Investigation of lactate dehydrogenase isoenzymes as candidate biomarkers of idiopathic pulmonary arterial hypertension

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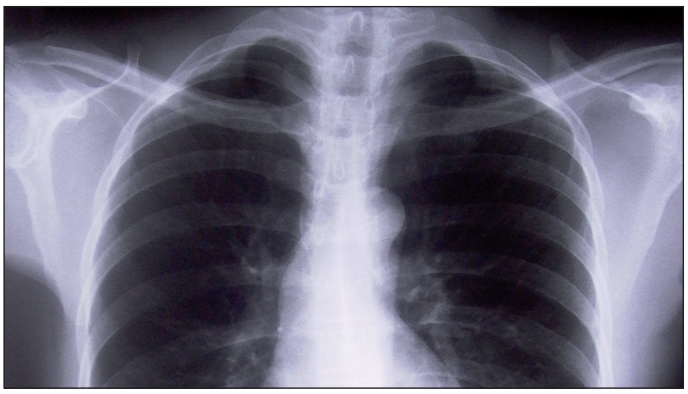
This study investigates the activity and expression of lactate dehydrogenase (LDH) in idiopathic pulmonary arterial hypertension (IPAH) patients. IPAH is a rare and highly fatal disease with a median life expectancy at diagnosis of only 2.8 years. Ideally a simple blood test for biomarkers could simplify the physician's diagnostic work-up, resulting in earlier diagnosis and successful institution of therapy. Recent publications suggest IPAH may behave like cancer, with monoclonal proliferation and a shared pathway of mitochondrial dysfunction. LDH is often upregulated in cancers, and a similar elevation is suspected in IPAH. Discovering similar patterns of flux in the cellular bioenergetics of IPAH and cancer would support the emerging theory that IPAH has a 'cancer phenotype'. Quantitative proteomic analysis of fourteen lung tissue homogenate samples (seven lobectomy, seven IPAH) was performed using liquid chromatography – tandem mass spectrometry (LC-MS/MS). The lung samples, as well as 30 plasma samples (ten normal, 20 IPAH) were analysed for LDH fractional isoenzyme activity and expression. A pyruvate-to-lactate spectrophotometric activity assay was performed on the 44 samples, followed by LDH isoenzyme separation on thin-layer agarose gel and densitometric analysis. A significant link exists between IPAH and increased plasma and lung levels of LDH-1 ($P = 0.0114$ and 0.0262 respectively on Mann-Whitney U test). Receiver Operating Characteristic analysis demonstrated plasma LDH-1 had biomarker sensitivity and specificity of 80%. Measuring plasma LDH-1 appears clinically useful in diagnosing IPAH. This work supports the re-evaluation of IPAH as a cancer-like disease and suggests a new biomarker.

Introduction

Idiopathic pulmonary arterial hypertension (IPAH), first characterized in 1951, is pulmonary arterial hypertension (PAH) of unknown aetiology. [1] Clinically, it is defined by elevated mean pulmonary arterial pressure (mPAP) ≥25mmHg at rest or ≥30mmHg in exercise, and normal pulmonary capillary wedge pressure (PCWP) of ≤15mmHg. [2] Although rare (1-2 cases per million), IPAH is devastating. [3] It is 2.3 times more common in females and characteristically strikes during reproductive years. [4] In the natural history of the disease, patients suffer extremely poor quality of life and a high mortality rate comparable to many cancers, with life expectancy only 2.8 years at diagnosis. [5]

IPAH is notoriously difficult to diagnose. Not only is it extremely rare, but it also occurs in people with none of the classic risk factors for pulmonary arterial hypertension (i.e. connective tissue disease or HIV). [6] The typical IPAH patient is an otherwise healthy female aged in her thirties presenting with very non-specific symptoms, usually 'shortness of breath.' Other common presentations include fatigue, weakness, angina, syncope, peripheral oedema and abdominal distension. [7] Due to these barriers to diagnosis, median time from presentation to diagnosis is two to three years. [8] By then, 80% of patients have deteriorated to NYHA functional class III or IV. [9]

The current gold standard for IPAH diagnosis is right heart



catheterisation (RHC). Although informative, RHC is expensive and also highly invasive, raising particular risks for the haemodynamically fragile IPAH patient. Ideally a simple blood test for biomarkers could simplify the physician's diagnostic work-up, resulting in earlier diagnosis and successful institution of therapy. While several IPAH markers have been suggested, none have yet made it to the clinical setting. [10,31]

IPAH : A cancer-like disease?

Recent re-appraisals of IPAH suggest it may be a cancer-like process in aetiology, pathology and therapeutic response. [14] While IPAH was first explained as a disease of unexplained increase in pulmonary arterial tone, it was then discovered that extreme vasoconstriction occurs only in the minority of patients. Scientific investigation next identified that IPAH was histopathologically defined by proliferation of pulmonary artery endothelial cells (PAECs) and pulmonary artery smooth muscle cells (PASMCs). Recently, this proliferation was found to be strictly monoclonal, whereas the vascular hypertrophy of secondary PAH is mixed-form. [32]

The scientific literature supports this reinterpretation of IPAH as a 'neoplasia of the pulmonary circulation.' [15] The BmprII mutation causing familial pulmonary arterial hypertension (fPAH) also causes colon cancer. [33] Survivin, an anti-apoptotic molecule upregulated in most cancers, is linked to pulmonary vascular remodelling. [34] Further, offering promise for new clinical therapies, chemotherapy can increase survival in IPAH by up to 25%. [35]

Most compellingly, a pathway common to both cancer and IPAH has now been identified. Otto Warburg proposed in 1924 that altered glucose metabolism was central to the origin and maintenance of cancers. [36] Whereas normal cells increase their rate of glycolysis only when triggered by hypoxia, cancer cells have increased glycolysis even in a normoxic environment due to their defective mitochondria. The 'Warburg phenotype' is characterised by mitochondrial hyperpolarization, and downregulation of pyruvate dehydrogenase activity and hydrogen peroxide (H_2O_2) production. [37] It is this concept of 'inappropriate glycolysis' which underpins innovative technologies such as fluorodeoxyglucose positron emission tomography (FDG-PET).

Both IPAH and cancerous cells share this Warburg phenotype due to their mitochondrial dysfunction, which disrupts the mitochondria-ROS-HIF-1a-Kv1.5 O2-sensing pathway. When the mitochondria become

dysfunctional, pyruvate dehydrogenase kinase (PDK) is activated. PDK phosphorylates and inhibits pyruvate dehydrogenase, such that most cellular pyruvate is metabolised by LDH into lactate. In the cytosol, this increases the rates of glycolysis and LDH activity. [15] In the mitochondria, decreased anaerobic respiration results in decreased production of reactive oxygen species such as hydrogen peroxide, triggering the activation of hypoxia-inducible-factor 1a (HIF-1a). By this stage, the mitochondria have become hyperpolarized, dysmorphic and anti-apoptotic. [38,39]

HIF-1a downregulates voltage-gated potassium channels such as Kv1.5, disrupting the body's oxygen-sensing system and worsening lung function. Reduced Kv1.5 also causes cell membrane depolarization, resulting in the cell flooding with cations (Ca2+ and K+). Increased intracellular Ca2+ causes vasoconstriction and activation of nuclear factor activating transcription (NFAT), promoting cellular proliferation. Increased intracellular K+ represses caspase activity, to decrease the rate of apoptosis. Consequently, in both IPAH PASMCs and cancer cells this dysfunctional pathway creates a pro-proliferative, apoptosis-resistant cell line. [40]

Proteomic analysis of IPAH samples was conducted to determine if any proteins associated with cancer were also upregulated in IPAH. Investigation of this putative 'cancer-like behaviour' may generate a double benefit, adding further data to the literature on IPAH's pathogenesis, as well as lighting the way to new diagnostic biomarkers.

In this study, proteomic work suggested LDH H was increased in IPAH lung samples. Lactate dehydrogenase (LDH) is a cytoplasmic enzyme catalysing the final step of anaerobic glycolysis. [37] The two major subunits of LDH are labelled M (coded for by the LDHA gene) and H (coded for by the LDHB gene). They assemble randomly to form 5 tetrameric isoenzymes: LDH-1 (H4), LDH-2 (H3M1), LDH-3 (H2M2), LDH-4 (H1M3), and LDH-5 (M4). The observed increase in LDH H was evaluated using an independently-performed assay and isoenzyme electrophoresis, with assessment of diagnostic utility in lung and plasma samples.

Methods

Lung Samples

Fourteen lung tissue homogenates used from Lobectomy (n=7) and IPAH (n=7) donors (Table 1). Lobectomy samples were healthy lung sections from lung cancer patients undergoing surgery. IPAH samples were hypertensive lung sections from transplant. One gram of lung tissue was immersed in 15mL 0.1M phosphate buffer (pH 7.4) with 1mM EDTA and 1mM DTT (dithiothreitol), and homogenised on ice with a PT-K Polytron® Stand Homogenizer (Kinematica AG, Switzerland).

Plasma Samples

Thirty plasma samples used from Control (n=10) and IPAH (n=20), matched for mean age and sex as far as was possible. Controls were healthy and medication-free. IPAH samples were obtained at the time of diagnosis, when patients were naïve to IPAH therapy.

Table 1. *Demographics of the 44 subjects (14 lung tissue and 30 plasma samples).*

	'Control' Group Plasma Samples (n=10)	'IPAH' Group Plasma Samples (n=20)	'Lobectomy' Group Lung Samples (n=7)	'IPAH' Group Lung Samples (n=7)
Gender Distribution	9 female 1 male	17 females 3 males	4 females 3 males	5 females 2 males
Mean Age	31.6 y.o.	46.8 y.o.	66.6 y.o.	39.1 y.o.
Age Range	22-57	29-68	50-79	28-51

1. Proteomics: Label-free LC-MS/MS

Lung samples in lithium dodecyl-sulfate (LDS) buffer underwent electrophoresis on bis-tris sodium dodecyl-sulfate (SDS) gels, then were destained with ammonium bicarbonate in acetonitrile (ACN) and digested with trypsin. Peptides extracted in formic acid/ACN were loaded on the LC-MS/MS. The program DeCyder MS identified and sequenced peptides based on ion intensity, LC retention time and m/z (molecular weight/charge) ratio, with inclusion cut-off 3-peptide validation. Amino acid structure was entered into Turbo SEQUEST search engine and compared against RefSeq human peptide sequence database.

2. Total LDH activity assay

The reagents for this stage were:

- 0.1M potassium phosphate buffer (PPB) (BDH Laboratory Supplies, Poole UK) at pH 7.4 and 30°C.
- NADH : 3.1 mM NADH (Sigma-Aldrich Company Ltd, Dorset UK) in PPB.
- Pyruvate in buffer: 11 mM pyruvate (Sigma-Aldrich Company Ltd, Dorset UK) in PPB.
- Sample (plasma, lung tissue): 10µL sample, 40µL PPB vortexed and on ice. Spectrophotometer: UV spectrophotometer (Hitachi U-3000) at 340nm, with 'UV Solution 2.0' software, and cuvette at 30°C.

440µL PPB, 30µL NADH solution, 10µL of diluted sample in cuvette. 30µL pyruvate solution added and solution inverted. Total LDH activity calculated through LDH-catalysed conversion of pyruvate to lactate, following decrease in NADH. Decreased absorbance recorded over 3 minutes, then Beer-Lambert's Law ($A\lambda = \varepsilon cL$) applied to determine sample activity. [41,42]

3. Separation of lactate dehydrogenase isoenzymes

The reagents for this stage were:

- Electrophoresis Buffer: 10.3g sodium diethylbarbiturate (Sigma-Aldrich Company Ltd, Dorset UK), 0.35g ethylenediaminetetraacetic acid (EDTA, BDH Laboratory Supplies, Poole UK), 7mL 1M HCl (BDH Laboratory Supplies, Poole UK) in dH2O, with pH 8.6 at 20°C, made up to 1.0L.
- Gel Buffer: 1.34g sodium diethylbarbiturate, 0.035g EDTA and 5g sucrose in dH2O, with pH 8.6 at 20°C, made up to 100mL.
- L+-lithium lactate solution: 2.4g L(+)-lithium lactate (Sigma-Aldrich Company Ltd, Dorset UK) in 50mL dH2O, pH 7.0 at 20°C.
- PMS incubating solution: 1mL 200mg/L phenazine methosulfate (PMS) (Sigma-Aldrich Company Ltd, Dorset UK) ; made up immediately prior to use in dark.
- β-NAD / lactate solution: 3mg β-nicotinamide adenine dinucleotide (β-NAD) (Sigma-Aldrich Company Ltd, Dorset UK), 7.2mg tetranitroblue tetrazolium (TNBT) (Fluka Biochemika, Switzerland) in 0.9mL L+-lithium lactate solution and 2.1mL dH2O.
- Samples (plasma or lung homogenate): based on total LDH activity measurements, samples were diluted with phosphate-buffered saline (PBS, Sigma-Aldrich Company Ltd., Dorset UK) to aliquots with constant total LDH activity. *:*

1% agarose/gel buffer solution poured into 0.75mm thin-layer gel, with 2µL of sample in each well. Gel run at 200V, 40mA for 1 hour 45 minutes on Pharmacia Fine Chemicals Flat Bed Apparatus FBE-3000, then incubated 30 minutes at 37°C in 1mL PMS solution, 3mL β-NAD/ lactate solution, 7mL dH2O, with isoenzymes reacting as follows: [43-46]

$$\text{Lactate} + NAD^+ \leftrightarrow \text{Pyruvate} + \text{NADH} + H^+$$

$$\text{NADH} + \text{PMS} \leftrightarrow NAD^+ + \text{PMS-H}$$

$$\text{PMS-H} + \text{TNBT} \leftrightarrow \text{PMS} + \text{TNBT-Formazan}$$

Gel was scanned on Kodak 440 Image Station Densitometer (Kodak,

USA), and intensity profile exported to The Scientific Figure Processor 6.0 program (Biosoft, UK). Each isoenzyme's area under the curve (AUC) as fraction of sample total plotted in GraphPad Prism 4.0 for Windows (GraphPad Software, Inc., USA).

4. Calculation of LDH subunit levels

To interpret relative LDH subunit expression, calculations were applied to data from total LDH activity assays and isoenzyme electrophoresis. For example, to determine relative proportion of LDH H, 'XLDHH % = 100 (ΣLDHH subunits) / (total LDH mean activity)'. The other LDH subunit's expression was then simply 'XLDHM % = 100 – XLDHH %'. Individual values were obtained for each sample, then group mean values calculated.

5. ROC analysis to determine sensitivity and specificity

Parameters showing significant changes were analysed in Receiver Operating Characteristic (ROC) curves in GraphPad Prism 4.0 for Windows with 95% confidence intervals.

Results

1. Proteomics

Seventy-three proteins were differentially expressed between IPAH and control groups; Table 2 presents data for LDH subunits. LDH H subunit was expressed 2.7-fold higher in IPAH compared to controls, and this change was statistically significant (P = 0.02).

Table 2. *Data obtained from proteomic analysis (liquid chromatography – tandem mass spectrometry) of fourteen lung tissue samples. Seventy-three proteins were differentially expressed between IPAH and control groups; lactate dehydrogenase was selected for further investigation, and data relating to its subunits is presented here. SD = standard deviation.*

Protein	Number of Peptides	Lobectomy Mean ion intensity ± SD	IPAH Mean ion intensity ± SD	Fold difference	P value (Mann-Whitney U test)
LDH H subunit	3	58.7 ± 27.1	163.9 ± 47.8	2.7	0.02
LDH M subunit	4	88.6 ± 44.4	137.9 ± 109.0	1.6	0.73

2. Total LDH activity assay

Lung Homogenate: Mean total LDH activity was 1.4-fold higher in 'IPAH' group (37 U/g) compared to 'Lobectomy' (26 U/g) (Figure 1A). However, on Mann-Whitney U test, this difference was non-significant (P > 0.05).

Plasma: Mean total LDH activity was only 1.15-fold higher in 'IPAH' group (310 U/L) compared to 'Control' (269 U/L) (Figure 1B); this difference was not significant (P > 0.05).

3. LDH isoenzymes

Lung Homogenate: Mann-Whitney U test showed both LDH-1 and LDH-2 were significantly upregulated in the 'IPAH' cohort (P=0.0262 and P=0.0379 respectively; Figure 1C).

Plasma: LDH-1 was increased by mean value 32 U/L (a 52% elevation in isoenzyme fraction), with proportional change significant on Mann-Whitney U test (P=0.0114). LDH-5 was significantly downregulated in IPAH (P=0.0186) from mean value 8 U/L to 4 U/L. (Figure 1D).

4. LDH H subunits

Lung Homogenate: Mann-Whitney U test showed the proportion of LDH H subunit was significantly increased in the 'IPAH' group (61.5%) compared to the 'Lobectomy' group (52.7%), with P = 0.0111 (Figure 1E). Data for LDH H and LDH M was also compared with respective levels determined using proteomics. In both cases, there was good correlation between levels determined by proteomics and levels determined using enzyme activity and electrophoretic separation (Figure 2).

Plasma: Mann-Whitney U test showed proportion of LDH H subunit activity was highly significantly increased in the 'IPAH' group (72.9%) compared to the 'Control' group (67.8%), with P = 0.0030 (Figure 1F).

5. Sensitivity and specificity of LDH measurements

Lung Homogenate: LDH H subunit proportion was the best marker,

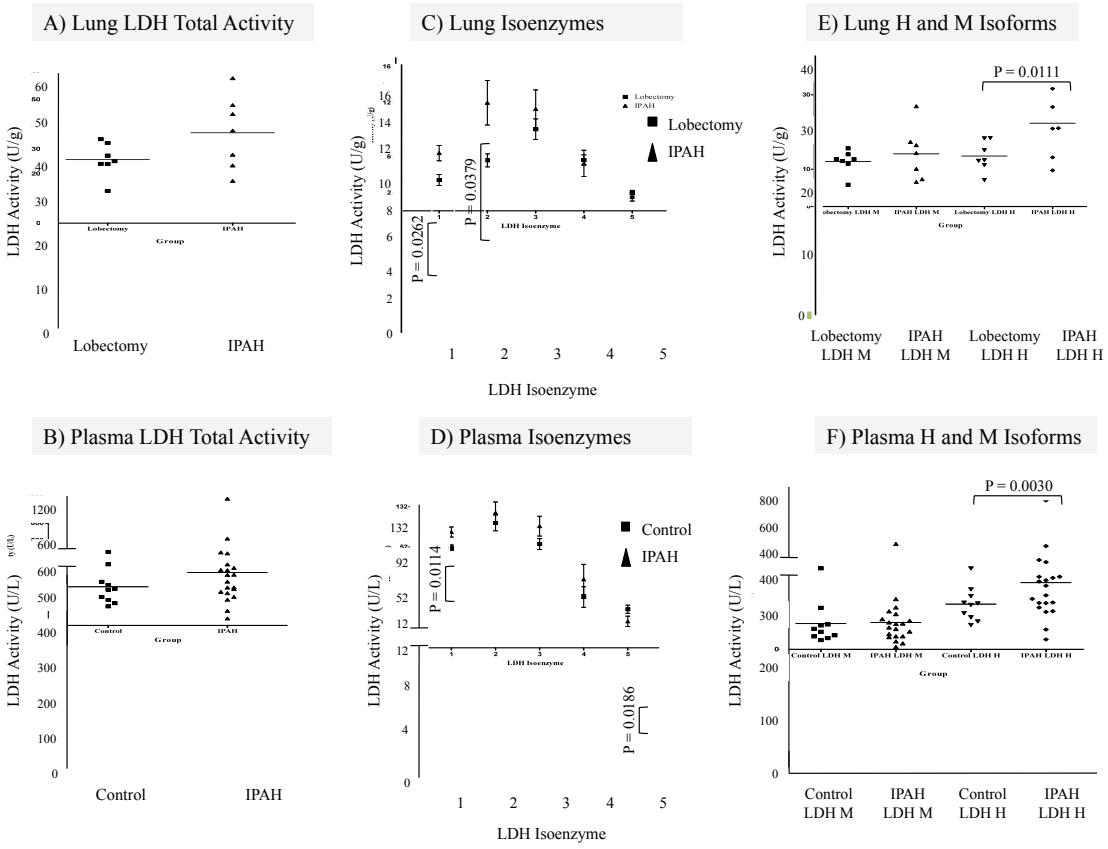


Figure 1. *Results of Total Activity Assay, Electroporesis and Isoform Quantification.*

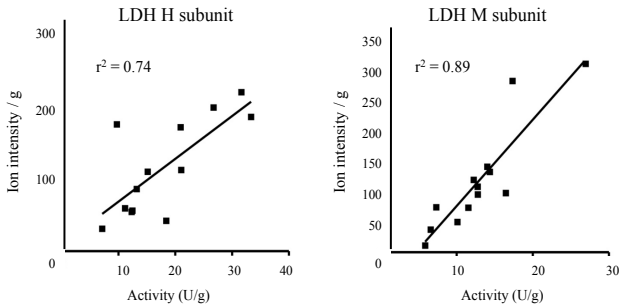


Figure 2. *Correlation of LDH isoform activity values from proteomics work with results from activity assay and electrophoretic separation.*

Table 3. *Receiver-Operating Characteristic values for lactate dehydrogenase parameters found to be differentially expressed between IPAH and control groups in lung tissue or plasma. In lung tissue, measuring proportion of LDH H subunit offers best discriminative value; in plasma samples, measuring LDH-1 activity is the best diagnostic marker. AUC = area under curve.*

	Enzymes	Cut-off for IPAH Diagnosis	Sensitivity	Specificity	AUC
Lung	LDH-1	≥ 4 U/g	86%	71%	0.86
	LDH-2	≥ 5 U/g	86%	71%	0.84
	LDH H subunit	*≥ 56%*	*86%*	*86%*	*0.90*
Plasma	*LDH-1*	*≥ 59 U/L*	*80%*	*80%*	*0.73*
	LDH H subunit	≥ 72%	50%	80%	0.58

with sensitivity of 86%, specificity of 86% and AUC of 0.90 at cut-off of 56% (Table 3).

Plasma: LDH-1 levels were the best discriminator for diagnosing IPAH, with sensitivity of 80%, specificity of 80% and AUC of 0.73 at cut-off of 59 U/L.

Discussion

From proteomic analysis of lung tissue from IPAH patients and control donors, an increase in LDH H levels in IPAH was observed. This finding was confirmed by measurement of LDH H activity, using electrophoresis to separate and quantify the LDH isoenzymes. In addition, LDH H levels were measured in plasma from IPAH patients and compared against healthy controls. Again, LDH H levels were found to be elevated.

It is possible that lung LDH H levels could be used as an IPAH marker. Indeed, in this study lung LDH H was the best-performing diagnostic biomarker with a sensitivity and specificity of 86%. However, in terms of invasiveness, performing lung biopsy is not an acceptable improvement on cardiac catheterisation. Thus, plasma LDH H (LDH-1) would be the preferable biomarker, with a sensitivity and specificity of 80% and requiring only venepuncture for sample collection.

Changes in LDH isoenzyme profile are already widely recognised as biomarkers in cancer and lung pathology. In cancer, LDH isoenzyme pattern can reflect underlying histology. Increased LDH-5 expression characterises many solid tumours, particularly carcinomas of the genitalia or digestive tract. In germ cell tumours however, there is a shift toward LDH-1 expression. [47-49] LDH activity can also differentially diagnose solitary pulmonary nodules as benign or malignant. [50]

Many lung pathologies also alter the isoenzyme profile. LDH-3 is most commonly affected, particularly when large numbers of alveolar macrophages are destroyed. [51] It is elevated in tuberculosis, pneumoconiosis, pulmonary alveolar proteinosis, pulmonary embolism, bacterial pneumonia and acute pulmonary oedema. [52-57] However, other patterns have also been found in pulmonary pathology. LDH-1 is elevated in both small-cell lung cancer and severe acute respiratory syndrome (SARS). It is sufficiently specific and sensitive to be a useful supporting biomarker in diagnosing SARS. [58,59] LDH-4 and LDH-5 increase in non-small-cell lung cancer (NSCLC) and transplant-related acute lung injury. [55] LDH-5 levels characteristically increase in pulmonary diseases where large numbers of neutrophils are undergoing apoptosis. [51]

Recent advances in elucidating the true pathogenesis of IPAH have paved the way for exploiting insights from foreign areas of medical research. There is now the exciting possibility of drawing upon decades of intense studies on cancer in order to rapidly improve outcomes in IPAH. Our finding that LDH H is upregulated in IPAH is consistent with PASMCs and PAECs exhibiting the 'Warburg phenotype.' It is possible that the increased LDH H levels are occurring as a result of cellular phenotypic shift to a state of increased glycolysis.

In this study, the LDH H increase was observed in lung tissue (a local sample) and corroborated in plasma samples - a logical finding given that the majority of the circulation passes through the lungs. The specific elevation in LDH H subunit fits well with the traditional paradigm that LDH H-dominant tetramers are usually of cardiovascular origin, while kidneys and liver mainly produce LDH-4 and LDH-5, the tetramers with high LDH M levels.

Genetic analysis would be useful to capture mutational or epigenetic changes to the LDHB gene underlying the increased expression of LDH H subunit. Immunohistochemical studies would also be desirable, as by identifying cells expressing LDH isoenzymes they could further expose the underlying disease process.

Greater evaluation of the glycolytic and mitochondrial pathways in IPAH is also required, since LDH is just one of many glycolytic proteins. Of note, the 73 proteins identified here did not include any of the other classic glycolytic proteins. However, other studies have validated components of the mitochondrial pathway, such as the Kv1.5 channel, in IPAH. [60]

Future studies should ideally enrol a greater number of subjects than the 44 studied here. While these results are promising, data is needed on the performance of LDH-1 in diagnosing IPAH with high sensitivity and specificity on a population level. It would also be logical in other follow-up studies to contrast the IPAH patients against other cardiorespiratory patients to ascertain the precise discriminatory powers of LDH-1 in a setting where baseline elevated LDH levels can be expected. Given the rarity of IPAH and consequent difficulty of enrolling subjects, these future directions may absorb many more years of research.

Conclusion

This is the first known investigation into LDH and its isoenzymes as candidate biomarkers for diagnosing IPAH. Investigations show that:

a) There is a significant increase in LDH H subunits in IPAH patients;

b) There is significant upregulation of LDH-1 and LDH-2 and downregulation of LDH-5; and

c) plasma levels of LDH-1 are the most accurate and clinically acceptable biomarker for diagnosing IPAH, with sensitivity and specificity both 80%.

This study supports the re-evaluation of IPAH as a cancer-like disease and suggests plasma LDH-1 (an LDH H homotetramer) may be useful in diagnosis.

Conflict of interest

None declared.

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