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Laboratory Diagnostic Testing of Inflammatory Biomarkers: Laboratory Approaches in Chronic Heart Failure

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Abstract

Heart failure (HF) is a progressive, multifaceted condition characterized by complex pathophysiological mechanisms, including chronic inflammation, which contributes significantly to disease onset and progression. Despite advancements in pharmacological therapies, such as angiotensin-converting enzyme inhibitors and ß-blockers, HF remains associated with high morbidity and mortality. Inflammatory cytokines have emerged as potential biomarkers for HF, offering insights into disease mechanisms, risk stratification, and therapeutic responses. However, their clinical utility is hindered by analytical variability, pre-analytical challenges, and limited reproducibility across studies.

This review explores the role of inflammatory cytokines in HF, evaluating their pathogenetic significance, analytical performance, and prognostic value. Biomarkers such as TNFα, IL-6, sTNFR1, and gp130 are assessed for their ability to enhance diagnostic precision and predict adverse outcomes. Furthermore, we examine the limitations of current cytokine assays, including sample stability, diurnal variation, and lack of standardization, which affect their transition into clinical practice. While natriuretic peptides and cardiac troponins remain the gold standard for HF biomarkers, multimarker approaches incorporating inflammatory cytokines may provide a more comprehensive understanding of HF pathophysiology.

The findings underscore the need for robust, standardized methodologies and novel biomarkers beyond established pathways. These efforts will enhance HF management by

improving risk stratification, guiding personalized treatment strategies, and advancing our understanding of inflammatory processes in HF progression.

Introduction

Heart failure (HF) is a multifaceted, progressive condition characterized by the involvement of numerous pathophysiological processes, including the activation of neurohormonal pathways. This understanding has led to the development of pharmacological therapies, such as angiotensin-converting enzyme (ACE) inhibitors, angiotensin II receptor blockers, and β-blockers, which have revolutionized HF management. However, despite advancements in cardiovascular (CV) therapies, chronic HF remains associated with significant morbidity and mortality, indicating the persistence of pathogenic mechanisms unaddressed by current treatments. Among these, chronic inflammation has emerged as a potential unmodified mechanism. Elevated levels of tumor necrosis factor (TNF) in HF patients marked the beginning of the "inflammation era" in HF research. Subsequent studies have underscored the activation of inflammatory pathways as pivotal in the onset and progression of HF (Hartupee & Mann, 2013; Hofmann & Frantz, 2013; Vistnes et al., 2010).

Biomarkers are extensively employed for risk stratification and evaluating therapeutic responses in CV diseases. In HF, markers such as N-terminal prohormone of brain natriuretic peptide (NT-proBNP) and high-sensitivity cardiac troponin T (hs-cTn) have been rigorously studied (de Antonio et al., 2013; McMurray et al., 2013, p. 201). Inflammatory cytokines and related mediators, which are directly involved in HF pathogenesis, have also been proposed as potential markers for risk stratification and prognostication (Bozkurt et al., 2010). Studies suggest that inflammatory cytokines can predict adverse outcomes in HF patients; however, many such studies suffer from small sample sizes and fail to adjust for established biomarkers like NT-proBNP, hs-cTn, and C-reactive protein (CRP) (Ueland et al., 2012). Moreover, pre-analytical and analytical challenges in cytokine measurement pose additional limitations. This review explores the technical, informative, and practical aspects of utilizing inflammatory cytokines as prognostic biomarkers in HF.

2. Inflammatory Cytokines as Biomarkers

2.1 Pathogenetic Role of Inflammation in HF

Numerous studies have demonstrated increased expression and secretion of inflammatory cytokines, including TNFα, IL-1, IL-6, IL-18, cardiotrophin-1 (CT-1), and Fas ligand, alongside chemokines like monocyte chemoattractant protein (MCP)-1/CCL2, IL-8/CXCL8, CXCL16, and CCL21 in HF patients. Plasma levels of these inflammatory mediators correlate with worsening functional class (e.g., NYHA classification) and cardiac performance (e.g., left ventricular ejection fraction [LVEF]). Experimental studies have shown that cytokines contribute to HF pathogenesis by promoting hypertrophy and fibrosis, impairing myocardial contractility via calcium transport and β-adrenergic signaling, inducing apoptosis, and influencing myocardial remodeling genes.

Inflammatory mediators may also exacerbate HF progression through indirect mechanisms such as impairing bone marrow function, activating endothelial cells, and inducing skeletal muscle catabolism, thereby causing systemic inflammation and reflex abnormalities. While inflammation is generally a protective process, its dysregulation can lead to tissue damage, dysfunction, and impaired repair mechanisms. Achieving a balanced inflammatory response in HF remains challenging. Trials targeting specific mediators like TNFα have largely been unsuccessful, as seen with the chimeric anti-TNF antibody infliximab, which can harm TNF-expressing cardiomyocytes. This underscores the dual-edged nature of inflammation in HF, where excessive or insufficient inflammatory responses can be deleterious.

2.2 From Pathophysiology to Plasma Biomarker

Key inflammatory mediators may not necessarily serve as optimal biomarkers. For instance, CRP's role as a biomarker in CV diseases stems not from its pathogenic significance but from its stability and capacity to reflect upstream inflammatory activity. While secreted cytokines often circulate at low levels, leading to analytical variability and necessitating costly high-sensitivity assays, their soluble receptors are more abundant and stable. Soluble TNF receptors (sTNFR1 and sTNFR2), along with other TNF receptor superfamily members such as CD27, FAS, and osteoprotegerin (OPG), are detectable in high levels in HF and offer reliable biomarker potential.

Similarly, soluble gp130 (sgp130), a receptor subunit for IL-6 family cytokines, and IL-1 receptor-like 1 (IL1RL1/ST2) have shown promise as biomarkers reflecting inflammation and hemodynamic stress in HF (Askevold et al., 2014; Broch et al., 2012). Other markers like CXCL16, which is induced by TNFα, IL-1β, and interferon-γ (IFNγ), have demonstrated prognostic value. Additionally, pentraxin 3 (PTX3), unlike CRP, is locally produced at inflammation sites and has been linked to increased cardiac event risks in HF patients (Latini et al., 2012).

2.3 Clinical Role of Inflammatory Biomarkers

Morrow and de Lemos proposed three essential criteria for evaluating biomarkers: measurement feasibility, provision of new information, and impact on patient management. Clinicians must assess biomarkers for analytical accuracy, cost-effectiveness, and reproducibility. Furthermore, biomarkers should add significant prognostic or diagnostic value to existing tests. Lastly, biomarkers must guide patient management by outperforming other diagnostics, identifying modifiable risks, or demonstrating the utility of biomarker-based care strategies.

Inflammatory cytokines face significant challenges in meeting these criteria. Despite numerous studies, most novel inflammatory biomarkers have not transitioned into routine clinical practice, with CRP being the notable exception. This review evaluates TNFα, sTNFR1, OPG, IL-6, sgp130, MCP-1, IL-8, CXCL16, CCL21, and PTX3 as potential inflammatory biomarkers in HF populations.

Analytical Performance of Inflammatory Cytokines

The analytical performance of cytokines is influenced by numerous features and conditions, which can limit their utility as biomarkers in routine clinical practice. Biological factors such as age, gender, and diurnal and postprandial variations contribute to both intra- and inter-patient variability. Furthermore, pre-analytical factors, including sample handling (e.g., collection methods, storage conditions, freeze-thaw cycles, and plasma versus serum preparation), as well as analytical factors associated with assay methodology and standardization, also impact cytokine measurement. For instance, established normal levels for most relevant cytokines are currently lacking, and their absolute levels vary significantly across studies. These factors collectively contribute to the variability observed in similar clinical studies, complicating direct comparisons of study outcomes.

3.1 Patient-Related Variability

Several studies have examined the effects of age and gender on circulating cytokine levels. Aging is typically associated with a 2–4 fold increase in circulating inflammatory cytokine levels, indicative of low-grade inflammation, which is attributed to changes in lifestyle factors, infections, physiological alterations (e.g., increased fat mass and physical inactivity), and a higher risk of age-related diseases. Aging and the development of cardiovascular (CV) diseases share common mechanisms, including inflammation, often referred to as "inflammaging" (De Araújo et al., 2013; Libby et al., 2010). Most cytokines and their corresponding secreted receptors exhibit increased levels with advancing age.

Additionally, estrogen deprivation may account for particularly elevated cytokine levels in postmenopausal women (Hage & Oparil, 2013).

Although age and gender can be adjusted for in survival models to evaluate the independent contribution of an inflammatory biomarker, the absence of standardized age-adjusted normal ranges complicates the interpretation of minor variations in cytokine levels in routine clinical settings. Other factors, such as diurnal variation and food intake, can also affect cytokine measurability. Many inflammatory markers follow a circadian rhythm, partly influenced by plasma cortisol and melatonin levels. However, limited data are available for certain chemokines. Ideally, variations related to diurnal patterns and food intake should be considered when assessing a marker for clinical application.

Cytokine production is influenced by multiple cell types, including muscle cells, with factors such as physical exercise and stress modulating levels of certain markers like interleukin-6 (IL-6), which is often termed a myokine due to its high expression in skeletal muscles. Adhering strictly to sampling protocols (e.g., fasting samples collected at standardized time points) may be more feasible in homogeneous monocenter studies than in multicenter trials, where variability in sampling procedures is higher. Such variability in multicenter studies can attenuate the predictive value of inflammatory markers influenced by the aforementioned factors. Ultimately, a biomarker intended for clinical use should demonstrate relative stability and minimal susceptibility to day-to-day, postprandial, and diurnal variations.

3.2 Pre-Analytical Considerations

The results of plasma or serum cytokine analyses can be significantly influenced by pre-analytical factors, such as blood sample collection, processing, and storage (Zhou et al., 2010). The choice between serum and plasma is crucial, as platelets activated during serum preparation can release substantial amounts of cytokines (Hosnijeh et al., 2010). However, in heart failure (HF) patients, this may not necessarily result in elevated cytokine levels. In fact, platelets in these patients are often activated in vivo, leading to lower cytokine release from degranulated platelets during serum coagulation, as illustrated by low serum levels of RANTES/CCL5 in various CV disorders.

Sample processing time also plays a vital role since cytokines, particularly ligands, have short half-lives. They may be produced by immune cells after collection, bound by receptors, or affected by enzymatic activity. Plasma cytokine measurements can also be influenced by the choice of anticoagulant. For instance, citrate and heparin plasma have been shown to alter levels of IL-6 and tumor necrosis factor-alpha (TNFα). Endotoxins can induce IL-6 and TNF release in contaminated vacutainer tubes, while ethylenediaminetetraacetic acid (EDTA) inhibits endotoxin-induced cytokine release. Additionally, heparin may release cytokines bound to heparin-sulfate on blood cell surfaces (Gilbertson-White et al., 2011). EDTA plasma has been shown to provide superior cytokine stability due to its protease-inhibiting properties. However, a recent study analyzing a large cytokine panel in spiked serum and plasma samples, using collection tubes with various additives, revealed recovery rates of 80–120% for all cytokines. This study also highlighted that serum might be preferred for certain cytokines, while plasma may be more suitable for others.

Quick processing is crucial for accurate cytokine measurement, with EDTA plasma offering the most consistent results, though no single sample type is optimal for all cytokines. These stringent requirements represent a limitation for the clinical use of inflammatory cytokines.

Most cytokines demonstrate stability during long-term storage at −80°C. Stability is more affected by repeated freeze-thaw cycles, with some reports indicating stability for up to three cycles, while others report significant variability. Certain cytokines, such as IL-6, remain

stable throughout multiple cycles, whereas others, like TNFα, may increase, and CXCL8 may decrease after one or more freeze-thaw cycles. While long-term storage stability may be less relevant for clinical applications, where bench life (i.e., stability at room temperature after separation) is more critical, it is vital for evaluating cytokines' predictive value in retrospectively analyzed prospective studies.

Markers that exhibit stability and durability are less affected by pre-analytical factors, which are essential for their transition into clinical practice. However, even if a candidate marker does not meet clinical usability standards, it may still provide valuable insights into the biological mechanisms of HF. Poor pre-analytical assay characteristics could obscure the pathophysiological relevance of a marker. Mono center studies with stricter adherence to specific sampling protocols may offer advantages for evaluating markers in biological contexts, despite their limited observational scope.

3.3 Analytical Considerations

Enzyme-linked immunosorbent assays (ELISAs) have become the most utilized and extensively validated method for quantifying circulating cytokine concentrations since their introduction in the 1970s. This widespread adoption is attributed to their ease of use, high sensitivity (enabling detection of most cytokines at picogram levels), and generally high specificity. Despite these advantages, several limitations and considerations surrounding ELISAs merit attention. First, the quality and precision of ELISA antibodies and kits can vary significantly depending on their origin, rendering direct comparisons of cytokine levels unreliable unless assays from the same manufacturer are used. Even within a single manufacturer, variability between production batches may lead to inconsistencies. Furthermore, the absence of international standardization for age-adjusted normal ranges of many cytokines poses additional challenges for interpreting ELISA results. This gap underscores a critical distinction between the routine, standardized tests conducted globally in hospital laboratories and the ELISA measurements frequently performed in research settings, particularly those focused on biomarkers.

Another limitation of immunoassays lies in what is measured. Although ELISA antibodies are often highly specific, they may not distinguish between free cytokines, cytokine-soluble receptor complexes, or cytokines bound to other proteins. The intrinsic characteristics of cytokines themselves can also affect the assays. For example, certain cytokines are biologically active only in their glycosylated forms, yet the antibodies used in ELISAs may target the non-glycosylated variants. Similarly, the biological importance of cytokine multimerization (e.g., monomeric versus multimeric forms) might not be discerned by the assay. Additionally, the dynamic range of ELISA assays, representing the linear association between cytokine concentration and absorbance readings, is often narrow, necessitating sample dilution. Such dilution can affect the measured levels of cytokines as well as their soluble receptors and natural inhibitors.

Multiplex assays have garnered attention for their ability to measure multiple cytokines simultaneously in a single specimen, which is advantageous for multi marker approaches. However, compromises related to incubation time, buffers, specimen dilution, and type are often necessary to accommodate the simultaneous measurement of multiple analytes. Consequently, multiplex assays are generally considered more suitable as screening tools rather than definitive diagnostic measures.

Currently, a major limitation for cytokine measurement, irrespective of the analytical method, is the limited availability of commercial assays. None of the cytokines or cytokine receptors discussed herein are presently available on automated platforms commonly used in hospital laboratories, although benchtop immunoassay analyzers offering assays for IL-1β, IL-6, IL-8, and TNFα are available. However, these analyzers often require large analyte volumes and offer only average sensitivity.

4. Cytokines and Cytokine Receptors as Predictors of Long-Term Adverse Outcomes in HF

4.1 Current Circulating Biomarkers in HF

For a biomarker to be suitable for clinical use, it must demonstrate consistent and robust associations with the disease or outcome of interest, while also improving upon or complementing existing diagnostic and prognostic tools. In heart failure (HF) management, only a limited number of biomarkers are currently used frequently (Böhm et al., 2011), with natriuretic peptides, specifically BNP and NT-proBNP, being the only markers endorsed by European Society of Cardiology (ESC) guidelines. Despite their prognostic efficacy, natriuretic peptides have notable limitations, including reduced performance in specific populations such as patients with obesity or renal impairment, as well as challenges in interpreting mid-range or "gray-zone" levels (Maisel & Daniels, 2012). Moreover, natriuretic peptides do not fully capture all the pathological processes in the failing myocardium and provide only modest enhancements to well-constructed multivariable risk models.

High-sensitivity cardiac troponin (hs-cTn) measurements have been shown to provide prognostic information independent of NT-proBNP in chronic HF. Additionally, simultaneous measurement of hs-cTn and NT-proBNP enhances mortality risk stratification in these patients.

4.2 Statistical Considerations

Evaluating potential biomarkers requires a robust statistical approach. Initial assessments may involve straightforward comparisons of patient and control groups, often using statistical methods such as the Student's t-test or Mann–Whitney U-test. However, more advanced statistical techniques are necessary to assess inherent properties of biomarkers. The Cox proportional hazards regression model remains the most widely used and accepted method for survival analysis in clinical medicine. Using a stepwise approach within this model allows for the evaluation of the effect or attenuation of variables on outcomes. It is essential to exercise caution when incorporating variables into multivariable regression models to maintain a reasonable ratio of events per variable, typically no lower than 10.

In many cases, biomarker evaluations are performed retrospectively on prospectively conducted studies, where endpoints and statistical approaches for assessing predictive power are pre-specified. This methodology provides advantages, as such trials typically include appropriate clinical and biochemical covariates (e.g., NT-proBNP), with the number of covariates adjusted to the event prevalence for specific outcomes.

An essential feature of diagnostic biomarkers is their ability to discriminate between diseased and non-diseased individuals, a capability quantified by the c-statistic or the area under the receiver operating characteristic (ROC) curve. However, in prognostic settings, where the disease has not yet occurred and can only be estimated as a probability or risk, the c-statistic may have limitations. Improving already robust risk prediction models is inherently challenging, and even a well-calibrated model may achieve c-statistic values significantly below the theoretical maximum of 1 (range: 0.5–1.0). Sole reliance on the ROC curve may hinder the clinical implementation of novel biomarkers with potential utility.

To address this challenge, alternative methods for evaluating the incremental value of new biomarkers have been proposed. One such method is the Net Reclassification Improvement (NRI), which evaluates how effectively a new marker reclassifies patients into higher or lower risk categories. While this approach offers insights beyond traditional multivariable regression and c-statistical analysis, it is not influenced by the calibration or goodness-of-fit of the baseline model. However, the range of meaningful improvements provided by NRI remains undetermined (Pencina et al., 2010).

4.4. Evaluation of Inflammatory Biomarkers

Miettinen et al. explored the prognostic relevance of TNFα and IL-6 using high-sensitivity assays in 465 acute HF patients, reporting that TNFα was an independent predictor of all-cause mortality in adjusted models, with a stronger predictive association observed in patients without severe cardiac and renal dysfunction. In contrast, Nymo et al., in a study on chronic HF utilizing the CORONA trial data, did not find TNFα levels to correlate with multiple adverse outcomes. This limitation was attributed to the use of a first-generation multiplex assay for TNFα, which exhibited poor sensitivity (Nymo et al., 2014). However, the same study identified an association between sTNFR1 levels and all-cause mortality, although it did not enhance discriminatory metrics such as the C-index or NRI. Regarding the TNF receptor superfamily member OPG, which circulates at high levels, Røysland et al. found no significant link between circulating OPG levels and all-cause death or CV-related hospitalization after adjustment for NT-proBNP and CRP in the GISSI trial. Conversely, in the CORONA trial, which featured a more homogenous population (elderly patients with ischemic HF), OPG levels were associated with HF-related hospitalization and the composite outcome of all-cause mortality and HF hospitalization, accompanied by an increase in discrimination as measured by the C-statistics (Ueland et al., 2011).

Within the IL-6 family, Miettinen et al. demonstrated that IL-6 levels above a specific cutoff predicted all-cause mortality in 465 acute HF patients. However, Liu et al. reported no association between IL-6 levels, measured via a proteasome array, and outcomes like all-cause mortality or HF hospitalization in 548 chronic HF patients (Liu et al., 2011). Similarly, Askevold et al. observed no significant link between serum IL-6 levels and various outcomes in the CORONA trial, although the findings were limited by the multiplex assay's low sensitivity. In contrast, Askevold identified that gp130 levels were predictive of multiple fatal outcomes in the same cohort (Askevold et al., 2013).

Hohensinner et al. reported an association between elevated levels of the chemokine MCP-1 and all-cause mortality in 351 HF patients, although discriminatory metrics were not evaluated. In the CORONA trial, IL-8 emerged as a significant predictor for all outcomes, except coronary endpoints, after adjustment. Moreover, IL-8 significantly improved net reclassification for all-cause mortality and CV hospitalization, though its impact on the primary endpoint, CV mortality, and the composite of HF hospitalization or CV mortality was only borderline significant. However, MCP-1 and CXCL16 levels showed no significant associations with outcomes following comprehensive multivariable adjustment, although mortality risk persisted when considering changes in CXCL16 levels from baseline to three months. CCL21 levels were associated with higher risks of all-cause and CV mortality across the combined GISSI and CORONA trials, displaying modest but significant effects on discriminatory metrics when analyzed independently (Ueland et al., 2013). Additionally, baseline and three-month changes in PTX3 levels correlated with increased risks of all-cause mortality, CV mortality, and HF hospitalization in combined analyses of the GISSI and CORONA trials, though the improvements in discrimination were marginal.

To summarize these investigations, several cytokines demonstrated associations with fatal outcomes and/or HF-related hospitalization. Diverging results between acute and chronic HF (e.g., for TNFα and IL-6) may reflect differences in assay type and sensitivity (e.g., high-sensitivity ELISA versus multiplex assays) as well as patient demographics, including age and etiology. For cytokines that remained significant in multivariable analyses alongside NT-proBNP and CRP, the improvements in discriminatory power were generally modest. This limited incremental value of inflammatory cytokines in HF progression aligns with the modest enhancement NT-proBNP itself offers over robust multivariable risk models.

5. Multimarker Strategies

Although NT-proBNP and hs-cTn are well-established biomarkers in HF, they do not encapsulate all pathogenic mechanisms underlying this complex condition. While individual cytokine measurements are unlikely to substantially enhance HF patient risk stratification in clinical practice, assessing global patterns of cytokines alongside other biomarkers may provide more comprehensive biological insights. Given that multiple mediators contribute to HF development and progression through distinct mechanisms at various levels, combining multiple circulating markers could enhance the accuracy of risk stratification and potentially aid in tailoring individualized therapies. For instance, Miettinen et al. demonstrated that combining cytokines such as IL-6 or TNFα with NT-proBNP facilitated a more thorough risk stratification in acute HF, whereas no significant enhancement in risk prediction was observed when IL-8 was combined with NT-proBNP.

Despite the promise of multimarker analyses for prognostic assessments in HF, caution is warranted in interpreting results. Firstly, the combined markers must demonstrate enhanced discriminatory power compared to individual markers. Secondly, when markers are combined based on cutoff values (e.g., tertiles of NT-proBNP and IL-6), any observed predictive improvement may arise from the correlation between the weakest and strongest markers, with the combined model often performing worse than one that optimally integrates the strongest marker in a continuous fashion.

6. The Search for New Inflammatory Biomarkers in HF

Many cytokines and ligands discussed in this review exhibit suboptimal analytical characteristics. For instance, low-level ligands such as TNF and IL-6 are challenging to measure accurately due to their significant diurnal and postprandial variability. However, the potential role of IL-6 as a mediator of HF progression, rather than merely a biomarker, remains compelling. While distinguishing between disease markers and mediators is challenging, identifying novel markers remains critical to elucidate disease mechanisms inadequately captured by existing biomarkers. Since most current biomarkers are involved in pathways known to contribute to HF progression, significant advancements in predictive value and novel insights may arise by exploring biomarkers outside established pathological pathways.

While certain cytokine receptors, such as gp130 and OPG, show potential, their utility in prognostication is limited compared to natriuretic peptides and cardiac troponins. This comparison, though clinically logical, might undervalue the mechanistic insights these receptors could provide. Despite their limitations, natriuretic peptides perform well in statistical evaluations, potentially discouraging further investigation into novel markers if statistical evaluations remain the primary criterion. HF, as a multifaceted clinical entity, cannot be fully characterized by a single marker. Therefore, even markers with inferior statistical profiles might be worth exploring if they advance our understanding of HF pathophysiology. Furthermore, as in oncology, not all therapies are suitable for every individual. Cytokine profiling may eventually become a component of personalized HF management, tailoring treatments to specific patient profiles.

Conclusion

Inflammatory biomarkers, while offering valuable insights into the pathophysiology of heart failure (HF), face significant challenges in transitioning from research to routine clinical diagnostics. Current laboratory techniques must contend with variability in sample handling, assay sensitivity, and the biological characteristics of cytokines. Despite these limitations, advancements in multimarker strategies hold promise for enhancing risk stratification and tailoring patient management. Continued exploration of cytokines and their receptors may uncover novel biomarkers that bridge the gap between mechanistic understanding and clinical

applicability, emphasizing the importance of laboratory research in driving diagnostic innovation.

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