia were included as non-related disease controls in the study design, it became apparent that LPC(18:3) was not a disease specific marker (Fig. 4C).

4. Discussion

A key aspect of a biomarker discovery study is to find disease specific markers. The results from the present study show that the number of general disease markers in serum may be larger than expected, making the discovery of truly unique markers more challenging. Our study cohort comprised serum samples from three diseases that had been selected to represent clearly separate disease categories: malignancy (non-Hodgkin lymphoma), intrinsic organ failure (congestive heart failure) and infectious disease (community acquired pneumonia). Considering that these diseases have very different etiology and clinical presentation, they would be expected to have a large fraction of unique serum metabolic markers when compared to healthy controls. Based on our results

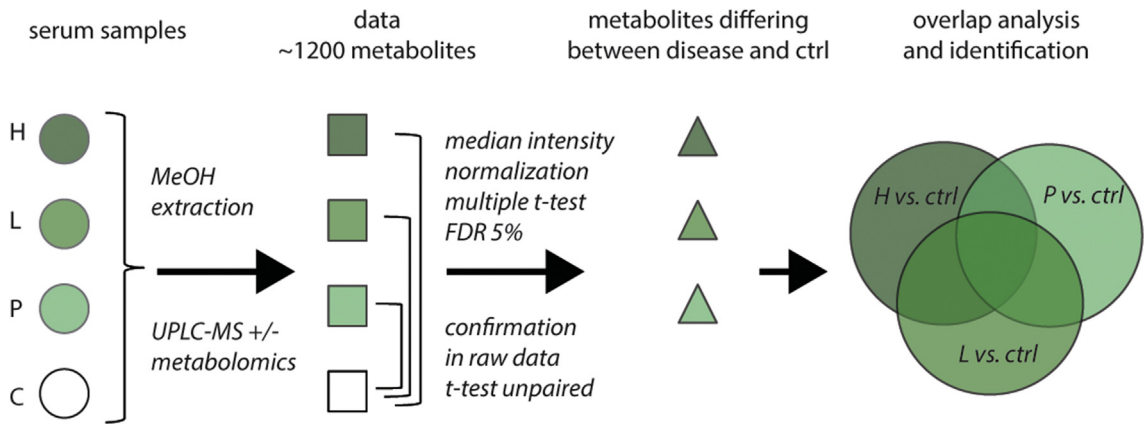

Fig. 1. Schematic representation of study design. Serum samples from the three unrelated diseases non-Hodgkin lymphoma (L), community acquired pneumonia (P) and congestive heart failure (H) and healthy controls (C) were analyzed by UHPLC-MS in positive and negative mode. All three disease groups were each compared to the healthy control group by Student's *t*-test with Benjamini Hochberg correction for multiple testing (FDR 5%). Selected metabolite features were examined and reintegrated in the raw data and statistical significance was verified by *t*-test. Metabolites were then identified and subject to overlap analysis to identify metabolites specific to or shared between different diseases.

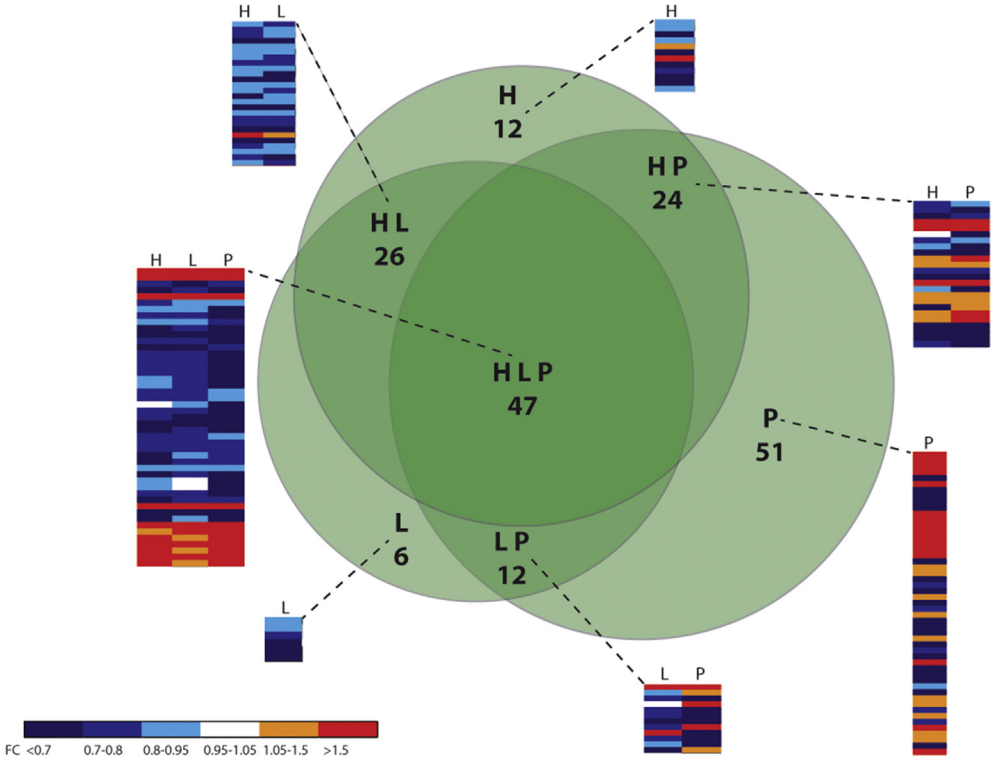

Fig. 2. Overlap analysis of metabolites differing between disease and healthy controls. Metabolites were selected by *t*-test with multiple test correction (Benjamini Hochberg FDR 5%) (H = congestive heart failure vs. controls, L = lymphoma vs. controls, P = pneumonia vs. controls). Of a total of 178 metabolites, 61% (109) were shared between two or all three diseases (sections HL, HP, LP and HLP in the Venn diagram). While 51 metabolites were specific to pneumonia, the corresponding numbers were only 6 and 12 for lymphoma and congestive heart failure, respectively. Fold change (FC) calculation showed that with few exceptions, overlapping metabolites were regulated in the same direction, with a majority being down-regulated in comparison to healthy controls. Bar indicates FC of the respective metabolite in the respective disease versus healthy controls.

this appears not to be the case; the number of disease specific markers seems in fact to be surprisingly small.

The metabolites phenylalanine, LPC(O-18:0), androsterone sulfate and LPC(20:5) were found down-regulated and specific to lymphoma, congestive heart failure and pneumonia, respectively (Supplementary Fig. 1). However, all four of these metabolites have also been found specific to other diseases in previous studies unrelated to the present one. For example, phenylalanine was found down-regulated in serum from patients with Alzheimer's disease compared to healthy controls [18]. The plasma levels of the phospholipid LPC(O-18:0) were lower in bacteremia patients than in controls [19]. Androsterone sulfate was found down-regulated in plasma from patients with lung adenocarcinoma [20] and plasma levels of LPC(20:5) were lower in women diagnosed with Gestational Diabetes Mellitus (GDM) [21]. These examples show that potential metabolite disease markers risk being repeatedly identified across a wide range of non-related disease states, and hence not disease specific.

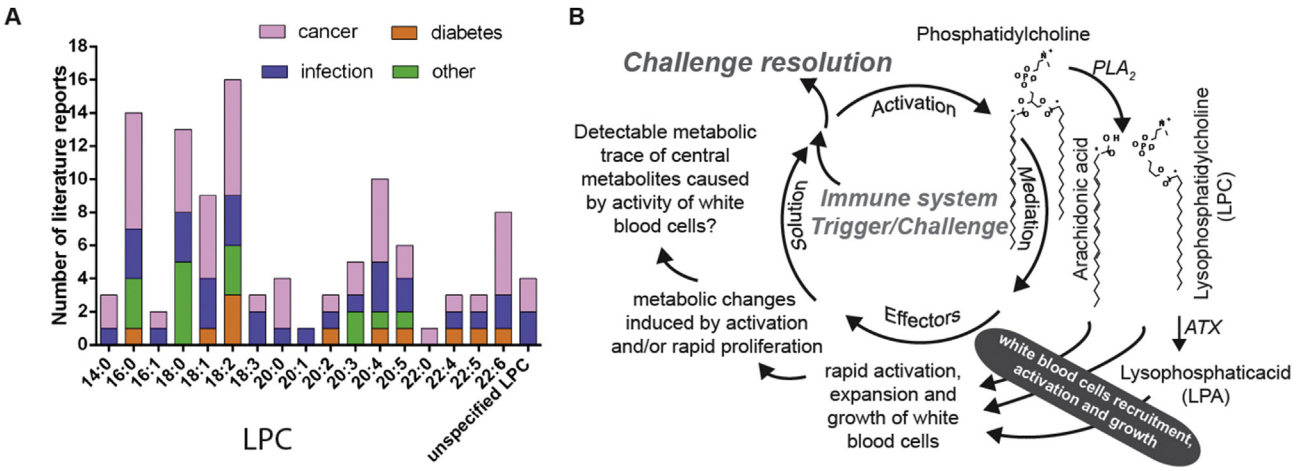

Fig. 3. A connection between serum LPC levels and immune responses. A. A number of LPCs found overlapping in this study (Supplementary Table 2) were associated with a wide range of disease states in the literature. This indicates that serum LPC levels are likely to be connected to secondary systemic responses rather than perturbations in a disease specific pathway. The y-axis gives the number of literature reports where a specific LPC is reported to be associated with a specific disease. PubMed and ISI Web of Science were queried using the following search phrases in combinations for the different LPCs: LPC(X:Y), biomarker, disease, lysophosphatidylcholine, cancer, Alzheimer, diabetes, infection, cardiovascular and serum. Studies involving humans were retained. See Supplementary Table 3 for references. **B**. LPCs play a central role in initiating and maintaining an inflammatory response. Upon activation of the immune system, LPCs are released from plasma membranes and act as potent inflammatory mediators. As such, serum LPC levels are likely to change with a range of pathological conditions.

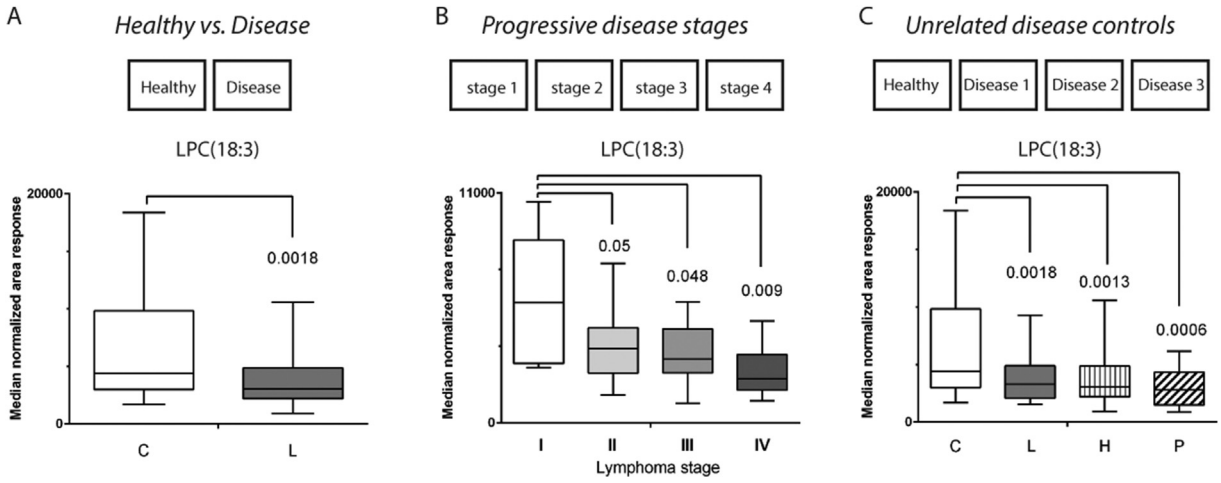

Fig. 4. Implications for biomarker discovery study design. A hypothetical biomarker study aiming to discover lymphoma specific markers serves to illustrate how marker specificity may be increased. **A**. Comparing healthy controls (C) with lymphoma (L) samples, LPC(18:3) is an example of a significant single marker. **B**. Likewise, LPC(18:3) levels correlate with lymphoma stage. **C**. However, when the non-related disease controls congestive heart failure (H) and community acquired pneumonia (P) are included it becomes clear that LPC(18:3) is not disease specific but rather a general disease marker.

When a disease of interest is compared to healthy control samples or progressive disease states, proteins and presumably metabolites that are part of a general stress- or inflammatory response will display altered levels [22,23]. In the present study, LPCs were in majority among the overlapping metabolites identified (Supplementary Table 2). A systematic literature search revealed that the levels of these phospholipids have repeatedly been found altered in a number of different pathological conditions including cancer, infection and diabetes (Fig. 3A, Supplementary Table 3). LPCs are released by the action of phospholipases A2 (PLA2s) on plasma membrane phosphatidylcholines resulting in an LPC and a free fatty acid (Fig. 3B). The free fatty acid, if being arachidonic acid, can be further metabolized into various prostaglandin-, thromboxane- and leukotriene metabolites which participate in inflammatory responses. LPCs and the downstream metabolite lysophosphatidic acid (LPA) are emerging as equally important inflammatory mediators [24]. For example, LPCs are potent attractors of monocytes [25] and activators of macrophages [26]. As LPCs are being released from apoptotic tissue or immune cells such as neutrophils attracted to the site of insult or inflammation, they will in turn attract and activate phagocytic cells [27]. Thus, LPCs play a central role in initiating and maintaining the inflammatory response creating a rationale for postulating that LPC levels in serum will change with a plethora of pathological conditions that trigger inflammatory responses.

Further, activation and expansion of white blood cells such as neutrophils and macrophages require substantial changes of their intrinsic metabolism to sustain their immune functions. These changes include increased glycolysis, glutaminolysis and fatty acid oxidation [28]. At a certain critical level and magnitude of immune cell activation, it is reasonable to assume that this altered metabolic activity can leave a trace detectable in the blood, and that this trace

will be specific to the immune cell status rather than to the cause of the inflammation (Fig. 3B). When healthy control subjects were compared to the entire lymphoma cohort in this study, LPC(18:3) was one promising biomarker (Fig. 4A). Comparing the serum level of LPC(18:3) in patients with different stages of lymphoma, progressive reduction of this metabolite appears to correlate with disease stage I to IV (Fig. 4B). However, when the level of LPC(18:3) in patients diagnosed with non-related diseases is included as well, it becomes clear that this particular metabolite marker most likely is indicative of disease in general rather than lymphoma specifically (Fig. 4C). Based on our results, non-related disease controls would help avoiding general disease markers and thus improve clinical usefulness.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2016.08.155.

Transparency document

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