Pulmonary Hypertension: Biomarkers

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Abstract Physicians look to biomarkers to inform the management of pulmonary hypertension (PH) at all stages, from assessing susceptibility through screening, diagnosis, and risk stratification to drug selection and monitoring. PH is a heterogeneous disorder and currently there are no accepted blood biomarkers specific to any manifestation of the condition. Brain natriuretic peptide and its N-terminal peptide have been most widely studied. Other candidate prognostic biomarkers in patients with pulmonary arterial hypertension (PAH) include growth and differentiation factor-15, red cell distribution width, uric acid, creatinine, inflammatory markers such as interleukin-6, angiopoietins, and microRNAs. Combining the measurement of biomarkers reflecting different components of the pathology with other modalities may enable better molecular characterisation of PH subtypes and permit improved targeting of therapeutic strategies and disease monitoring.

Keywords Pulmonary hypertension • Biomarkers • Pulmonary arterial hypertension • Brain natriuretic peptide • Cytokines • Inflammation • Iron • microRNA

Contents

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1   Biomarkers

The US National Institutes of Health Biomarkers Definitions Working Group have defined a biomarker as "any characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" (Atkinson et al. 2001). That is to say, a biomarker is any objective measure that informs on the state of health of an individual.

To be useful it has to satisfy a number of criteria. The strength and consistency of the relationship between the biomarker and the disease parameter it is reporting are an important consideration; associations must be convincing (statistically significant) and reproducible across different cohorts before a relationship can be considered true. A biomarker's *specificity* is the proportion of true negatives over the true negatives and false positives—and so a measure of the ability of a biomarker to accurately rule out disease. *Sensitivity*, conversely, is the proportion of true positives over the true positives and false negatives—and so the ability of a biomarker to predict disease. The *temporality* of a biomarker is also important; some biomarkers respond rapidly to events, while others may accumulate and integrate information. The *biological gradient* refers to the dynamic range of the measurement and together with the quality of the assay used must be sufficient to differentiate accurately between groups.

Biomarkers may help in the management of PH at several stages, from susceptibility, screening, diagnosis, and risk stratification to therapeutic selection and monitoring.

1.1   Biomarkers of Susceptibility to PH

Genomic information holds the greatest potential for identifying susceptible individuals, i.e. individuals at risk but with no manifestations of the disease. Family members of patients with PAH can be tested for mutations in known risk genes,

namely *BMPR2*, *ALK1*, or *ENG* (see Sect. 2.1). Following appropriate counselling, those harbouring mutations can be seen at regular intervals to detect and manage the disease at its earliest occurrence.

Other scenarios in which biomarkers for susceptibility to PH may be identified include identifying those individuals most likely to develop PH following pulmonary embolism/thromboendarterectomy, or in other at-risk populations including but not limited to those where PAH is associated with connective tissue disorders, HIV, or schistosomiasis.

1.2 Biomarkers for Screening for PH

A considerable proportion of the pulmonary vascular bed needs to be 'lost' before pulmonary vascular disease becomes symptomatic (Lau et al. 2011), and even in patients with mildly symptomatic PAH, there is already a marked increase in pulmonary vascular resistance (Galië et al. 2008). Screening individuals for subclinical disease requires a non-invasive test and the most practical tool for this purpose is the echocardiogram. No blood biomarker currently identified is sensitive enough to detect subclinical disease.

1.3 Biomarkers for Making the Diagnosis of PH

The insidious onset and non-specific nature of presenting symptoms in PH means that the diagnosis is delayed while other pathologies are excluded. The definitive diagnosis of PH is dependent on demonstrating a raised resting mean pulmonary artery pressure at cardiac catheterization. While a normal level of the cardiac stress-marker brain natriuretic peptide (BNP) or its N-terminal peptide (NT-proBNP) may help rule out PH, there is no blood biomarker that is diagnostic of the condition. The cost and invasive nature of cardiac catheterization drive the search for alternative non-invasive tests. Further developments in other imaging technologies such as cardiomagnetic resonance (CMR) imaging or 3D echocardiography may provide a solution but remain research tools at present. Cardiac output is a prognostic marker in PH patients and inert-gas rebreathing may represent a non-invasive means of measuring this important parameter (Clinical Trials.gov Identifier NCT01606839), detecting for example treatment responses in patients with PAH (Lee et al. 2011).

1.4 Biomarkers for Risk Stratification in PH

Risk stratification can inform not just prognosis but may help optimization of treatment and the achievement of specific goals aimed at improving longer-term survival. Multicentre patient registries—National Institutes of Health (NIH), French PAH registry, and US-based Registry to Evaluate Early and Long-term Pulmonary Arterial Hypertension Disease Management (REVEAL)—have been

very useful in identifying independent factors that are associated with prognosis of PAH, including age, sex, disease aetiology, World Health Organization (WHO) functional class, 6 min walk distance (6MWD), and haemodynamic parameters (D'Alonzo et al. 1991; Rich et al. 2000; Humbert et al. 2010; Benza et al. 2010). But these are blunt instruments and we remain poorly equipped to make judgements at the individual patient level (Thenappan et al. 2012). That is, to predict exactly which individuals will survive for years with their condition versus those who will succumb within months or weeks of their original diagnosis and may have benefitted from earlier intervention with combination therapy or more aggressive treatment (Hoeper et al. 2005; Nickel et al. 2011a).

Measurements of circulating BNP and NT-proBNP levels remain the most commonly used blood biomarkers for stratifying PH patients and are recommended in the European guidelines for the diagnosis and treatment of PH (Galie et al. 2009). It is recognised, however, that some patients with advanced disease have circulating levels within the normal range and the levels for an individual patient have to be interpreted in the context of all the clinical information available. Repetitive measurements may add information and improve their utility in disease management (Nickel et al. 2011a).

Novel candidates for prognostic biomarkers in PH, which will be described in more detail below, include growth and differentiation factor-15 (Nickel et al. 2008; Rhodes et al. 2011a), red cell distribution width (Hampole et al. 2009; Rhodes et al. 2011a), inflammatory markers such as interleukin-6 (Humbert et al. 1995; Soon et al. 2010), creatinine (Leuchte et al. 2007; Shah et al. 2008), uric acid (Nagaya et al. 1999), angiopoietins (Kumpers et al. 2010), and microRNAs (Caruso et al. 2010; Courboulin et al. 2011).

1.5 Biomarkers for Therapeutic Selection and Monitoring

There is interest in the PH community in goal-orientated treatment strategies, whereby treatments are titrated according to response. The biomarkers used to assess response are 6 min walk distance, cardiopulmonary exercise testing, and BNP levels (Hoeper et al. 2005).

With the increase in number of drugs available to treat PH, the possibilities for individualising treatment have increased. Biomarkers that can identify which patients will respond best to particular therapies enable expensive treatments to be used more cost effectively and reduce unnecessary drug exposures. This point is well illustrated by the use of acute vasodilator testing at cardiac catheterization to identify patients suitable for treatment with calcium antagonists. A small number (<10 %) of patients with PAH who show an acute reduction in pulmonary artery pressures, when exposed to nitric oxide or adenosine, respond well to calcium channel blockers with excellent haemodynamic recovery and long-term survival (Sitbon et al. 2005; Tonelli et al. 2010). The recent finding that over 60 % of patients with PAH are iron deficient (in the absence of anaemia) may identify a

subgroup of patients who will benefit from iron, but this awaits formal testing (Rhodes et al. 2011b).

While there is most interest in biomarkers of efficacy it is important not to forget toxicity and the role of safety biomarkers. For PH patients on warfarin, the International Normalised Ratio (INR) is used to monitor the clotting tendency of blood and thereby ensure the correct dosage. Patients on endothelin antagonists also have their liver enzymes monitored for early signs of liver toxicity, which is generally reversible upon discontinuation of the drug. A new protein kinase inhibitor-induced toxicity has been identified, pre-capillary PH having been associated with dasatinib therapy (Montani et al. 2012). Cardiac biomarkers such as Troponin I and BNP may represent an early means of detecting cardiac toxicity in patients exposed to protein kinase inhibitors (ClinicalTrials.gov Identifier NCT00532064).

1.6   Statistical Evaluation of Novel Biomarkers

Key to the discovery and validation of biomarkers is access to well-phenotyped patient populations from cohort studies and clinical trials. The data and/or samples are mined based on plausible candidates or using unbiased screens. These datasets can be very large, particularly if fed from platform technologies, such as proteomics or metabolomics.

Before an analysis can be started, general requirements for statistical testing must be met. Whether the biomarker of interest is normally distributed (i.e. fits to a "normal" bell-shaped curve) will determine whether parametric (for normal or Guassian data) or non-parametric tests can be deployed. Normality can be assessed informally by box and whisker plots/histograms and formally by the Kolmogorov–Smirnov test. If required, distribution of a marker can be transformed by logging, square-rooting, or inverting all numbers to achieve normality.

Tests for two groups (e.g. control versus patient) of continuous data (i.e. not categories) include the Student's *t*-test and Mann–Whitney *U*-test. Multiple groups require analysis of variance (ANOVA or Kruskal–Wallis) followed by post hoc testing. Correlations can be assessed by Pearson's test or Spearman's rank test. Chi-squared analysis is required when assessing relationships between categorical variables, for example, levels of a marker above or below a cut-off level against WHO functional class, or diagnosis (see Fig. 1).

The first step in evaluating a novel biomarker is to understand its association with the disease of interest. This usually involves comparing levels measured in a patient population with appropriate control subjects. In PH, appropriate controls may simply be healthy subjects, but could include non-PH patients with a chronic disease (e.g. asthma, COPD without PH) or different diagnostic classes of PH to that being investigated (e.g. chronic thromboembolic PH versus idiopathic PAH).

Once an association with a disease state has been demonstrated, the meaning and importance of this association can be further investigated by assessing correlations
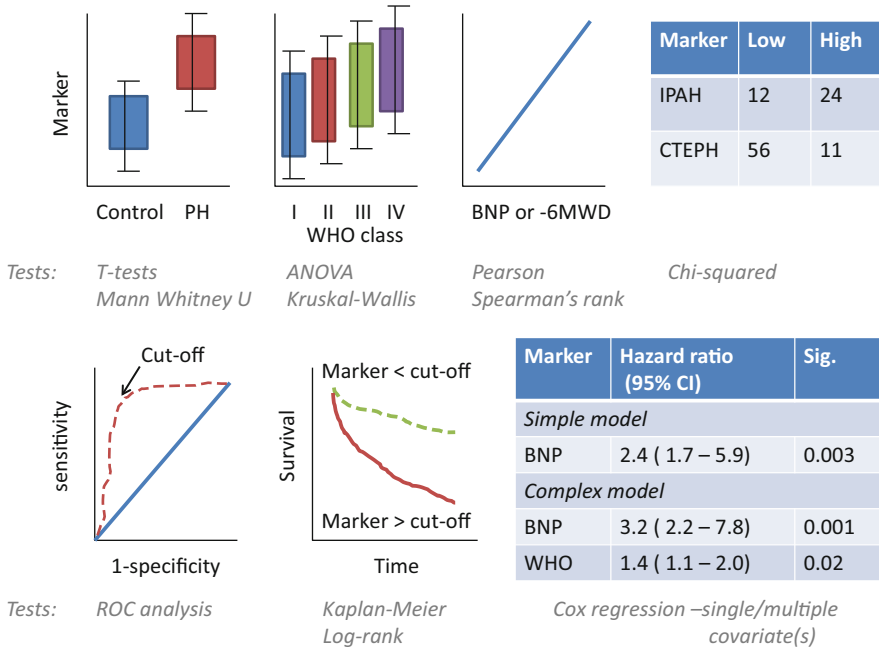
Fig. 1 Common data types and statistical analyses employed in biomarker assessment

between markers of disease severity (e.g. 6MWD, WHO functional class, pulmonary haemodynamic measurements) and circulating levels of markers such as BNP. Demonstrating the relevance of a marker to disease severity can also be hypothesis-generating; for example a marker that strongly correlates to BNP may be more likely to reflect myocardial stress than one that did not. Unless these investigations are hypothesis-driven, considerations for multiple testing may be necessary (see below).

Ultimately, if a biomarker truly reflects the severity and progression of disease, then its levels should reflect the prognosis of an individual patient. For this it is necessary to censor the survival/mortality of patients in a cohort at one given time point, and to calculate the time between biomarker measurement and the censor date. Receiver operating characteristic (ROC) analysis can then be used to assess the relationship between potential cut-off levels of a biomarker and survival over a set period, for example 1-, 2-, or 3-year survival. It plots the sensitivity versus 1-specificity for each possible cut-off level of a marker, and the area under the curve represents the power of the marker to predict survival over this time period. ROC analysis also allows for direct comparisons of performance between candidates, as those with larger area-under-the-curve (AUC) values (also known as *c*-statistic) will best discriminate survivors and non-survivors. The cut-off furthest deviating from the line x=y will represent an optimum cut-off for this purpose (Fig. 1) and may be determined by plotting biomarker concentrations against their respective

sensitivity and specificity data (Schumann et al. 2010). Cut-offs can also be derived from distributions in the general/healthy populous or simply by taking median/quartile values.

The relationships of biomarker cut-offs to survival are frequently illustrated by Kaplan–Meier survival estimates and tested statistically by the log-rank test. The predictive value of stepwise changes in biomarker levels (i.e. 4–5, or 5–6, or 7.4–8.4) to the hazard rate (relative risk of mortality in a set period of time, i.e. year 1–2, year 2–3, year 3.4–4.4) can be assessed by Cox regression analysis. If a biomarker performs well by ROC, Kaplan–Meier, and Cox analysis then it can be said to have prognostic power in the population studied.

To determine whether a prognostic biomarker might add to current clinical practice, it must be compared to established gold standards, and multiple variable Cox regression analysis is the test of choice for this purpose. If a biomarker is still significantly related to survival in a Cox model including the best current prognostic measures (e.g. haemodynamic values, exercise capacity, BNP levels), then it can be said to be an "independent" prognostic marker which adds to the information currently available.

Important assumptions must be met for simple Cox analysis including that the hazard rate stays constant over time (so a high BNP would imply the same increased risk from year 1 to 2 as from year 3 to 4) and the same amplitude of change has the same hazard whatever the starting level of biomarker is (so an increase in BNP from 300 to 400 is as bad as an increase from 700 to 800). Normalisation of biomarkers prior to Cox analysis is standard and most likely aids the meeting of the latter criteria.

If a biomarker appears useful following the above tests, then the reproducibility of any findings must then be validated in further independent cohorts (see below), and eventually, its use in a clinical setting tested in specifically designed prospective clinical trials.

1.6.1 Cautionary Points

Over-fitting. An important consideration in any regression model, including Cox analysis, is not to over-fit the model. A general rule of thumb in regression is to only consider 1 covariate for each 10 data points and in Cox analysis this applies to the events (e.g. death, transplantation, hospitalisation) being considered. So a study with 100 patients with only 10 deaths would not be sufficient to assess the predictive value of a novel biomarker in comparison to BNP on death by Cox regression analysis.

Multiple Testing. This is a particular issue with the advent of high-throughput methodologies, such as genomics, proteomics, and metabolomics. If corrections are severe (such as the simplistic Bonferroni method) they may mask true differences but if ignored then false positives will arise. Estimations of the false discovery rate of a methodology may help gauge confidence in hits, but there remains no substitute

for validation of hypotheses in independent cohorts. Intrinsic to a discovery study is that identified candidates will be those that best perform, leading to over-estimation of their value compared to established markers previously identified. Hence discovery and validation cohorts are required to prove the value of a novel biomarker, and as the validation cohort will be used to assess an a priori defined hypothesis, then corrections for multiple testing will no longer be required.

Missing Data. Another problem commonly faced in biomarker discovery and development is that of missing data. This may reflect the retrospective nature of many studies which are considered secondary in importance to more orthodox clinical studies of novel therapeutics (Jenkins et al. 2011).

Incident and Prevalent Disease. It is notable that the recent REVEAL and French registry studies included prevalent as well as treatment naïve patients with incident disease (Benza et al. 2010; Humbert et al. 2010). Survival modelling with both groups of patients has its limitations and statistical techniques were used to take account of potential survivor bias arising from the inclusion of patients with prevalent disease. These methodological issues may still have resulted in overoptimistic survival rates due to an underestimation of the proportion of patients with severe disease who died soon after diagnosis (McLaughlin and Suissa 2010). Nonetheless, it is the response to treatment, captured through clinical observations and biochemical measurements, rather than the presence of treatment itself that is important in determining patient prognosis. For biomarker studies, careful analysis of survival from the date of blood sample collection is required in order to stratify risk at clinical follow-up appointments (Rhodes et al. 2011a).

1.6.2 Additional Criteria

Several groups have proposed criteria for evaluating new biomarkers and emphasised the need for prospective validation (Hlatky et al. 2009; Pletcher and Pignone 2011; Wang 2011). The lack of prospective clinical studies directly designed to assess the efficacy of adding in biomarker measurements to treatment decisions demonstrates that we are still some distance from incorporating any of the novel biomarkers proposed into standard guidelines. An appropriate trial design could be the randomization of front-line therapeutic strategies following grouping of subjects by biomarker measurement, or the randomization of patients to either normal treatment algorithms or algorithms that incorporate biomarker measurements into treatment decisions (Fig. 2).
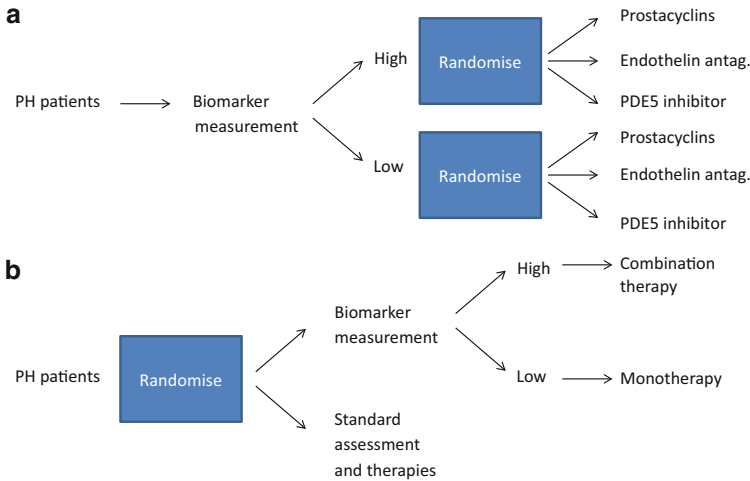
Fig. 2 Potential trial designs to test the value of biomarkers in treatment decisions in PH. (**a**) Comparison of treatment efficacies in "high" and "low" biomarker groups. (**b**) Comparison of example treatment algorithms with and without biomarker measurements. *Antag.* antagonists, *PDE5* phosphodiesterase type 5. Modified from Sargent et al. (2005)

2 Individual Biomarkers for PH

2.1 Genetic Biomarkers

Mutations in the bone morphogenetic receptor type 2 gene (*BMPR2*) are found in 50–80% of hereditary PAH (Deng et al. 2000; Lane et al. 2000; Machado et al. 2001; Morisaki et al. 2004), 10–20 % of idiopathic PAH patients (Newman et al. 2008; Girerd et al. 2010), and rarely in secondary PAH associated with anorexic drugs or congenital heart disease (Humbert et al. 2002; Roberts et al. 2004). Most predict loss of function (Machado et al. 2006). Patients with *BMPR2* mutations present approximately 10 years earlier than PAH patients without a mutation and have more severe haemodynamic disturbance leading to death at a younger age (Sztrymf et al. 2008). These patients are also less likely to respond to vasodilator therapy (Rosenzweig et al. 2008).

Two other genes in the tumour growth factor (TGF)-β receptor superfamily, activin receptor-like kinase 1 (*ALK1*) and endoglin (*ENG*), have also been causally linked to the development of PAH in hereditary haemorrhagic telangiectasia (Harrison et al. 2003). *ALK1* mutations are also found in hereditary PAH patients, leading to earlier presentation and rapid clinical decline even in comparison to *BMPR2* mutation carriers (Girerd et al. 2010). In addition, gene mutations in downstream intermediaries of the TGF-β/BMP-signalling pathway, namely Smads 1, 4, 8, and 9, have been identified as an infrequent cause of PAH (Nasim et al. 2011; Shintani et al. 2009).

Penetrance of PAH in *BMPR2* mutation carriers is generally around 20% and can vary depending on TGF-β polymorphisms (Phillips et al. 2008). Reduced penetrance suggests the presence of modifying factors that influence disease risk and the need for a so-called second hit to make the disease clinically expressed. Several genetic variants have been investigated, including polymorphisms of serotonin transporter, prostacyclin synthase, endothelial nitric oxide synthase, angiotensin-converting enzyme type 1, angiotensin II receptor type 1, carbamoyl phosphate synthetase 1, smooth muscle potassium channels (KCNQ, KCNA5), and cytochrome P450 1B1 (Fessel et al. 2011). With the cost of genome sequencing falling, the opportunities to investigate the genomic influence on PAH will increase. The sizable datasets that accompany these studies will however require careful analysis.

2.2   Clinical and Haemodynamic Indices

Before a novel marker can be considered useful it must be compared against the current gold standard. In PH, definitive diagnosis is dependent on invasive haemodynamic measurements by right heart catheterization—mean pulmonary artery pressure, mean right atrial pressure, pulmonary vascular resistance, and cardiac index all reflect disease severity and have been shown to hold prognostic information (D'Alonzo et al. 1991; McLaughlin et al. 2002; Sitbon et al. 2002). The current therapies for PAH have been approved on the basis of improvements in 6MWD as a surrogate for survival (Galie et al. 2005, 2008; Rubin et al. 2002). Indeed this measure has been shown to predict survival in PH (Miyamoto et al. 2000; Sitbon et al. 2002), although its variability between centres limits its use. WHO functional class is a simple clinical assessment of the symptoms of right heart failure that has been consistently demonstrated to predict survival in PH (McLaughlin et al. 2002; Sitbon et al. 2002; Humbert et al. 2010). All these measures should be considered, together with parameters such as age, diagnosis, sex, disease duration, and therapies, when assessing the potential value of a putative new biomarker in PH.

An empirically derived equation, based on baseline haemodynamic measurements, was developed to estimate survival of patients with primary PAH in the NIH registry (D'Alonzo et al. 1991). Clinical trials have used this equation to compare observed and predicted survival, but the management and treatment of PAH have changed dramatically over the last decade and contemporary registries show that the NIH equation significantly underestimates current survival (Benza et al. 2010; Humbert et al. 2010; Thenappan et al. 2010). New risk prediction equations have been produced in order to better model survival in patients with category 1.1–1.3 PAH (Humbert et al. 2010; Thenappan et al. 2010) or all groups of PAH (Benza et al. 2010). These equations have since been applied to prospectively collected data from PAH cohorts and a simplified risk score calculator developed

that may be more applicable to general clinical practice (Benza et al. 2012; Thenappan et al. 2012).

2.3   Natriuretic Peptides

The natriuretic peptides—atrial and brain, ANP and BNP—are synthesised by myocardium and secreted into the circulation, regulating natriuresis, diuresis, and vasomotor tone (Yoshimura et al. 1991; Nakao et al. 1992). Pressure and volume overload increases secretion and circulating levels of ANP and BNP and acts to reduce the workload of the heart, albeit ineffectively in PH.

   BNP is produced as a 108 amino acid prohormone, which is cleaved and secreted as a biologically active sequence (BNP-32) and inactive N-terminal proBNP (NT-proBNP) peptide. The former has a short half-life of around 20 min and NT-proBNP a longer half-life of several hours, but both fragments are cleared by the kidney and influenced by renal function (Holmes et al. 1993; Yandle et al. 1993). Other non-cardiac factors (e.g. age, obesity, diabetes mellitus) are also known to influence NT-proBNP/BNP plasma levels.

   Circulating levels of ANP, BNP-32, and NT-proBNP correlate with haemodynamic measures in PAH (Nagaya et al. 1998; Leuchte et al. 2005; Souza et al. 2005) and reduced BNP levels following long-term vasodilator therapy also correlate strongly ($p < 0.001$) with falls in PVR, suggesting that BNP measurements may reflect treatment efficacy (Nagaya et al. 1998).

   Natriuretic peptide levels are elevated by left as well as right ventricular dysfunction and therefore not specific to PH. Nonetheless, the right ventricle is a major determinant of prognosis in PH (van de Veerdonk et al. 2011) and circulating BNP/NT-proBNP levels predict survival and can be used to risk stratify patients (Nagaya et al. 2000; Fijalkowska et al. 2006; Nickel et al. 2008; Hampole et al. 2009; Rhodes et al. 2011a). In a study of 55 PAH patients with a mean follow-up time of 36 months, a cut-off NT-proBNP level of 1,400 pg/ml predicted mortality with 88 % sensitivity and 53 % specificity (log rank, $p < 0.01$) (Fijalkowska et al. 2006). The influences of renal insufficiency were also examined in a later study of 118 patients, which showed in a multiple covariate analysis that NT-proBNP levels and creatinine clearance outperformed BNP, and were independent predictors of mortality (Leuchte et al. 2007).

2.4   Growth and Differentiation Factor-15

Growth and differentiation factor (GDF)-15 is a member of the TGF-β superfamily (Bootcov et al. 1997) that protects mice from developing ventricular hypertrophy following pressure overload (Xu et al. 2006). It is ubiquitously expressed at low levels in most tissues and is induced in response to tissue injury and inflammation.

Mechanical stretch, as well as other stimuli, triggers its release from cardiomyocytes and it is thought that GDF-15 has a role in protecting myocytes against apoptosis and induction of hypertrophy (Heger et al. 2010).

Circulating GDF-15 levels have prognostic power in several cardiovascular diseases (Kempf and Wollert 2009; Khan et al. 2009) and hence this biomarker is not specific to PH. GDF-15 expression has also been localised to plexigenic lesions in PAH patients (Nickel et al. 2011b) but, as it expressed elsewhere, the source and major stimulus for GDF-15 production in PAH are unclear. This may in part explain why GDF-15 levels added prognostic information to BNP in one study (Nickel et al. 2008), but appeared redundant in another where other markers of inflammation and general cardiovascular health were also measured (Rhodes et al. 2011a).

2.5 Red Cell Distribution Width and Other Iron Parameters

Anaemia is a recognised predictor of survival in both chronic heart failure and PAH (Rhodes et al. 2011b). Iron deficiency, measured using a number of indicators—red cell distribution width (RDW), serum ferritin, and soluble trans-ferrin receptor—predicts a poor survival in PAH even in the absence of anaemia (Rhodes et al. 2011c). Inappropriately raised hepcidin levels suggest impaired absorption of iron from the gut. Interestingly, hepcidin is regulated by BMP6 and the possibility that dysregulated hepcidin production and PAH may be linked through a perturbation of BMP signalling merits further investigation. Neverthe-less, hepcidin is an acute phase protein and hepatic expression is induced by circulating pro-inflammatory cytokines such as interleukin-6 (IL-6, an inflamma-tory cytokine linked to the development of PH, see below).

Inflammation can induce iron deficiency, but the relationship of iron deficiency to survival in PAH seems independent of measures of inflammation. In 139 IPAH patients (Rhodes et al. 2011a), RDW was assessed as a prognostic biomarker alongside several other previously identified plasma biomarkers: NT-proBNP, GDF-15, creatinine (a marker of renal dysfunction, see below), and IL-6, as well as baseline haemodynamics and coincident clinical measurements. All 5 plasma markers predicted survival, though RDW was the strongest performer in ROC analysis, with an area under the curve of 0.82 (GDF-15 and 6 min walk distance 0.76/0.75, NT-proBNP, and IL-6 0.67/0.66). Again, a "normal" RDW level of 15.7 % was the best cut-off, levels above identifying a group of patients with around 40 % 3-year survival compared to over 70 % in those with lower levels. Haemodynamic measurements (mPAP, mRAP, CI, and PVR) also predicted sur-vival, as did WHO functional class, but a Cox regression model comprising just 6 min walk distance, RDW, and NT-proBNP levels could not be improved by the addition of any further measure identified as prognostic in single variable regressions.

2.6 Inflammatory Markers

Inflammation has long been appreciated as an element of PH pathology (Hassoun et al. 2009) and patients with PAH associated with connective tissue disorders generally exhibit a poorer prognosis and increased mortality risk when compared with other forms of the disease (Condliffe et al. 2009; Benza et al. 2010). The inflammatory marker C-reactive protein (CRP) has been shown to predict outcome, the normalisation of CRP levels being associated with improved haemodynamic and functional capacity as well as long-term survival (Quarck et al. 2009; Sztrymf et al. 2010). The levels of interleukins (ILs) such as IL-1β and IL-6 are also raised in PAH patients (Humbert et al. 1995) and a study characterising circulating endothelial progenitor cells in PAH demonstrated elevated tumour necrosis factor (TNF)-α, as well as the levels of IL-6 (CRP) in both IPAH and Eisenmenger congenital heart disease PAH patients (Diller et al. 2008). Increased IL-6 has also been demonstrated in inoperable congenital heart disease patients (Lopes et al. 2011). Interestingly, IL-6 can be induced by loss of BMPR2 expression, interacts with BMP signalling (Hagen et al. 2007), and has been implicated in the development of PH in experimental models (Savale et al. 2009; Steiner et al. 2009). Furthermore, high IL-6 levels may contribute to the reduced BMPR2 expression seen in IPAH through activation of the microRNA (miR)-17/92 cluster (Brock et al. 2009).

A recent screen of serum TNF-α, interferon-gamma, and several interleukins (IL-1β, -2, -4, -5, -6, -8, -10, -12p70, and -13) in 60 idiopathic and heritable PAH patients showed that all the cytokines measured (except IL-5 and IL-13) were elevated when compared with healthy volunteers (Soon et al. 2010). In addition, Kaplan–Meier analysis indicated that levels of IL-6, -8, -10, and -12p70 predicted survival. Another study of 139 IPAH patients also found that raised IL-6 was a good predictor of survival, but may not offer prognostic information above that provided by NT-proBNP, RDW, and 6MWD (Rhodes et al. 2011a). However, a direct measure of the inflammatory component in PH may yet prove useful in guiding therapeutic decisions.

2.7 Creatinine

Failure of the right ventricle is associated not only with reduced cardiac output but also back-pressure on the liver and kidney. In a study highlighting the dependence of NT-proBNP levels on renal function, estimates of creatinine clearance were shown to predict survival in over 100 PH patients of mixed aetiology (Leuchte et al. 2007). In one of the largest studies of biomarkers in PH to date, serum creatinine levels not only correlated with pulmonary haemodynamics, functional class, and exercise capacity but also predicted survival, particularly in a subgroup of patients with lower mRAP (<10 mmHg) measurements (Shah et al. 2008). In a more recent study of idiopathic PAH patients, creatinine levels correlated with baseline mRAP and CI, predicting survival in single variable analyses but not independently of NT-proBNP, RDW, and 6MWD (Rhodes et al. 2011a).

2.8 Uric Acid

Uric acid is the final degradation product of purines and an endogenous free radical scavenger that is elevated following tissue ischaemia and hypoxia. Raised levels have been described in chronic obstructive pulmonary disease (Braghiroli et al. 1993), chronic heart failure (Leyva et al. 1998), and cyanotic congenital heart disease (Hayabuchi et al. 1993; Leyva et al. 1998). Circulating uric acid levels are also widely reported to be elevated in PAH, with values around 50 % higher than seen in controls (Hoeper et al. 1999; Nagaya et al. 1999; Bendayan et al. 2003; Voelkel et al. 2000). Correlations of uric acid serum levels with cardiac output, PVR, WHO functional class, and mortality suggested its potential usefulness as a biomarker in PH (Nagaya et al. 1999). This was also underlined by reductions of uric acid levels in response to chronic vasodilator therapy. Potential confounding factors however include renal dysfunction and diuretic use and hence uric acid levels must be interpreted in the context of these factors.

2.9 Angiopoietins

Angiopoietin (Ang)-1 and its antagonist Ang-2 are ligands for the Tie2 tyrosine kinase receptor on endothelial cells and, in balance with vascular endothelial growth factor (VEGF), they act to control vascular development and maturation (Augustin et al. 2009). Overexpression of Ang-1 has been proposed as a murine model of PH (Chu et al. 2004), whereas other studies have suggested that the pathway is protective (Kugathasan et al. 2009). Circulating levels of Ang-1, Ang-2, VEGF, and soluble Tie2 (which inhibits circulating Ang-1) were all shown to be elevated in a study of IPAH patients (Kumpers et al. 2010). Of these four markers only Ang-2 levels correlated with disease severity (as assessed by mRAP, PVR, CI, and WHO functional class) and also correlated with elevated NT-proBNP levels. Furthermore, Ang-2 levels predicted disease outcomes; the best cut-off for predicting death or transplantation at 3 years was 2.9 ng/ml (sensitivity 85 % and specificity 58 %), a level rarely reached in controls measured. A small second cohort also demonstrated changes in Ang-2 following the initiation of treatment that correlated with alterations in parameters such as mRAP, PVR, and 6MWD (Kumpers et al. 2010). The usefulness of this interesting new biomarker, which might potentially reflect the dysfunctional endothelium in PAH, must be validated in independent studies.

2.10 MicroRNAs

Recently, several non-protein coding microRNAs (miRs) have been identified that modulate gene expression at the post-transcriptional level, regulating a wide range of physiological and pathological processes in the cardiovascular system, and circulate in the bloodstream (Creemers et al. 2012). Several miRs have been implicated in the pathogenesis of PH and could have utility as biomarkers as well as therapeutic targets in PAH (McDonald et al. 2012). These include the miR-17/92 cluster—mainly miR-20a and miR-17—(Brock et al. 2009, 2012; Pullamsetti et al. 2012), miR-21 (Caruso et al. 2010), and miR-204 (Courboulin et al. 2011). Most of these studies focused on changes in miRs in hypoxia- and monocrotaline-induced experimental models of PH, but miR-21 was shown to be reduced in human lung tissue and serum from patients with IPAH (Caruso et al. 2010). Downregulation of miR-204 has also been described in lung tissue, cultured pulmonary artery smooth muscle cells, and circulating buffy coat cells from patients with PAH (Courboulin et al. 2011). Interestingly, a recent in silico investigation, using a network-based bioinformatics approach, identified these miRs as ones predicted to be disease modifying in PH (Parikh et al. 2012). In addition to their potential role as biomarkers, circulating miRs are now thought to have a role in mediating intercellular communication. For example, miR-150 released from monocytic cells affects endothelial cell function and miR-143/145 from endothelial cells modulates smooth muscle cell phenotypes (Hergenreider et al. 2012). Circulating miRs might provide new insights into the pathogenesis of PH, particularly as they are accessible, relatively stable, and in some cases tissue-specific. Research in circulating miRs is however still at an early stage and the methodology is challenging, requiring careful consideration of study design, standards, statistical analysis, and validation in independent cohorts (Zampetaki et al. 2012).

2.11 Other Potential Biomarkers in PH

While the focus of this chapter has been primarily on blood biomarkers which represent possibly the simplest objective measure of disease at relatively low cost, other methodologies relevant to PH are being improved and may in future become more commonly adopted in disease management. *Cardiopulmonary exercise testing (CPET)* is an improvement upon more standard exercise measures recommended in Europe for risk stratification, which can discern between PH of different aetiologies (Zhai et al. 2011) and has prognostic value (Ferrazza et al. 2009; Deboeck et al. 2012), but requires expertise and specialist equipment that are not available at all centres. *Radionuclide imaging*, such as positron emission tomography (PET), and the availability of novel radiotracers for studies of relevant signal transduction pathways (Jakobsen et al. 2006) offer the potential to monitor disease progression and response to therapy in PH. There has been

particular interest in the use of [18]flurodeoxyglucose PET in PAH (Xu et al. 2007), providing a surrogate marker of changes in glycolytic activity, vascular structure, and immune/inflammatory activation in the lung (Marsboom et al. 2012) as well as a marker of right ventricular dysfunction in PAH (Bokhari et al. 2011). Imaging techniques such as *3D echocardiography* (Amaki et al. 2009; Grapsa et al. 2012a, b) and *cardiomagnetic resonance (CMR) imaging* (Vonk Noordegraaf and Galie 2011; Bradlow et al. 2012) also offer greater accuracy and reproducibility in the assessment of the right ventricle and can provide volumetric measurements that reflect its function. CMR in particular has been utilised to assess the efficacy of therapeutics in recent clinical trials (Wilkins et al. 2005, 2010) and provides prognostic information in PAH (van Wolferen et al. 2007; van de Veerdonk et al. 2011), but the expense and the expertise required to operate the machinery and interpret results may have inhibited its more widespread use.

Many other blood biomarkers have also been associated with PH and are summarised in Table 1.

2.12   Comments on Biomarkers in PH

While many different biomarkers have been proposed for use in the management of PH, most have been inadequately validated in larger, independent cohorts and few have met the criteria to support routine measurement and use in clinical practice. The evidence for the utility of BNP and NT-proBNP as indices of right ventricular stress in PH is strong and their measurement is recommended by European guidelines (Galie et al. 2009). Despite this, the lack of validated specific cut-offs and an understanding of the meaning of proportional changes in BNP levels over time limits their use in practice. GDF-15 and RDW have been particularly strong performers in recent biomarker studies (Nickel et al. 2008; Rhodes et al. 2011a), possibly because they report on multiple facets of the disease pathology. Other markers such as IL-6, creatinine, uric acid, and angiopoietins may reflect more specific components, inflammation, renal dysfunction, oxidative stress, and endo-thelial cell dysfunction, respectively. The relatively recent discovery of microRNAs opens up a new avenue for biomarker discovery in PH. But before any of these biomarkers will be used routinely, circulating levels have to be shown to provide additional prognostic information that aids clinical decision-making and the necessary laboratory tests are readily available and reliable. The use of multiple markers, reflecting different aspects of PH pathology and incorporating imaging, may best represent the overall status of a patient and help guide future therapeutic decisions as more specific therapies become available. A critical question however is whether such a panel of biomarkers can identify not just a cohort but an individual patient with PH who is likely to derive benefit from a particular treatment?

Table 1 Other potential biomarkers in pulmonary hypertension

Biomarker	Biology	Evidence
Insulin resistance	Control of blood glucose levels	Insulin resistance twice as common in females with PAH (Zamanian et al. 2009)
Soluble TWEAK	Member of tumour necrosis factor (TNF) receptor superfamily	Predicts hemodynamic impairment and functional capacity in patients with PAH (Filusch et al. 2011)
Osteoprotegerin (OPG)	TNF/apoptotic pathway	Serum OPG elevated in IPAH patients and predicts survival (Lawrie et al. 2008; Condliffe et al. 2012)
Apelin	Vasodilator	Plasma levels decreased in IPAH, chronic lung disease, and heart failure (Goetze et al. 2006)
Endothelial progenitor cells (EPCs)	Endothelial repair/dysfunction	Heterogeneous population; altered circulating cell number and/or function of cultured cells in PAH (Diller et al. 2008, 2010; Asosingh et al. 2008; Toshner et al. 2009)
Circulating microvesicles	Endothelial function	Increased endothelial microvesicles predict haemodynamic severity and outcome in PH (Amabile et al. 2008, 2009)
Von Willebrand factor (vWf)	Endothelial function	Elevated levels prognostic in a small study of PAH associated with congenital heart disease (Lopes et al. 2011)
Cyclic GMP	Nitric oxide (NO) pathway	cGMP levels increased in PH (Bogdan et al. 1998), decreased by iloprost (Wiedemann et al. 2001), and correlate with PVR (Ghofrani et al. 2002)
Asymmetric dimethyl arginine (ADMA)	Inhibitor of NO synthase	Upregulated in PAH associated with congenital heart disease (Gorenflo et al. 2001). Levels correlate with haemodynamics and mortality in IPAH (Kielstein et al. 2005) and CTEPH (Skoro-Sajer et al. 2007)
Osteopontin	Inflammatory cytokine	Raised levels in IPAH correlate with 6MWD, mPAP, and functional class and predict survival (Lorenzen et al. 2011)
Isoprostanes	Oxidative stress	Increased in PAH and urinary levels associated with survival (Cracowski et al. 2001, 2012)
Nitric oxide (NO)	Vasodilator	Exhaled NO and urine NO metabolites reduced in IPAH and reversed by bosentan treatment (Girgis et al. 2005)
Serotonin	Vasoconstrictor	Raised levels in PAH correlate with PVR, but unresponsive to vasodilator treatment (Kereveur et al. 2000)
Endothelin-1	Vasoconstrictor	Raised plasma levels correlate with RAP, PVR, and disease severity in PAH (Stewart et al. 1991; Cacoub et al. 1993; Nootens et al. 1995; Rubens et al. 2001)
Cardiac troponins T & I	Myocardial injury	Troponin T & I levels predict disease severity and survival in PAH (Torbicki et al. 2003; Heresi et al. 2012)

(continued)

Table 1 (continued)

| Biomarker	Biology	Evidence
Fatty acid binding protein	Myocardial injury	Negative prognostic marker in CTEPH (Lankeit et al. 2008) and pulmonary embolism (Puls et al. 2007)
Complement C4a des Arg	Complement cascade	Elevated levels of C4a des Arg (C4a activation fragment) in IPAH (Abdul-Salam et al. 2006)
Matrix metallo-proteinases (MMPs)	Tissue remodelling	Elevated plasma levels of MMP-2, MMP-9, and tissue inhibitor of MMP (TIMP)-4 in PH (Elias et al. 2008; Hiremath et al. 2010; Schumann et al. 2010)
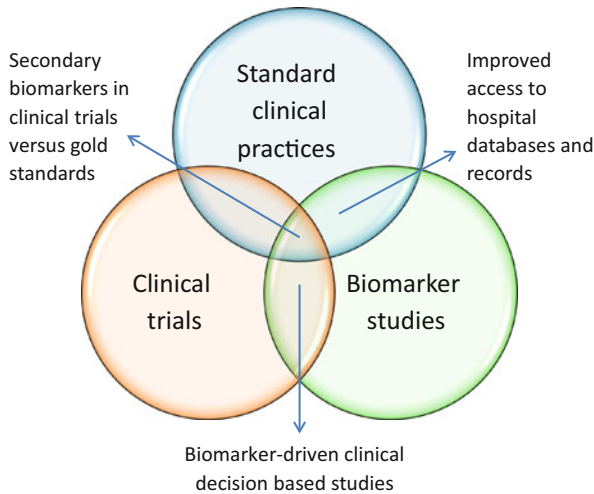
Fig. 3 Areas where improvements are required to take biomarker science forward in PH

3 Future Directions and Conclusions

The advent of "unbiased" genomic, metabolomic, and proteomic screening methodologies has not yet provided a major improvement in our understanding of disease biology or identified many new biomarkers. As the technologies are refined and improved upon, however, they will inevitably generate novel targets for therapy or biomarker use. MicroRNA screens in particular may generate novel hits, with panels of miRNAs being used to assess disease progression and response to therapy. The replication of findings using independent cohorts from other centres is vital in order to validate a potential biomarker and comparisons against current gold standards may be accelerated using hospital databases and registries in research protocols.

Genetic factors in PH can be useful in estimating susceptibility but currently apply only to a relatively small proportion of the overall PH population. More biomarker-focused clinical studies and better integration of pre-clinical biomarker studies into standard practices and clinical therapy trials are needed to take biomarkers in PH to the next level (Fig. 3).

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