The search for biomarkers in the critically ill: a cautionary tale

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ABSTRACT

The search for biomarkers has been described as a dismal patchwork of fragmented research. We review biomarkers in sepsis in the critically ill in terms of conventional single circulating proteins. Despite sepsis biomarker publications trebling over the past 6 years, currently only one, procalcitonin, has materialised promise.

We survey genomic biomarker initiatives, single nucleotide polymorphisms (SNPs) and gene signatures. Despite many SNP associations with sepsis susceptibility and a limited number of genome-wide association studies, the status of these associations is that of genomic signposts only. The standing of gene signatures in the paradigmatic discipline, breast cancer, is described. Uncertainties in the understanding of the sepsis process are documented — the dissociation between blood and tissue element activity, or compartmentalisation. The paradox of the active search for gene signatures to refine the sepsis phenotype and discover target subtypes for new therapies in the absence of such therapies is presented.

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The search for biomarkers in the critically ill is a phenomenon that needs both explanation and assessment, more so as this is an instance of the so-called new directions in critical care evidence.[1] Biomarkers may be broadly defined as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention".[2] For our purposes, we will follow Ballman[3] and characterise biomarkers as diagnostic, distinguishing between two states (infected or non-infected); prognostic, informing of a likely outcome (mortality); or predictive, if a treatment effect is differential between biomarker positivity and negativity.

We undertake biomarker exploration in the critically ill from the perspective of a dominant paradigm in the critically ill: that of sepsis. First, in terms of the traditional[4] notion of "single" circulating protein,[5] we briefly overview the many putative biomarkers. Second, we consider current genomic outputs: single nucleotide polymorphisms (SNPs) (differences in a single nucleotide within the DNA building block consequent upon replacements) and the complementary genome-wide association studies (GWAS) (the systematic assessment of whole genomes to demonstrate SNP association with traits[6]), and variants,[7] the majority of which are non-coding and manifest effects via regulation of gene expression;[8] and gene signatures (a gene group with a combined expression pattern[9] defining a particular patient phenotype[10]), as frontier biomarker exemplars. We note in passing some ambiguity in the notion of gene.[11]

Definitions

Sepsis is a "… concept — that of disease arising from the host response to infection — rather than a measurable pathological process,"[12] a sentiment echoed by Seymour and Angus,[13] reflecting on the new consensus definitions for sepsis and septic shock.[14] We may contrast this with the case of breast cancer, which is currently viewed not as a single disease with variable morphology and biomarkers, but as a group of molecularly distinct neoplastic disorders (with four main classes: basal-like, luminal A, luminal B and human epidermal growth factor receptor 2 [HER2]-positive[15]).

Biomarkers

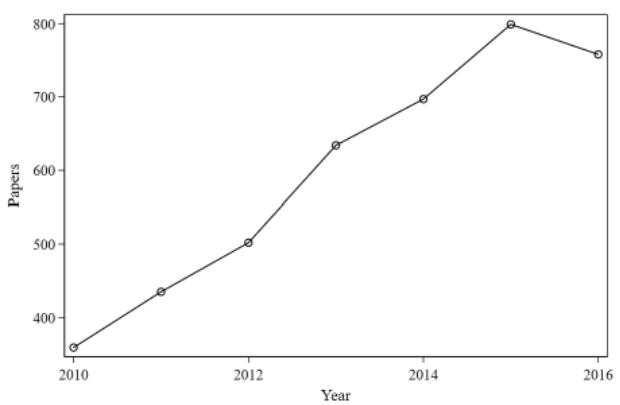
A review of sepsis biomarkers in 2010, using 3370 references covering 178 different biomarkers, concluded that "… none has sufficient specificity or sensitivity to be routinely employed in clinical practice".[16] This salutary assessment was also reflected in a wider general review, in 2011, of disease-associated biomarkers as a "dismal patchwork of fragmented research," whereby an estimated 150 000 articles documenting thousands of biomarkers had returned fewer than 100 validated for routine clinical practice.[17] The current situation has not substantially advanced from the previous 2010 survey, albeit the number of articles referencing "biomarkers" and "sepsis" has increased almost linearly from 2010 to 2016 (Figure 1; search conducted using Web of Science, 20 August 2017). Many reviews have summarised multiple single biomarkers (eg, C-reactive protein,[18] presepsin,[5] proadrenomedullin,[19] cytokines,[20]

monocyte human leukocyte antigen-DR expression,[21] interferon (IFN)-β,[22] cell-surface markers[23] and biomarker panels[24]), and more novel recently described markers, such as monocyte distribution width[25] (see also ClinicalTrials.gov NCT03145428), circulating microRNA,[26] haemoglobin subunit-β[27] and the endocannabinoid system,[28] both diagnostic and prognostic; but except for procalcitonin, no candidate has yet materialised promise.[29,30] With respect to procalcitonin,[31] we have recently reviewed its role[32] and concluded that there was little apparent benefit in guiding decision making for diagnosis and therapy of infection or sepsis, and the benefits or otherwise of procalcitonin-guided antimicrobial therapy appear uncertain and dependent on the cost and medical structure of each jurisdiction. The reasons for the variable sensitivity/specificity of putative biomarkers are manifold: patient cohort composition and severity of illness, sepsis type, assay variability and varying biomarker cut points across different sepsis populations[30] and this has been recently formally addressed.[33] Lactate has been used as a predictive biomarker in sepsis by Jones and colleagues,[34] who showed non-inferiority between lactate clearance and central venous oxygen saturation (Δ = –10% in-hospital mortality) as early sepsis resuscitation goals, but no differences in administered therapies in the first 72 hours.

Genomic perspectives

The impact of the Human Genome Project has been heralded as one generating great biomedical progress, none the least in the cost of implementing genomic studies: in 2000, the time to sequence a human genome was 13 years, at a cost of US$2.7 billion, and in 2013, the time was 2 weeks, at less than US$5000.[35] The great leap forward was to be propelled by systems biology and P4 medicine (ie, predictive, personalised, preventive and participatory), with a claimed spin-off, the extension of normal life by 10–30 years.[36,37] Surprisingly, in a recent somewhat fulsome embrace of this perspective from the respiratory medicine viewpoint,[38] no formal reference was made to the P4 medicine originator: Leroy Hood.[37]

Figure 1. Number of papers referencing biomarkers and sepsis, 2010–2016



Polymorphisms

A large amount of literature has attended the description of genetic susceptibility to infections,[39] sepsis and septic shock,[40] and the acute respiratory syndrome.[41] A commentary on genetic association studies noted that single SNP associations may not identify causal SNPs, but may be markers in linkage disequilibrium with underlying causal genetic variants, and very large studies (10 000 participants) are required to identify key genes;[42] $P < 5 \times 10–7$ is appropriate for discovery in GWAS.[43] In such a GWAS[44] of patient blood samples (*n* = 1446) from the PROWESS (recombinant human activated protein C worldwide evaluation in severe sepsis) study,[45] markers to delineate a subgroup with improved 28-day mortality response to drotrecogin-α (activated) (DAA) was undertaken, addressing 1 199 187 SNPs and using massive computational facilities. Single treatment response markers (defined by a subgroup with > 20% of the PROWESS population and an absolute risk reduction of > 12.5%) were only evident for one SNP (rs17513961) at $P < 5 \times 10–7$; for three SNP genetic combination markers, an absolute risk reduction of up to 41.7% was defined, compared with an absolute risk reduction of 6.1% in the original trial. However, there was no replication cohort.[46] Of interest, in the placebo group (*n* = 700), there were few "interesting genetic markers" and no SNPs were identified that had previously been associated with outcome in severe sepsis. Two other GWAS in sepsis have been recently published using 28-day mortality as outcome:

- Scherag and colleagues,[47] in 740 adult patients with sepsis recruited from two German randomised controlled trials with varying infection sites, identified an SNP (rs117983287; $P = 8.16 \times 10–8$) localised to the *VPS13A* gene, which has an important role in autophagic degradation; this finding appears not to have been validated;[46] and
- Rautenen and colleagues,[48] in the pneumonia subcohort (*n* = 1553) of a three-cohort group (*n* = 2534), reported an SNP (rs4957796; discovery $P = 9.7 \times 10–8$) in the *FER* gene, important in regulation of cell cytoskeleton and cellular adhesion, migration and chemotaxis. These latter findings were not validated either in the initial Scherag and colleagues GWAS[47] nor in a subgroup of patients with pneumonia (*n* = 298) reported by Schoneweck and

colleagues.[49] At best, the current GWAS in sepsis would appear to be genomic signposts.[46]

A recent novel finding[50] in humans, replicated in murine experiments, that loss-of-function genetic variants of proprotein convertase subtilisin/kexin type 9 (PCSK9) was associated with improved survival in patients with septic shock, has generated much interest. PCSK9 binds to low density lipoprotein receptors (LDLR) on hepatocytes and increases clearance of LDLR-binding lipids from the blood, with consequent decrease in cholesterol. Inhibition of PCSK9 may increase pathogen lipid clearance via LDLR to reduce inflammation. That is, patient PCSK9 status could be a predictive biomarker, and therapeutic inhibition of PCSK9 in patients with PCSK9 gain-of-function variant rs505151[51] could improve sepsis outcomes.[52] The further comments by dos Santos and Marshall[51] that "the extent to which biological responses observed in relatively simplistic murine models reflect human biology is controversial," referring to articles by Seok and colleagues[53] and Takao and Miyakawa,[54] deserve some consideration.

Of mice and women and men

The validity of rodent, in particular mouse, experiments to reflect genomic responses in humans, that is, the assumption that "… molecular results from current mouse models developed to mimic human diseases translate directly to human conditions," has recently been questioned in the 2013 publication by Seok and colleagues,[53] using a comparative analysis of gene expression datasets, although such response differences had been previously suggested.[55,56] Seok et al[53] found that in inflammatory stress (burns, trauma and endotoxaemia), the murine orthologs to genes demonstrating significant change in humans were found to be close to random in matching to their human counterparts (R^2, 0.0–0.1).[53] Such a finding created some disquiet in both the scientific and lay literature.[57] Using the same datasets as Seok and colleagues,[53] Takao and Miyakawa,[54] on the basis of both methodological and analytic grounds, challenged these null findings and concluded that "… gene expression patterns in mouse models closely recapitulate those in human inflammatory conditions".[54] We detail these concerns in the Appendix (online at cicm.org.au/Resources/Publications/Journal) for the interested reader. Subsequent exchanges with the authors, Warren et al,[58] and an independent commentary by Shay and colleagues,[59] led to a modification of the position of Takao and Miyakawa[54] that: "… at least some genomic responses on inflammation show statistically significant similarities between humans and mice, regardless of subjective and opposing views on whether 5.9–15.0% …".[60]

Gene signatures: cancer

As comparisons have continued to be drawn between the ongoing biomarker pursuit in the critically ill and the cancer discipline,[12,61] it is useful to briefly review the terrain of an established biomarker-endowed paradigm: that of breast cancer, where predictive biomarkers, oestrogen hormone receptors[62] and HER2,[63] have been firmly established. A number of different prognostic gene signatures have also been described and at least six are commercially available.[64] The gene overlap between these signatures has generally been quite low[65] and many sets of genes can be assembled as prognostic signatures; that is, we may contrast the search for a survival-related master gene set and the construction of various prognostic "short lists".[66] These issues are not uncommon under the conditions of large numbers of highly correlated variables (gene expressions), where different selections can build similarly accurate classifiers[15] and tumour cell proliferation is the common denominator,[67] although the specific biological meaning of most signatures is unclear.[68] Prognostic concordance, based on predicted risk categories, has varied,[67,69] depending on the actual comparison and whether this comparison encompassed conventional clinical variable classifiers;[70,71] for example, Adjuvant!Online (www.newadjuvant.com/default2.aspx). That these signatures may have a prognostic or predictive advantage beyond, say, the TNM staging system — T (tumor size), N (lymph node status) and M (distant metastasis) — may be understood in that the TNM system has offered limited patient survival predictions for a number of cancers: receiver operating characteristic scores for breast cancer, 0.69–0.72; colorectal cancer, 0.74; and prostate cancer, 0.53.[72] Critical review and recommendations for gene expression publication have been undertaken from within the cancer paradigm.[73,74] Two recent large-scale trials of predictive biomarkers have been conducted using a 21- (Oncotype DX, Genomic Health[75]) and 70- (MammaPrint, Agendia[76]) gene signature to identify patients who could forgo adjuvant chemotherapy therapy (favourable gene expression profile), which might have otherwise been recommended on the basis of clinico-pathological criteria (clinical "high risk"). A 2008 review of the performance of both (first generation) signatures had suggested that they were suboptimal in terms of the potential of the technology, despite good metric performance and undoubted ability to improve quality of care in the subsequent decade.[77] Both trials realised this promise and identified appropriate patient groups, albeit the outcomes were somewhat critically received.[78,79] The relevance of these two trials, as illustrations of the potential use of predictive biomarkers for the critically ill, must be interpreted cautiously as they were undertaken using current recommended adjuvant

chemotherapy. With respect to the potential of gene-signature predictive biomarkers, it is instructive to read that the gain in overall survival for anticancer drugs approved by the Food and Drug Administration, 2002–2012, has been modest, measured in months (a range of 0.3–5.6 months[80]) rather than years; a more sobering assessment is provided from a consideration of the European Medicines Agency approvals, 2009–2013: "… most drugs entered the market without evidence of benefit on survival or quality of life".[81]

Genes and sepsis

Pathophysiology

Sepsis as a dynamic time-compressed process presents two problems: how is it understood and by what means do we generate this evidence?[82] The dominant interpretation is a two-phase process, initial hyperinflammation followed by secondary immune suppression or paralysis,[83] and the source tissue has been blood and its constituent cellular elements for both gene expression[84] and cytokine studies,[85] notwithstanding the role of bronchoalveolar lavage as a "direct" source of lung elements. The two-phase process has been challenged in the sense that the acute sepsis event has been shown to involve both inflammatory and immunosuppressive elements.[86-89] This being said, although both states may be simultaneous at the transcriptional level, there may be differences at the translational phase by virtue of variable cytokine and chemokine kinetics and receptor affinity constants.[90] With respect to the importance or otherwise of the blood and its constituents being a dominant effector and an accurate reflection of tissue process, this has been variously commented on,[91,92] but more formally contested by invoking the notion of compartmentalisation,[93-95] whereby there is a variable dissociation between blood elements (cytokines, cellular elements and their gene-expressions) and tissue element activity. That is "… the immune status of leukocytes in the peripheral blood might be quite different from those present in inflamed tissues,"[96] and further, gene signatures are cell-type-specific.[97]

The solution to these paradoxes is fundamental to establishing predictive biomarkers. The failure of inflammatory suppression therapeutics has swung the balance towards the promise of immunomodulators — such as granulocyte colony stimulating factor, granulocyte-macrophage colony-stimulating factor, IFN-γ, inhibitors of programmed cell death protein type 1, recombinant human interleukin (IL)-3, IL-7 and IL-15[83,98] — yet this new therapeutic direction is by no means guaranteed.[96,99]

Signatures

As assessed by micro-assay hybridisation,[100,101] septic shock provokes a global reprogramming of the whole leukocyte transcriptome affecting multiple functions and pathways related to cellular death and apoptosis, with > 71% of the whole genome being modified within 48 hours of onset.[102] Numerous articles have investigated this storm and we highlight the most pertinent to our concerns, noting that the discovery of sepsis subtypes has been identified as a key goal in sepsis research.[101] Maslove and colleagues,[103] combining the data from two previous investigations by Tang and colleagues,[104,105] used (unsupervised) class discovery methods,[106] partitioning around medoids clustering, to identify sepsis subtypes (S1, which significantly increased expression of genes involved in inflammatory and toll-like receptor-mediated signalling pathways, and S2, which was associated with "less" expression) within gene expression profiles (using microarray techniques[100]). Clinical differences between subtypes were established for severe sepsis (S1, 36% *v* S2, 9%; $P = 0.009$) but not for septic shock (S1, 44% *v* S2, 64%; $P = 0.13$). The authors did not offer an explanation as to why there was an increased prevalence of the S1 subtype in severe sepsis but not in septic shock, although altered genomic pathways are not necessarily different between sepsis severity classes.[102] Similar studies have been reported by Johnson et al,[107] Pachot et al,[108] Davenport et al,[89] Burnham et al[109] and Scicluna,[110] and we summarise details of these studies in Table 1. What is evident from these studies is the variability of patient number (for discovery and validation cohorts), sepsis type, gene signatures (and number of constituent genes) and invoked pathways, albeit with a common theme. The use of unsupervised clustering methods is common to the class discovery in these studies, but will "… invariably identify clusters";[103] and the set of most differentially expressed genes will not necessarily yield the best predictive accuracy.[111] Instability of diagnostic labels makes supervised methods to detect expression differences between groups (class comparison[112]), problematic[101] — illustrated in this context by the new taxonomy of the third international definition of sepsis (Sepsis-3), where the term "severe sepsis" is no longer used, although "septic shock" is retained.[14]

Inference regarding various genetic signatures must attend to potential confounders, such as patient age, gender, ethnicity, basal state of immune activation, diagnostic label, infecting organism and site, timing of the insult, and therapy. Gene signature generation in sepsis enables increased resolution of the sepsis phenotype, independent of clinical variables, such as severity of illness scores, and identification of subgroups dependent on

Table 1. Summary of sepsis gene signature studies

Study	Pachot et al[108]	Johnson et al[107]	Maslove et al[103]	Davenport et al[89]	Burnham et al[109]	Scicluna et al[110]
Date	2006	2007	2012	2016	2017	2017
Tissue	Whole blood	Whole blood	Blood leukocytes	Blood leukocytes	Blood leukocytes	Whole blood
Patient type	Septic shock	SIRS + infection *v* SIRS	SIRS + infection	CAP	CAP and FP	Sepsis, including Davenport et al[89]
Time frame	Day 2–4 after admission		Within 24 hours of admission	After admission	First through Day 5 after admission	Within 24 hours of admission
Discovery set (*n*)	31	90 (45 in each group)	55	265	CAP, 73 FP, 64	306
Validation set (*n*)	7		71	106	CAP, 53 FP, 53	216 and 265
Analytic method	Supervised	Various	Clustering (PAM)	Clustering (USH)	Clustering (USH) and assignment based on 7-gene set of Davenport et al[89]	Clustering (USH)
Initial gene number		7453 meeting acceptance criteria	365, based on GenBank search	3080 differentially expressed between groups	1075 differentially expressed between groups	5000 top ranked
Gene set	28 assigning correct mortality outcome	459 amenable to pathway annotation	170	7	6 Strong concordance between 7- (Davenport et al[89]) and 6-gene set	Initial, 140; final, 8 Low risk M3 highly correlated with low risk SRS2 (Davenport et al[89])
Expression: downregulated		85.8%				65%
Expression: upregulated	▪ Survivors: 18 upregulated ▪ Non-survivors: 10 upregulated	14.2%				35%
Sepsis subtypes	Survivors and non-survivors		S1 and S2	▪ SRS1 (41%) and SRS2 (59%) ▪ 3080 genes differentially expressed	▪ SRS1-FP (46%) and SRS2-FP (54%) ▪ 1075 genes differentially expressed	▪ MARS types 1 (29%), 2 (34%), 3 (23%), and 4 (14%) ▪ MARS 3 and MARS 4 common in CAP
Pathways	Toll-like receptor, cytokine and chemokine	Ten pathways related to inflammation (innate immunity, cytokine receptors, T cell differentiation, protein synthesis regulation); one to nicotinate and nicotinamide metabolism	▪ Inflammation: chemokine and cytokine ▪ Toll receptor signalling	T cell activation, cell death apoptosis, cytotoxicity phagocyte movement endotoxin tolerance	T cell activation, cell death apoptosis, cytotoxicity endotoxin tolerance	▪ MARS 1: UR, haemebiosynthesis ▪ DR, cytokine and lymphocyte signalling and PRR ▪ MARS 2: UR, PRR and cytokine signalling ▪ MARS 4: UR, PRR and cytokine signalling ▪ MARS 3: UR, adaptive immune pathways (T cell)
Subset mortality difference	By definition	Not applicable	No	Yes	Yes	▪ MARS 1, worst survival ▪ MARS 3, low risk

CAP = community-acquired pneumonia. DR = downregulated. FP = faecal peritonitis. MARS = Molecular Diagnosis and Risk Stratification of Sepsis project. PAM = partitioning around medoids. PRR = pattern recognition receptor. S1 = sepsis subtype 1. S2 = sepsis subtype 2. SIRS = systemic inflammatory response syndrome. SRS = sepsis response signatures. SRS1 = sepsis response signatures subtype 1. SRS2 = sepsis response signatures subtype 2. SRS-FP = sepsis response signatures in faecal peritonitis. UR = upregulated. USH = unsupervised hierarchical clustering.

response state,[46,113] but the prognostic performance of genomic variables compared with conventional clinical variables appears unclear. Davenport and colleagues[89] (Table 1) found mortality outcome prediction for models using the most informative gene expression superior to that of various combinations of clinical covariates, including the SOFA (Sequential Organ Failure Assessment) and APACHE (Acute Physiology and Chronic Health Evaluation) II scores. However, in their validation cohort, the misclassification rate of the best predictive gene set for 14-day mortality was 34%. Sweeney and colleagues[114] reported four 30-day mortality predictive models, assessed by area under receiver operator character operating curves (AUROC), generated by three co-investigator groups using public-domain transcriptomic data. There was little gene overlap between models (in aggregate, 58 genes: 31 up regulated and 27 downregulated), model AUROCs ranged from 0.75 to 0.89 with no significant difference from AUROCs generated using clinical severity scores, including SOFA, APACHE II and SAPS (Simplified Acute Physiology Score) II scores. Not surprisingly, joint prediction by gene signatures and severity scores showed superior performance, but this was somewhat varied across the four models, as assessed by the net reclassification index (NRI) (see the TRIPOD [transparent reporting of a multivariable prediction model for individual prognosis or diagnosis] statement[115]), with significant improvements in the NRI reported in 17–50% of the validation datasets. Both the NRI and the integrated discrimination improvement index (IDI) were used by Scicluna and colleagues[110] to establish improvement of a clinical-molecular model; APACHE IV score[116] + Molecular Diagnosis and Risk Stratification of Sepsis project (MARS) endotype membership, over the APACHE IV scores alone.

Conclusions

The repeated claims of potential novel target groups for new sepsis therapies[117] belies the fact that, as opposed to the cancer paradigm, no such treatments are currently available for sepsis.[118] Enthusiasm must be tempered by reflection upon the multiplicity of biomarkers, including genomic signatures; as Hudis[78] said of the Oncotype DX signature: "The world did not (and still does not) need yet another prognostic factor".[78]

Competing interests

None declared.

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