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Introduction to the ‘omics
Talking ‘omics

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"I remember seeing an elaborate and complicated washing machine for automobiles that did a beautiful job of washing them. But it could do only that, and everything else that got into its clutches was treated as if it were an automobile to be washed. I suppose it is tempting, if the only tool you have is a hammer, to treat everything as if it were a nail."


The philosophy and psychology that determines our interests and what we do on a daily basis is fascinating, to me at least. As a general (non-interventional) cardiologist with an interest in acute cardiac care I enjoy teasing my interventional colleagues by accusing them of one-dimensional management and belittling their skills with the phrase “to a man with a hammer everything looks like a nail.” However, to confine such criticism to the ocular-stenotic reflex is itself belittling. As Wikipedia attests, the concept of distorting one’s view towards a nail, if holding a hammer, is known as the “Law of the instrument.” The concept is delightfully embodied by the French transmutation of “formation professionnelle” to “déformation professionnelle.” Although perhaps a little unfair, I believe a scientific hypothesis, especially when much has already been invested, can also act like a hammer. You may ask what this has to do with the current issue of our journal.

This issue is devoted to the “omic” discovery technologies. Inherently these technologies do not rely on a hypothesis and therefore provide an unbiased report of variations in gene sequence (genomics), RNA abundance (transcriptomics), protein abundance or modification (proteomics) or metabolite concentration (metabolomics) that are associated with a particular disease (phenotype). As a result, endeavors using these techniques are often likened to “fishing trips” since it is unknown at the start of the journey what will be caught, if anything. This criticism is becoming less valid as sophisticated bioinformatic approaches are used to analyze, organize and visualize the huge amount of data these techniques generate. To extend the fishing trip analogy, increasingly, modern bioinformatic approaches combine with improved machinery and computing power to effectively trawl through the data and capture a more complete shoal rather than an individual fish. Thus, by their nature the ‘omic techniques bear little, if any, resemblance to the man with the hammer.

Nonetheless, it could be argued by confining an investigation of a particular disease to one ‘omic technology, investigators are in effect using a hammer. This is exactly the point made in the Basic Article about ‘omic technologies by Manuel Mayr et al. Using cardiac fibrosis as the example, it is clear the proteome is determined by the transcriptome. However, this relationship need not be the simple linear relationship between mRNA transcript and the protein it encodes. Rather some RNA species can have more wide-ranging effects on multiple mRNAs and therefore proteins, providing an introduction into the exciting and rapidly evolving world of microRNAs. In the past, combining genomics and transcriptomics has enabled novel insights into gene regulation [1]. What Manuel Mayr is proposing is a wider extension of such
technologies to embrace the spectrum from genetic code to metabolic substrate. A generic term often used for such integration of unbiased information at multiple levels is “systems biology.”

The topic of systems biology is further elaborated in the Main Clinical Article by Grainger et al. Here the main focus is on metabolomics, the measurement of small molecules that are products of metabolism. As Grainger points out the advantage of this approach is that it incorporates the influence of environment. In contrast, genetic information is fixed at conception (meiosis). While this is probably true for most diseases relevant to the cardiovascular system, it is not true of diseases where clonal proliferation of cells is important. A nice example of how metabolomics can provide information not directly dependent on genotype is illustrated by the recent high-profile studies on vascular risk prediction. These studies are nicely summarized by Grainger and have concluded that a major proportion of vascular risk is both indicated and determined by circulating choline metabolites that are produced by the bacteria we carry in our colons.

Thankfully, the metabolic imaging article brings us back to the heart! Eykyn et al. provide an introduction to an extraordinarily powerful MRI technique that allows visualization of the fate of a single atom in a cardiac substrate. The technique of dynamic nuclear polarization uses science fiction-like quantum physics to line up spins on asymmetric stable isotopes, in most cases carbon-13. In the case illustrated by Eykyn pyruvate is labeled with $^{13}$C on its first carbon. The spin of this atom is then aligned/polarized at absolute zero before rapid warming and injection in the circulation. The fate of $^{13}$C can then be tracked by its chemical shift according to whether it remains in pyruvate, or the pyruvate flows through competing metabolic pathways to convert its first carbon atom to CO$_2$, HCO$_3^-$, lactate or alanine. The only caveat is that once the pyruvate has been warmed, spin alignment/polarization decays with a half-time of 30s! As is apparent from the article, experiments of this type must be as immensely expensive as they are powerful.

For this issue of *Heart and Metabolism*, we have veered from our usual New Therapeutic Approaches. Instead we have focused on the topic of personalized medicine and how cheap sequencing can provide genetic information that informs the therapeutic approach. Luisa Mestroni provides an extremely thorough review that ties together threads of information from varied cardiac therapies where response is determined by genotype. This article provides a glimpse of the cardiologist of the future where a DNA sequencing machine accompanies the ECG machine, to provide an index of cardiac repolarization and the arrhythmia risk of medication.

Finally, the Case Report and Hot Topics focus on the measurement and prevention of myocardial ischemic injury. At the end of this exciting issue they bring us back down to earth. We have amazingly sensitive ways to measure myocardial infarction. Unfortunately, despite enormous effort and expense, we have not found all the ways to totally control it. Perhaps those hammer-wielding interventional cardiologists found the right nail after all?

Reference

Proteomics and microRNA profiling in cardiac fibrosis

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Abstract

Cardiac fibrosis is characterized by the deposition of extracellular matrix (ECM) and mediated by cardiac fibroblasts via the renin-angiotensin system (RAS) and transforming growth factor beta (TGFβ) signaling pathways. Recognition of TGFβ by fibroblasts leads to myofibroblast differentiation as well as induction of ECM protein expression. An imbalance between pro- and anti-fibrotic signals results in excessive ECM deposition, which is linked to arrhythmogenicity, and defective systolic and diastolic function. Since ECM proteins accumulate over time, the balance of protein synthesis and degradation is particularly important in the context of cardiac fibrosis. Additionally, microRNAs (miRNAs) target messenger RNAs (mRNAs) and control their degradation or translation into proteins, affecting both the dynamics and the composition of cardiac ECM. Proteomics and miRNA profiling are novel research tools for discovering biomarkers and therapeutic targets in disease. Bioinformatic integration of proteomics and miRNA profiling experiments will further our understanding of the balance between repair and pathological processes in cardiac fibrosis.

Keywords: cardiac fibrosis; fibroblasts; proteomics; miRNAs.

Introduction

Cardiac fibrosis is a common complication of cardiovascular diseases, such as cardiomyopathy and myocardial infarction. Histologically, cardiac fibrosis is characterized by extracellular matrix (ECM) deposition by cardiac fibroblasts. Initially, ECM synthesis is part of a repair process. However, an excessive fibrotic response results in perivascular and interstitial ECM deposition, which is linked to arrhythmogenicity, and impairment of both systolic and diastolic function [1]. High-throughput approaches such as proteomics and microRNA (miRNA) profiling can provide an integrated readout that will improve our understanding of the molecular basis of this disease.

Molecular mechanisms in cardiac fibrosis

Following cardiac injury, matrix metalloproteases (MMPs) are secreted by cardiac fibroblasts and by infiltrating inflammatory cells and induce extensive ECM remodeling. After this acute phase, fibroblasts deposit ECM, eventually leading to cardiac fibrosis [2]. The dysregulated response of cardiac fibroblasts is mainly mediated by the renin-angiotensin system (RAS) and...
transforming growth factor beta (TGFβ) signaling pathways (Fig. 1A). The effector molecule of the RAS system is angiotensin II (Ang II), which in turn induces the expression of TGFβ [3]. TGFβ acts in an autocrine and paracrine manner leading to myofibroblast differentiation and induction of ECM protein expression, the hallmark of cardiac fibrosis [3,4].

TGFβ is secreted as a latent, inactive complex consisting of the mature, TGFβ protein, which is non-covalently bound to a dimer of its propeptide, the latency associated peptide (LAP). This complex is associated with certain proteins of the ECM to prevent the activation of downstream pro-fibrotic pathways [5]. For instance, small leucine-rich proteoglycans (SLRPs) (i.e., decorin or biglycan) have TGFβ-binding domains and modulate its distribution and bioavailability [6]. In contrast, other extracellular proteins such as thrombospondins or MMPs are capable of activating latent TGFβ.

**Fig. 1 A**) Regulation of cardiac fibrosis. After its induction by Ang II, TGF activates the expression of a range of ECM proteins (transcriptional activation). ECM proteins regulate TGF bioavailability. MiRNAs play a key role in controlling the translation of mRNA to proteins that are important for cardiac fibrosis (translational repression). **B**) Bioinformatics combined with integrated analysis of mRNA and proteomics profiles will be key for understanding the molecular processes underlying cardiac fibrosis.
of activating TGFβ by cleaving the inactive LAP-TGFβ complex (Fig. 1A) [5].

Both macrophages and fibroblasts produce TGFβ. Upon ligation to its receptors (TGFBRs), TGFβ activates SMADs (mothers against decapentaplegic homolog) and other signaling pathways resulting in the expression of ECM proteins [7], MMPs and TIMPs (tissue inhibitors of metalloproteases) [2]. Besides TGFβ, the master regulator of cardiac fibrosis, molecules such as connective tissue growth factor (CTGF) are induced either by TGFβ/SMAD-dependent mechanisms or stretch-activated signaling [8] and act synergistically with TGFβ [9]. In contrast, various chemokines, cytokines and growth factors contribute to cardiac fibrosis independently of TGFβ, i.e., MCP-1 (monocyte chemoattractant protein 1) [10] or endothelin-1 [11].

Proteomics for an integrated readout of cardiac fibrosis

The proteome represents the entire set of proteins expressed in an organism, tissue, cell or subcellular fraction at a particular time point. Proteomics is an unbiased discovery approach, which is not limited to known molecules of presumed importance but enables the comprehensive assessment of protein expression in tissues and can be applied to clinical samples as well as pre-clinical models of disease. In comparison to transcriptomics (the analysis of messenger RNA - mRNA), proteomics offers certain advantages [12]. While transcript analysis can provide information on cellular activity at the time of harvest, the actual protein content depends on the balance of protein synthesis and degradation. This balance is particularly important when studying the ECM and its associated proteins because of their accumulation over time. Second, proteomics can identify changes that are not detectable at the mRNA level [13] but affect protein function in disease, including post-translational modifications (i.e., phosphorylation, oxidation, etc.) as well as proteolysis. Some proteolytic cleavage products of ECM proteins have known biological effects. Based on observed and predicted protein-protein interactions, co-expression patterns and functional similarities between proteins [14], bioinformatics can aid the identification of potential novel mediators of disease and biomarkers.

MicroRNAs regulate ECM expression

miRNAs have recently emerged as important regulators of disease processes, including cardiac fibrosis. miRNAs comprise a group of small non-coding RNAs that target mRNAs and control their degradation or translation into proteins. miRNAs are encoded by introns and processed by Drosha and Dicer as reviewed elsewhere [15]. To date, almost 1000 different miRNAs have been identified in the human genome [16]. A single miRNA usually targets multiple mRNAs, often within the same biological pathway [17]. For example, miR-29b, miR-30c and miR-21 have been implicated in cardiac fibrosis (Fig. 1A). The role of miR-21, however, is controversial. In the heart, miR-21 is primarily expressed in cardiac fibroblasts and its pharmacological inhibition in adult mice was associated with attenuated cardiac fibrosis [18]. However, no effect was observed in miR-21 deficient mice [19]. In addition, miRNAs regulate glucose and lipid metabolism and insulin sensitivity [20], suggesting a role in cardiac metabolism.

Conclusion

Proteomics and miRNA profiling are novel research tools for discovering biomarkers and therapeutic targets in cardiac fibrosis. Since a single miRNA can regulate hundreds of transcripts, comprehensive proteomics profiling is the method of choice for miRNA target identification. Bioinformatic integration of proteomics and miRNA profiling (Fig. 1B) will further our understanding of the balance between repair and pathological processes in cardiac fibrosis.

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References


Abstract

Improvements over the last decade in treatment of vascular disease, to reduce future myocardial infarction events, have not been matched by improvements in diagnostic technology, to predict who will suffer from such events. As a result, targeting limited healthcare resources so that increasingly effective drugs are given to the people most at risk remains the weak link in clinical cardiovascular care. It is now more than a decade since the promise of metabolic profiling to aid diagnosis of vascular disease was first uncovered, but such techniques have yet to deliver real improvement to the clinical management of heart disease. Here, we review the progress that has been made, and examine what remains to be done before the promise of metabolomics as a clinical diagnostic tool can be fully realized.

Keywords: diagnostics; atherosclerosis; profiling; multivariate statistics.

The diagnostic problem

Coronary heart disease (CHD) remains single biggest cause of morbidity and mortality in the United Kingdom, with over 25,000 premature deaths (defined as death prior to age 75) attributed to it each year [1]. Over the past two decades we have seen considerable improvements in therapeutic options (particularly with the widespread use of cholesterol-lowering drugs of the statin class [2]), with dramatic improvements in outcome now evident in the statistics [2]. However, the maximum benefits of these improved interventions are difficult to achieve in practice because of difficulties in selecting targets for preventative interventions (whether pharmaceutical in nature, or public health interventions such as dietary modification).

Existing diagnostic tests are generally focused on detecting cardiac ischemia (resulting, for example in chest pain, shortness of breath or edema) or coronary artery stenosis, and as a result do not necessarily direct preventative treatments to the majority of individuals who would otherwise go on to suffer a myocardial infarction (MI). Optimizing strategies to make sure that the majority of those who will suffer an MI are receiving the most aggressive treatment (while treating the minimum number of people who would not otherwise suffer an MI) has therefore become a major task facing the healthcare profession, and any steps that improve identification of these individuals will pay a large public health dividend.

The scale of the problem is best illustrated by the diagnostic performance of the gold standard: coronary angiography. Five-year follow-up data from the MaGiCAD cohort [3,4] shows that individuals with angiographically defined CHD have an annual death rate from cardiovascular causes of around 3%. This compares to about 0.3% in the general population of the same age and gender distribution, from whom the MaGiCAD subjects were drawn. At first
sight, a ten-fold enrichment of the at-risk individuals looks impressive, but the problem is that a little less than 0.1% of the relevant population has an angiogram. As a result, less than 1% of the deaths from cardiovascular causes occur in the population selected to undergo angiography. Indeed, the majority of cardiovascular deaths occur among people unaware that they had CHD and who are receiving no treatment for it.

Faced with a problem of this magnitude, it becomes clear that improvements in prognosis will require a step-change in the nature of the tests that are used (Fig. 1). Firstly, discrimination of cases from healthy subjects will need to improve: sensitivities and specificities of 90%, or even 99% are insufficient as part of a population screening paradigm. Secondly, the test needs to be suitable for use on a large fraction of the population. Clearly, angiography would not meet that criterion, since it is both invasive (carrying risk of morbidity and even mortality) and costly. In addition to a dramatic improvement in performance, then, a new test that could significantly address this key problem is also going to have to be non-invasive, high throughput and low cost.

Using profiling assays to meet the challenge

It is inherently unlikely that any single measure (certainly any single molecular measure) can yield the required degree of diagnostic performance. Several solutions to this problem have been adopted. For example, physiological rather than molecular measures effectively integrate multiple pathways into a single measurement. Hence, measuring blood pressure reflects properties of many cell and organ systems, and provides a better indication of future cardiovascular risk than most molecular measures. A similar integration is also achieved with measures such as low-density lipoprotein (LDL)-cholesterol, where assays for classes of lipoprotein particles provide a better snapshot of lipid metabolism than any single lipid component. The limitation is that a single measure, even integrating multiple pathways, has insufficient information density to achieve reliable risk stratification.

An alternative approach is to measure different markers in sequence, using low cost, non-invasive methods as an initial screen followed by more intensive work-up of the selected sub-population. This is at the heart of current clinical strategy for the management of heart disease. Patients are prioritized for more aggressive diagnostic work-up or therapeutic intervention on the basis of "risk factors," such as elevated plasma lipids, the presence of diabetes or hypertension or, more recently, inflammatory markers such as hsCRP. The limitation of the approach, however, is usually the performance of the initial "risk factor" screens—few if any approach even 90% sensitivity and specificity and consequently the majority of CHD events occur outside the "high-risk population" they define.

The solution to both these problems may lie in profiling diagnostics. The principle here is that many measures are made at once, on the whole population, without stratifying on the basis of individual risk factors. This profile is then sufficiently information-dense to allow a clinically useful prediction of future risk of a CHD event [5,6].

There are multiple profiling approaches, each focused on a different type of analyte: genomics (genetic polymorphisms), transcriptomics (mRNA) proteomics (proteins), immunomics (antibodies) and
metabolomics (metabolites). There are advantages and disadvantages to each, but for cardiovascular disease, with defects in lipid metabolism at its core, metabolomics seems particularly promising. Unlike genetic profiling (which can only identify baseline risk, since your genotype is fixed at conception), metabolomics offers the promise of integrating genetic and environmental influences, which are both known to contribute to the development of CHD in almost equal measure.

**Metabolic profiling and CHD**

During the 1990s, Professor Jeremy Nicholson and his team at Imperial College, London, perfected the methodology required to apply nuclear magnetic resonance (NMR) spectroscopy directly to complex biological fluids, in order to obtain a fingerprint of the collection of low-molecular weight metabolites present. This approach exploited the exquisite reproducibility of NMR spectroscopy (with co-efficients of variation between replicate measures as low as 1%), although the lack of sensitivity restricts the method to examining the most abundant metabolites (perhaps as many as 1000 of the most abundant metabolites contribute to the spectra), and considerable additional work is required to assign changes in the spectrum to particular compounds.

Following a demonstration that this technique was able to robustly and completely discriminate between mice of different strains [7], it was applied to blood samples collected from subjects with and without CHD, defined by angiography [8]. The results were very striking: the two groups were completely distinguished on the basis of their NMR-derived metabolic profiles. The metabolite(s) responsible for this discrimination were not uniquely identified, but the key region of the spectrum association with the separation (around chemical shift $\delta^r 3.22$ppm) indicated the involvement of choline-like species [8].

Despite such a positive first study, attempts to exploit these findings have been slow. This, in part, may be explained by a poorly designed study [9] that used samples from different sources combined into a single cohort. As a result, the variability between samples was larger, diluting the signal due to differences in CHD health status. Despite this issue, statistically significant separation of the groups was achieved, and the spectral regions responsible were the same as in the original study. Inexplicably, this was interpreted by the authors as a negative outcome, when (to the extent possible given the limitations inherent in the design) it replicated all the key findings of the original study and additionally demonstrated that the diagnostic power of the NMR-derived metabolite profile, even in this mixed cohort, was greater than any combination of existing risk factors [9].

A final resolution to these opposing conclusions came in 2011, with the publication of a study using a different technique to obtain a metabolic profile. Using chromatography linked to mass spectrometry, Wang et al. [10] conclusively demonstrated that metabolites of choline differ between individuals with CHD and those without, consistent with both the 2002 and 2006 publications [8,9]. Moreover, they went on, in mice, to demonstrate that these metabolic differences are due to the interaction between the host metabolome and gut flora, and that these differences actually contribute to the development of atherosclerotic disease [10].

Separately, a Finnish group published the largest clinical metabolomics study reported to date, with NMR spectroscopy of serum on more than 4,300 subjects [11]. In contrast to the earlier studies, their definition of CHD was based on carotid intima-media thickness in asymptomatic subjects, and therefore examined the metabolic phenotypes associated with sub-clinical atherosclerosis. This study also concluded that the multi-metabolite signature obtained by NMR spectroscopy was strongly associated with vascular disease, and provided a clinically useful extension to conventional risk factor measures in predicting the presence of atheroma.

Indeed, all the studies published to date demonstrate a significant association between the metabolite profile and CHD, irrespective of the methodology used to derive the profile or the phenotypic definition of cardiovascular disease [8–14]. The strength of the association varied, with the quality of the study design a major factor in the outcome.

However, none of these studies have properly quantified the additional diagnostic power of the multi-metabolite profile compared to conventional (and very low-cost) analytes such as HDL-cholesterol, triglycerides and hsCRP combined with demographic information such as gender, age and body mass index, and physiological measures such as hypertension. It will be important to understand the extent to which the apparent improvements in diagnosis stem from the
extra information in the metabolite profile rather than from the improve statistical analysis framework that ‘omics’ studies typically employ compared to conventional risk factor modeling.

Additionally, these studies use phenotypic definitions based on imaging techniques, and are therefore associated with a structural, rather than functional, definition of the disease. Improving clinical management of CHD, however, requires better prediction of events rather than simply less invasive methods to diagnose the presence of atheroma. The presence and severity of vessel stenoses is a poor predictor of long term outcome: by 5 years after angiography the annual risk of a clinical event among those with multiple, severe stenoses is almost back to the risk among individuals with no detectable stenoses [3]. It is completely unknown at present whether the metabolite profile, either alone or in combination with other readily available clinical data, can better predict future risk of a cardiovascular event.

Exploiting metabolic profiling to improve clinical management of CHD

Having burst onto the scene with a powerful demonstration of the association of the metabolic profile with angiographically defined CHD in 2002 [8], it has taken more than a decade for the robust nature of that association to be demonstrated by multiple laboratories in different studies using a range of techniques (both analytical and statistical) [10–14]. The next challenge is to translate this basic science into improved clinical management of CHD.

All the current methods of generating a multi-analyte metabolite profile depend on expensive hardware with limited throughput. They do not (and for the foreseeable future seem unlikely to) meet the criteria set out in Fig. 1. While metabolite profiling may have good diagnostic performance and is non-invasive, it is neither high throughput nor low cost.

Over a longer timescale (perhaps another decade) advances in analytical technology, for example using sensors based on nanotechnology, may allow extensive multi-analyte profiles to be captured quickly and cheaply. In the shorter term, however, it may be possible to exploit the new information by extracting from the multi-analyte profiles just those measurements that contribute the greatest fraction of the diagnostic performance. Conventional clinical chemistry assays for choline metabolites or for triglyceride species selectively associated with atherosclerosis may find utility in a clinical setting.

The key next step, then, will be to test the ability of the most promising new biomarkers to emerge from these metabolomics studies to predict clinical events. The predictive power of both NMR and gas chromatography-mass spectrometry (GC-MS) derived metabolite profiles for events up to five years after testing will be evaluated in the MaGiCAD cohort during 2012, which should provide the first indication of the likely clinical utility of these biomarker signatures. More likely, the best predictions will come by combining the best metabolites with other measures (both conventional risk factors and the winner’s from other ‘omics’ investigations into CHD, such as genomics, transcriptomics and proteomics). As we move into this new phase of pragmatic testing, it will be as important to determine the relative contribution of different analytes to the power to predict clinically relevant events so that available resources can be targeted most efficiently. Simply demonstrating that metabolite profiles, or individual analytes that compose them, are different between those with CHD compared to disease-free controls will no longer be sufficient.

Conclusions

Early, encouraging pilot scale studies suggesting that metabolite profiles differ in those with heart disease [8] have, despite early concerns [9], now been extensively replicated [10–14]. It is now time to take the next step, and address some important questions: how much additional diagnostic information can metabolite profiles contribute compared to existing risk factor modeling? Can metabolite profiles predict clinically relevant events rather than just replicate the diagnostic power of invasive imaging techniques? And does enough predictive power reside in a small enough fraction of all the analytes to allow a cost-effective screening test to be developed, without relying on the impractically expensive technology used to generate multi-analyte metabolic profiles today?

Based on the growing amount of data available, it is tempting to guess that all three questions will be answered in the affirmative. The challenge for the next decade is to deliver that promise into the hands of clinicians as quickly and cost-effectively as possible.
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References

Dynamic Nuclear Polarization and MRI for the study of cardiac metabolism

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Abstract
Dynamic Nuclear Polarization (DNP) is an emerging technique in magnetic resonance imaging (MRI) that is able to enhance or “hyperpolarize” the signal of 13C by many orders of magnitude in a wide range of endogenous metabolites. This enables us to image not only an injected parent molecule such as pyruvate but also its metabolic fate due to enzymatic conversion. The technology is being used for kinetic studies in solutions of viable cells, ex-vivo in perfused organs, in-vivo in preclinical disease models and now with a successful phase I clinical trial there is the potential to translate these technologies into the clinic. DNP has great potential for the study of altered cardiac metabolism under pathophysiological conditions.

Keywords: dynamic nuclear polarization; MRI; cardiac metabolism; 13C.

Introduction
The heart consumes and recycles approximately 6 kg of adenosine triphosphate (ATP) every day, deriving energy for contraction and ionic homeostasis. Most of this extraordinary energy demand is supplied by continuous mitochondrial oxidation of fatty acids and pyruvate derived from glycolysis [1]. It would be highly desirable to be able to non-invasively visualise and quantify these metabolic processes, to provide new insight into how these change in disease. Access to such information would be invaluable for diagnosis, prognosis, and assessment of response to therapy. Cardiovascular magnetic resonance (MR) is a powerful non-invasive technique to assess cardiac morphology and function and to distinguish regions of infarct from viable tissue of myocardium. Magnetic resonance spectroscopy (MRS) employing 31P can be used to measure intracardiac concentrations of ATP and phosphocreatine [2], while 1H can be used to measure triglyceride accumulation in ischemic regions [3]. With higher sensitivity 3-tesla (3T) clinical MR systems becoming more widely available, both 31P and 1H MRS have been extended to detect metabolic changes in patients affected by aortic stenosis, cardiomyopathy, ischemia and diabetes [4–8]. Despite the advances, the limited sensitivity of MRS leads to long scan times, and hence only steady-state metabolite concentrations can be measured.
The recently introduced concept of dissolution dynamic nuclear polarization (DNP) provides a method to enhance the MRS signal and thus the MR sensitivity of biologically important nuclei such as $^{13}$C or $^{15}$N by greater than a factor ten thousand [9]. Using this technique, injectable hyperpolarized molecules can be used to probe metabolism in vivo [10]. For example, pyruvate is situated at a metabolic crossroads between glycolysis and oxidative phosphorylation (Fig. 1), and in combination with DNP, is proving a valuable tool in the study of metabolism in cancer [11] and in cardiovascular disease [12]. Pyruvate metabolism in the heart is largely driven by high activity of the mitochondrial pyruvate dehydrogenase (PDH) complex, under tight phosphorylation-mediated regulation by pyruvate dehydrogenase kinase (PDK) and pyruvate dehydrogenase phosphatase (PDP). Pyruvate has various competing fates, either entering the TCA cycle for catabolism, anaplerotic conversion to oxaloacetate by pyruvate carboxylase, transamination to form alanine, or under ischemic conditions or mitochondrial dysfunction, to the production of lactate by lactate dehydrogenase (LDH). The interplay of fatty acid β oxidation vs pyruvate oxidation is controlled by the Randle cycle, and is sensitive to up- or down-regulation of fatty acid and glucose transport, to changes in PDH activity, the availability of enzyme cofactors such as NADH/NAD+, and endogenous concentrations of metabolic precursors or products. Modulations in glucose metabolism arising from nutritional status, response to insulin in diabetes, lipid overload due to obesity or changes in mitochondrial function due to ischemia lead to significant shifts in metabolism between fatty acid oxidation and anaerobic glycolysis [13]. Conditions such as cardiac hypertrophy lead to metabolic remodeling, upregulated glucose metabolism, increased activity of lactate dehydrogenase (LDH) and excretion of lactate. Unchecked, these metabolic alterations result in exhaustion of intracellular ATP, acidosis, loss of ionic homeostasis, and apoptosis or necrosis [14].

Dynamic nuclear polarization and hyperpolarized MRI

Nuclei with spin-quantum number $I = 1/2$ (e.g. $^1$H and $^{13}$C) possess a magnetic moment that can orient either parallel or antiparallel when placed in an external magnetic field. Hyperpolarization aims to greatly enhance the population difference or polarization and thereby boost MR sensitivity by many orders of magnitude (Fig. 2). The technique utilizes the high polarization of unpaired electrons at very low temperatures. Samples isotopically enriched in $^{13}$C or $^{15}$N are dissolved in a glass forming solvent containing a low-concentration

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**Fig. 1** Schematic diagram of key metabolic reactions in the heart and some of the pathways that can be interrogated using hyperpolarized pyruvate labeled in the C1 position (blue).

**Fig. 2** The sensitivity of MRI is determined by the very small population difference between nuclear spin states when placed in a strong magnetic field $B_0$ (top). This population difference is typically of the order $10^{-6}$ or parts per million making MRI an inherently insensitive technique. Hyperpolarization techniques aim to significantly boost this small thermal population difference by many orders of magnitude (bottom) and thereby dramatically enhance MR sensitivity.
of a free radical. They are then frozen in liquid helium within a superconducting magnet (B₀ = 3.35T) and pumped under vacuum to reach temperatures of the order 1.2-1.4K. Electron polarization is transferred to nuclear polarization under the action of a microwave field, and typically takes of the order of 1-2 hours to polarize a sample. The frozen substrate is rapidly melted and dissolved in a volume of hot physiological buffer to yield a biologically compatible solution retaining its hyperpolarized signal. Fig. 3 shows a typical time-series of 13C spectra acquired for a 50mM solution of hyperpolarized [1-13C] pyruvate, and a single acquisition per spectrum acquired on a 9.4T spectrometer. The signal decay for [1-13C] pyruvate at this field strength, governed by the longitudinal 13C relaxation time (T₁) is ~54s, and is typically in the range 20-60s for most molecules of biological interest. The primary limitation of the technique is the decay of the hyperpolarized signal back to thermal polarization. Nonetheless the time-window is sufficient to carry out real-time measurements following injection of the hyperpolarized solution into biological systems.

**The perfused heart—a pharmacological tool**

Metabolism can be studied in Langendorff perfused hearts with a range of hyperpolarized substrates in real-time, and mathematical models can be developed to estimate enzyme kinetics. Injection of hyperpolarized [1-13C] pyruvate into the perfused rat heart [15-16], figure 4, leads to conversion of pyruvate into lactate, or alanine, or to the production of CO₂ via PDH, which is in turn converted to bicarbonate by carbonic anhydrase. This exchange of bicarbonate and CO₂ is in pH-dependent equilibrium, and their relative ratios can be used to calculate myocardial pH[17]. The presence of the soluble fatty acid octanoate [15] leads to significant reduction in the conversion to bicarbonate and increased conversion to lactate, reflecting the metabolic switch between fatty acid and pyruvate oxidation. Likewise, global ischemia leads to increased lactate production and decreased PDH flux measured with [1-13C] pyruvate [16], to decreased citrate and glutamate measured with [2-13C] pyruvate [18], and to changes in carnitine buffering measured with hyperpolarized [1-13C] acetate and [1-13C] propionate [19], reflecting reduced TCA cycle oxidation and increased anaerobic metabolism.

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**Fig. 3** Time series of hyperpolarized 13C spectra acquired for a 50mM solution of [1-13C] pyruvate acquired with a small flip angle pulse (1deg) and a temporal resolution of Δt = 2s. Top shows the thermal 13C signal acquired for the same sample after the signal has decayed, acquired with co-addition of 32 scans. The difference in peak areas between the first hyperpolarized spectrum and the thermal signal is about 15,000 fold in this example.

**Fig. 4** 13C spectrum acquired following injection of a solution of hyperpolarized [1-13C] pyruvate into a Langendorff perfused rat heart. The injected pyruvate peak is clearly seen as well as a number of metabolites, including CO₂, bicarbonate, lactate and alanine. Temporal variations of each metabolite peak are measured and the time dependences correlate with the respective enzyme activities in the heart. Reprinted from Merritt et al [16] with permission of the publisher. Copyright © John Wiley and Sons.
Cardiac metabolism *in vivo*

Experiments can be extended *in vivo* through intravenous injection of hyperpolarized solutions. The *in vivo* rates of PDH flux in rats measured with hyperpolarized [1-13C] pyruvate, correlate with PDH activity measured by enzymatic assay [20], demonstrating marked reductions in PDH flux following overnight starvation and in streptozotocin (STZ) induced type I diabetic rats [21]. Decreased PDH flux was also observed in response to triiodothyronine (T3) induced hyperthyroidism and hypertrophy, with increased expression of PDK4, decreased PDH flux as well as increased conversion to lactate via LDH [22]. These metabolic changes were renormalized on treatment with the PDK inhibitor dichloroacetate (DCA).

Non-spatially selective spectroscopy is sufficient to estimate global metabolic changes *in vivo*; however, to distinguish regional changes, spatially resolved measurements employing 13C chemical shift imaging are required. The approach was first demonstrated in the pig heart [23] where it was possible to observe clear regional reductions in pyruvate to bicarbonate conversion following 15min or 45min coronary occlusion, and at the later time-point correlated with a region of infarct observed in the late Gad enhanced images. Using newly developed MR sequences and improvements in hardware, imaging gradient and coil design, it is now possible to acquire rapid multi-slice cardiac-gated 13C images in the pig heart using spectral-spatial excitation pulses combined with single-shot spiral readout for rapid whole-heart imaging [24] (Figs. 4, 5).

Clinical translation

The instrumentation required to carry out DNP is largely experimental or home-built apparatus and currently not suitable for clinical use. A phase I first in man study has been carried out in a collaboration between GE Healthcare and UCSF using modified hardware to carry out the DNP process under FDA approval within a clean room and incorporating quality assurance steps to remove the free radical and to verify parameters such as solution temperature and pH. This first trial has been completed in 31 men with confirmed prostate cancer and metabolic imaging achieved using [1-13C] pyruvate [25]. Initial experience is very encouraging for further development of the technique. Commercialization of a DNP polarizer for sterile use intent [26] will enable the technology to be expanded to other centers for further phase I studies and validation of the technology. We await the first demonstration of DNP applied to cardiac studies in humans and the next few years promise an exciting period in the development of these novel technologies.

Conclusion

DNP shows enormous potential for the study of metabolic changes associated with cardiac disease in both the laboratory setting for basic research but also in a clinical setting. Progress over the coming years will be driven on the one hand by technological advances and overcoming the necessary regulatory approvals, but also by the demands or unmet needs of cardiologists and oncologists in the stratification of disease or in the
development of novel therapies. DNP is uniquely placed to interrogate the biochemical changes that influence the balance between glucose or fatty acid oxidation and anaerobic metabolism in the healthy or failing heart.

Acknowledgements
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References
Genomic prediction of individual drug response

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Abstract
A novel medical approach, personalized medicine, seeks to use genetic information to “personalize” and improve diagnosis, prevention and therapy. The personalized management of cardiovascular disease involves a large spectrum of potential applications, from diagnostics of monogenic disorders, to prevention and management strategies based on modifier genes, to pharmacogenomics. Several lines of evidence suggest that common polymorphic variants of modifier genes can influence the response to drug response in cardiovascular disease. Using pharmacogenomics approaches to affect management of heart failure, arrhythmias, dyslipidemia and hypertension, warfarin anticoagulation and anti-platelet therapy appears very promising. In heart failure, common genetic variants of beta-adrenergic receptors, alpha-adrenergic receptors and endothelin receptors among others significantly alter the response to heart failure therapy. This knowledge could be used to personalize and optimize cardiovascular therapy based on the patient’s genetic profile.

While the advances in technologies will continue to transition personalized medicine from the research to the clinical setting, physicians and in particular cardiologists need to reshape clinical diagnostics paradigms, learn how to use new genomic information to change management decisions, and provide the patients with appropriate education and management recommendations.

Keywords: pharmacogenomics; genetic variation; cardiovascular genetics; drug response; heart failure.

Introduction
The International Human Genome Project was completed in 2003 after 13 years of extensive work by a network of laboratories and an approximately $3 billion investment [1]. The sequence of the 3 billion base pairs of the human genome became publicly available: this and the extraordinary change in technology have changed the way we perceive medicine.

Today, with the novel sequencing technologies (next-generation sequencing), the cost of sequencing a human genome is less than $5,000 and can be completed in about a week. Furthermore, it is also possible to sequence only the coding portion of the genome, the exome, for approximately $1,000. It is expected that the combination of improved technology and improved computational methods to handle the huge amount of data generated by the new sequencing platforms will rapidly push the cost of genome sequencing below the $1,000/genome as projected by the National Human Genome Research Institute (The Road
to the $1000 Genome — A Roundup of Sequencing Technology Developments, http://www.genome.gov). Meanwhile, taking advantage of the next-generation sequencing technologies, the “1000 Genomes Project” is currently sequencing the genomes of a 2,500 people of various ethnic origin, to provide a comprehensive resource on human genetic “common” variation [2].

The advances in genomics and high throughput technologies will soon have a profound impact in the management of cardiovascular medicine [3, 4]. Genomics, the science studying the genes of a genome and how they interact with each other, is at the foundation of personalized medicine, a form of medicine that uses the patient’s genomic information to improve diagnosis, prevention and therapy. Out of 3 billion base pair in the human genome, there are likely over 10 million common genetic variations that occur every 100-300 base pair of genomic sequence, and there are at least 100 new polymorphic variations per person [5]. Common variations of the nucleotide sequence are called genetic polymorphisms. Many of these polymorphisms are frequent in the general population and have no clinical effect. However, some genetic variation in genes functionally important account for variations in susceptibility to diseases and different response to drugs (pharmacogenomics) observed in population studies and even within families. Genes that modify the individual response to disease or therapy are called modifier genes.

Pharmacogenomics

More than 100,000 deaths each year are due to adverse drug reactions, and pharmacogenomics may contribute in reducing this number by better tailoring the therapy on the genetic profile of each individual patient [6]. Pharmacogenomics is defined as the study of genes that influence the response to drugs, and has the purpose of maximizing the benefits and minimizing the side effects of therapies based on the individual’s genetic profile.

Genes associated with different drug response have been identified using two approaches. The first one is the candidate gene approach based on the identification of “candidate variants” in pharmacokinetic pathways. The second and most recent approach is based on genome-wide association studies (GWAS). The Catalog of Genome-Wide Associations currently lists 1305 published GWAS at P≤5×10^-8 for 210 traits (accessed 2/2012). In GWAS, the approach is based on the genome-wide screening of hundreds of thousands of selected polymorphic variations (SNPs) rather than candidate genes. Once an association is discovered between a polymorphism and the disease, the modifier gene in proximity to the SNP is identified. This novel approach, developed thanks to the technological advances in high throughput sequencing methods and bioinformatics approaches, is based on the screening of large populations of patients and controls, and has already been successfully utilized in cardiovascular medicine in complex common disorders such as hypertension and coronary artery disease. GWAS studies are demanding in terms of cost, technology and size of the study population, but they are important since it is expected that they will provide innovative personalized genomic information about risk of disease and generate novel therapeutic targets.

Pharmacogenomics of heart failure: a paradigm

Heart failure (HF) is one of the most serious and expensive conditions in health care worldwide due to its high prevalence (1–1.5% of adult population) and high morbidity (frequent hospitalizations). In the United States, HF affects approximately 4 million people and causes about 200,000 deaths per year; and it has a generally rapid course with a mean survival of only 1.7 years for males and 3.2 for females after diagnosis [7]. In Europe, data are substantially similar and together it suggests that in spite of the improvement of HF therapy, disease progression has not changed and HF still remains one of the most important health problems in the world.

HF is a syndrome characterized by primary pathophysiological processes, which interact with a wide number of complex secondary interrelated pathophysiological mechanisms: rare mutations in single Mendelian genes, common genetic variations (polymorphisms) in modifier genes, which can modify the natural history of the disease, such as genes of the renin-angiotensin-aldosterone (RAAS) system and adrenergic system, genetic polymorphisms that can modify the response to therapy (pharmacogenomics), gene-gene interactions, such as β1 and α2 adrenergic
receptors, and environmental factors, such as ischemic heart disease, viral infections, hypertension, infiltrative diseases, toxins, diabetes [8].

Several studies have provided evidence of the existence of modifier genes in HF that can modify the severity and progression of the disease [9–15]. A more comprehensive answer toward the question of identification of modifier genes in HF is expected to come from GWAS, which are currently ongoing, including the Framingham study. Two recent papers have reported a large metanalysis of the risk of heart failure and mortality in the CHARGE Consortium [16, 17]. These studies, which included over 20,000 subjects of various ethnicities, found 2 loci (USP3 and LRG3) associated with risk of developing HF and one locus (CMTM7) associated with the risk of HF mortality. To have clinical impact, these studies should be replicated in independent prospective studies, and the functional significance of the modifier genes identified elucidated.

In HF, pharmacogenomics has already shown a promising role. Indeed, in spite of the improvement in the natural history of HF thanks to the therapeutic advancement in the last 20 years and the development of practice guidelines, large trials such as BEST (Beta Blocker Evaluation Survival) and AHeFT (African American Heart Failure Trial) have suggested that some patients have a different response to treatment (responders versus nonresponders) due to underlying genetic differences [18]. The most important genetic variations associated with a different pharmacological response are listed in Table 1.

In the BEST trial, the anticipated effect of bucindolol, a β-blocker/sympatholytic agent, on patients with HF in class 3 and 4 was disappointing and did not reach statistical significance. However, when the investigators analyzed the response to treatment based on the β1 adrenergic receptor genotype, they found a strong association with the Arg389Gly polymorphism. Arg389Arg homozygous carriers responded significantly better than Gly389 carriers to the treatment with a 38% reduction in mortality. The Arg389Arg carriers’ response was even better than the response previously reported for carvedilol [11]. The different behavior of the two allelic variants is explained by the fact that the Arg389 variant is more responsive to the agonist stimulation (isoproterenol) than the Gly389 allele [11], a behavior confirmed by other studies involving different β-blockers including metoprolol and carvedilol [18].

More recently, the BEST investigators reported the results of a substudy on the pharmacogenetic effect of the α2C-adrenergic receptor, whose role is to inhibit norepinephrine release in the prejunctional adrenergic nerve terminals. The polymorphism α2C Del322-325 had previously been associated with a worse prognosis in HF, with evidence for a synergistic effect with the β1 Arg389 allele in Black patients [13, 19]. In the current study, Bristow et al. showed that the norepinephrine-lowering and clinical therapeutic responses to bucindolol were strongly influenced by the α2C receptor genotype: α2C Del322-325 carriers had an excessive sympatholytic effect and had no evidence of any therapeutic benefit from bucindolol, whereas wild-type α2C carriers had a 30% reduction in mortality [20].

Other polymorphisms, such as the β1 extracellular adrenergic receptor-Ser49Gly and polymorphisms of β2 adrenergic receptor, can modify HF (Table 1) [18]. In the RAAS system, patients with the ACE DD genotype had a worse prognosis but at the same time were the best responders to β blocker therapy compared to the other genotypes (II and ID) [21]. It is interesting to note that the evidence of a genetically driven response to therapy in HF dates back to the AHeFT study. In this trial, the investigators found that African-American patients had a much better response to the therapy with hydralazine and isosorbide dinitrate compared to Caucasian patients. Indeed, this is the first FDA approved therapy for HF based on racial differences and therefore on genetic background [22]. Preliminary data of the GRAHF substudy (Genetic Risk Assessment of Heart Failure in AHeFT) suggests that at least one of the genetic causes lies in the -344C/T polymorphism located in the promoter of the aldosterone synthase gene, and that this is associated with a worse prognosis but with a better response to the hydralazine/isosorbide dinitrate therapy in carriers [23]. The same polymorphisms had previously been associated with higher enzymatic activity, hypertension and myocardial remodeling [18].

The studies on β1 – α2C receptors indicate the existence of complex gene-gene interactions in the genetic determinants of HF. The gene-gene interaction and the functional effect in the case of the adrenergic receptors are of particular interest. The β1 Arg389 receptor is more responsive to the adrenergic stimulation:
<table>
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<tr>
<th>DISEASE</th>
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<th>POLYMORPHISM</th>
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<td>Aldosterone synthase Promoter -344 T/C -344 C: increased transcriptional activity and aldosterone production</td>
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<td>NOS3 Asp298Glu Asp: associated with lower NOS3 activity</td>
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<td></td>
<td>EDN1 IVS-4 G/A Lys198Asn Unknown</td>
<td>Beta-blockers</td>
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**Table 1**: Pharmacogenomics of cardiovascular disease. ACE angiotensin-converting enzyme, RAAS renin angiotensin aldosterone system, NOS3 Endothelial nitric oxide synthase, EDN1 endothelin 1; A II angiotensin II, D deletion, I insertion. From McNamara et al., 2008 [18], and Roden et al., 2011 [4].
patients homozygous for the Arg389 allele carrying also the α2C Del322-325 receptor characterized by decreased uptake of norepinephrine seem to have an enhanced adrenergic response, worst prognosis but the greatest improvement in ejection fraction with β-blocker therapy [13, 19].

Finally, we have studied the association of polymorphisms of the endothelin system with HF in the BEST cohort [15]. Two genetic variations (IVS-4 G/A and Lys198Asn) on a common haplotype in the endothelin-1 gene were associated with differential response to bucindolol in terms of a combined endpoint of HF hospitalization and all cause death (Fig. 1). The effect of the endothelin-1 haplotype was only evident in the treatment group, supporting a pharmacogenetic interaction between bucindolol and the haplotype. Ultimately, these types of data could be used to tailor β-blocker therapy for individuals based on their underlying endothelin-1 haplotype [15].

Pharmacogenomics of lipid disorders

Another important area of research is the pharmacogenomics of HMG-CoA reductase inhibitors (statins). Both in vitro and in vivo (human) studies have shown

![Graphs showing event-free survival](Fig. 1 Time to the combined event of first heart failure hospitalization or death for endothelin-1 polymorphisms Lys198Asn (top) and IVS-4 G/A by genotype. Common homozygotes (G/G-Lys198; and IVS-4 G/G, respectively), heterozygotes (G/T-Lys198Asn; IVS-4 G/A), and rare homozygotes (T/T-Asn198; IVS-4 A/A) are depicted by the red, turquoise and dark blue lines respectively. The data are separated by treatment group, with placebo treated and bucindolol treated subjects on the left and right, respectively. Reproduced with permission from Taylor et al. [15].)
that the HMGCR H7 polymorphism and the LDLR L5 polymorphism are associated with a lower efficacy of the statin therapy in a number of ethnically different populations [4]. Similarly, various polymorphisms of the CYP3A4 have been associated with either lower or higher LDL level with statins (Table 1). Also statin-related myotoxicity has been associated with genetic variations in modifier genes, CYP3A5 and SLCO1B1, initially identified as candidate genes and then, in the case of SLCO1B1, replicated also in GWA studies [4]. Current studies are evaluating the clinical impact of genetic variations in pharmacogenomics of lipid therapy and risk of cardiovascular events.

Pharmacogenomics of arrhythmias
Several drugs can induce arrhythmias by causing a prolongation of the QT interval (drug-induced long QT syndrome): these include antiarrhythmic drugs (sotalol, dofetilide, quinidine), anti-psychotics, antibiotics, and methadone. Subclinical LQT syndrome appears to be the cause of a large proportion of these cases and seems to be associated with genetic variations of LQT genes [4]. Other modifier genes have been found in association with LQT by GWAS approach, such as NOS1AP, implicated in the nitric oxide synthase pathway, although the modifier mechanism is unknown. The identification of patients at risk of potentially life-threatening arrhythmia induced by drugs has significant clinical impact.

Pharmacogenomics of warfarin anticoagulation
A major field of interest has been the study of pharmacogenomics of warfarin therapy. The genes that appear to play the most important role are CYP2C9 and VKORK1. CYP2C9*2 and CYP2C9*3 are associated with lower warfarin dose requirement and increased risk of bleeding. Despite of the several studies on warfarin pharmacogenomics, the clinical utility of a pharmacogenomics approach over anticoagulation control is not established [24]. Large ongoing randomized trials are currently addressing this question.

Pharmacogenomics of antiplatelet therapy
Another field of interest in cardiovascular pharmacogenomics is the dose-response of antiplatelet agents. Clopidogrel shows a wide range of dose-responses, and this variability has been associated with the loss-of-function CYP2C19*2 polymorphism, which causes a lower conversion of clopidogrel (prodrug) into its active metabolite [4]. Carries have an increased risk of cardiovascular events and in-stent restenosis. The Food and Drug Administration has recently approved a warning alerting about these pharmacogenetic findings and the opportunity of alternative therapies in CYP2C19*2 carriers.

Conclusions
A new era in which personalized medicine will enter in our clinical practice and the $1,000 genome will be available is just around the corner [25]. Tailoring therapy based on pharmacogenomics tests may save lives and improve patients’ care. Advances in technologies continue to transition from the research to the clinical setting, reshaping clinical diagnostic paradigms and challenging the healthcare team to consider how new genomic information may be leveraged to influence management decisions and to approach the promise of personalizing medical care. The problem is then for the physician and in particular the cardiologist, to understand and manage this new genomic information and provide the patient with appropriate education and management recommendations. Finally, the possibility to study large cohorts of patients with cardiovascular disease will provide fundamental data for the profiling of risk factors and the optimization of the therapy.

References
Efficacy of trimetazidine: lessons from meta-analyses

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Abstract
Trimetazidine, a metabolic agent with anti-ischemic properties, has been used for the management of angina for more than a decade. The focus of this paper is the efficacy of trimetazidine as unveiled by recent meta-analyses. Six meta-analyses have been published on the effects of trimetazidine in the treatment of heart failure and in the management of stable angina, including nearly 20,000 patients. In patients with heart failure, trimetazidine improves ventricular function, functional capacity and may also reduce mortality. In stable angina, all meta-analyses indicate that trimetazidine improves exercise capacity and reduces angina. The stage is set for the development of large, long-term clinical trials on the effects of trimetazidine on clinical outcomes in patients with heart failure and in patients with stable angina.

Keywords: coronary artery disease; angina; heart failure; left ventricular function; functional capacity.


Introduction
In most countries, stable angina pectoris is currently managed with anti-platelet agents, statins, β-blockers, angiotensin converting enzyme and anti-anginal medications, but a high percentage of patients also receive revascularization procedures, particularly stent implantation [1]. However, recent randomized clinical trials have consistently shown that stent implantation has no significant impact on clinical outcomes for this patient population [2]. Moreover, despite improvement in angina at one-year, stent implantation seems to have no significant effect in the control of angina in the long-term [3]. Therefore, only patients with high-risk profiles may derive benefit from percutaneous or surgical revascularization with reduction of clinical events [4]. Despite these well-established concepts, most patients with stable angina do not receive optimal medical therapy and are referred to revascularization procedures [5].

In patients with stable angina, optimal medical therapy should include medications that may impact in disease progression, such as anti-platelet agents and statins, β-blockers and angiotensin converting enzyme as well as agents that are used for the control of angina. Very few clinical trials have been conducted to evaluate the efficacy of anti-anginal agents with enough power to evaluate clinical outcomes in patients with chronic coronary artery disease without a recent myocardial infarction [6,7]. Therefore, current guidelines for the management of these patients are mostly based on meta-analysis of small, randomized
trials in which the main outcome is angina, evaluated by exercise testing, the frequency of angina attacks or the use of short-acting nitrates.

Trimetazidine, a metabolic agent with anti-ischemic properties, has been used for the management of angina for more than a decade. Several clinical trials have demonstrated its efficacy as monotherapy as well as in combination with one or even more than one anti-anginal agent [8]. Recently, several systematic reviews and meta-analysis have summarized the efficacy of trimetazidine in patients with coronary artery disease [9–14]. The efficacy of trimetazidine on quality of life has been reviewed by Marazzi et al [15] and the focus of this paper will be the efficacy of trimetazidine as unveiled by recent meta-analyses.

**Meta-analyses of trimetazidine in chronic heart failure**

Since patients with coronary artery disease associated with heart failure have a high incidence of clinical events, the two meta-analyses recently published were able to evaluate the effects of trimetazidine on functional variables as well as in clinical outcomes [11,14].

Gao et al. [11] reviewed 17 trials involving 955 patients and were able to demonstrate improvement in left ventricular dimensions, left ventricular ejection fraction, and functional capacity with the administration of trimetazidine. The mean improvement in left ventricular ejection fraction of 7.5 % (95% CI 6.3 to 8.7; p<0.01) is clinically relevant and favorably compares with the effects of beta-blockers. Interestingly, ejection fraction was found to improve not only in patients with ischemic etiology, but also in patients with non-ischemic etiology, raising the hypothesis that trimetazidine might exert its effects independently of the improvement of myocardial ischemia. Mean improvement in functional class was –0.41 in New York Functional Class (CI –0.51 to –0.31, p<0.01), which also compares favorably with the improvement obtained by other medications, such as angiotensin converting inhibitors. Finally, the meta-analysis showed significant reductions in mortality (RR 0.29; 95% CI 0.17 to 0.49; p<0.01) and hospitalizations (RR 0.42; 95% CI 0.30 to 0.58, p<0.01), the most important outcomes in heart failure. The favorable effects of trimetazidine on left ventricular function was recently confirmed in the meta-analysis by Hu et al. [13], who evaluated 11 trials with 545 patients to compare trimetazidine with placebo, using as outcomes functional variables obtained by echocardiography or radionuclide angiography. In this later study, which did not select patients with the diagnosis of heart failure, there was improvement in ventricular volumes and ejection fraction, but trimetazidine also improved wall motion score index.

The meta-analysis by Zhang et al [14], which included 16 trials with 884 patients, presented results similar to those obtained by Gao et al [11], confirming the significant reduction in hospitalization for cardiac causes (RR: 0.43, p=0.03), but not the reduction in mortality. Moreover, New York Heart Association functional class and total exercise time on exercise testing, as well as resting left ventricular ejection fraction (mean change 6.46%, p<0.0001) were improved by trimetazidine. This meta-analysis also evaluated the effect of trimetazidine on B type natriuretic peptide levels, showing a mean reduction of 203 pg/ml (p<0.01).

**Meta-analyses of trimetazidine in stable angina**

Patients with chronic stable angina have a good prognosis and, therefore, there is little evidence that any anti-anginal intervention may alter outcome. For instance, the ACTION trial failed to show improvement in survival of patients with angina treated with long-acting nifedipine [6]. Likewise, a recent meta-analysis of 26 trials, including 6,108 patients, showed no significant survival benefits of beta-blockers (OR 0.92, 95% CI 0.62 to 1.38) when compared to placebo in patients with stable angina [16]. One study in which an anti-anginal agent was shown to reduce events was the BEAUTIFUL trial [7], which evaluated the effects of ivabradine, a heart rate reducing agent, in patients with coronary artery disease and left ventricular systolic dysfunction. However only patients with a resting heart rate higher than 70 bpm presented a significant reduction in admission to hospital for fatal and non-fatal myocardial infarction (0.64, 95% CI 0.49-0.84, p=0.001) and coronary revascularization (0.70, 95% CI 0.52-0.93, p=0.016). Therefore, the three meta-analysis available on the effects of trimetazidine in stable angina have evaluated as outcomes stress-induced myocardial ischemia, number of weekly angina attacks, and use of short-acting nitrates.

In 2003, Marzilli and Klein [9] published the first meta-analysis of 12 trials, including 868 patients. In this early meta-analysis, trimetazidine was found to
increase exercise duration to 1 mm ST segment depression on the exercise test, and to reduce weekly episodes of angina, both as monotherapy and as add-on therapy. Later, Ciapponi et al. [10] conducted a meta-analysis of 23 trials with 1,378 patients with stable angina. Compared with placebo, trimetazidine reduced the number of weekly angina attacks (mean difference -1.44, 95% CI -2.10 to -0.79; p<0.0001), reduced weekly nitroglycerin tablet consumption (95% CI -1.47 to -2.20, -0.73; p<0.0001) and improved exercise time to 1 mm segment depression on the exercise test (p=0.0002). At that time, only 4 small trials were available in which trimetazidine was compared to other anti-anginal agents and confidence intervals were too large for appropriate interpretation.

Since current guidelines recommend beta-blockers as first choice for the treatment of stable angina [17] and ivabradine has a clinical-trial-based indication [7], it is important to compare the effects oftrimetazidine with other anti-anginal drugs which do not act by reducing heart rate. This may be particularly useful when one is considering the addition of a second drug for those patients already taking beta-blockers, or for those who do not tolerate beta-blockers. In the largest meta-analysis conducted on the effects of trimetazidine, Danchin et al. [12] evaluated 218 trials with a total 19,028 patients, including the results a previously unpublished study, the VASCO trial. In agreement with the meta-analysis of Ciapponi et al. [10], trimetazidine improved exercise tolerance, weekly angina episodes, and use of short-acting nitrates when compared with placebo. For the comparison of other anti-anginal agents without an effect on heart rate, network meta-analyses were performed. This strategy allows for the evaluation of direct comparisons (A vs. B from head-to-head comparisons) and indirect comparisons (A vs. C is extrapolated from A vs. B and B vs. C), giving information about the relative efficacy of treatments that have not been compared in head-to-head trials. In these analyses, trimetazidine when compared to dihydropyridines, long-acting nitrates, nicorandil, and ranolazine, both as monotherapy and as add-on therapy, had similar anti-ischemic effects. The findings of this robust meta-analysis support the indication of trimetazidine as an effective agent for the management of stable angina.

One specific point should be mentioned on these data. Among the numerous studies included in this network meta-analysis, the VASCO trial [12] is the largest randomized study conducted with trimetazidine. In this trial, patients with stable angina receiving 50 mg q.d. of atenolol were randomized to the addition of trimetazidine MR 35 mg b.i.d., or trimetazidine MR 70 mg b.i.d., or placebo b.i.d. for a 12 week-period. In the global population of VASCO, there was no significant difference between treatment with trimetazidine and placebo on exercise test and clinical parameters. These data, which are not consistent with results reported in all other trials, were attributed to the fact that most of the patients in the VASCO trial had only mild angina, which was treated adequately by monotherapy with atenolol. This was confirmed when complementary analyses were performed in the more symptomatic patients of the VASCO trial, and showed that differences with placebo became statistically significant. Thus, these results suggest that the population of that study did not adequately represent the target population for trimetazidine. The large number of patients included in the VASCO trial confirmed the safety profile of trimetazidine MR. Indeed, there were no significant differences in adverse effects among the three groups. However, the short duration of the study (12 weeks) did not allow for the evaluation of the incidence of, for instance, extrapyramidal disorders, which have been recently associated with long-term use of trimetazidine [18].

Conclusion
Over the last decade six meta-analyses have been published on the effects of trimetazidine in the treatment of heart failure and in the management of stable angina, in total including almost 20,000 patients. In patients with heart failure, trimetazidine improves ventricular function, functional capacity, and may also reduce mortality. In stable angina, all meta-analyses indicate that trimetazidine improves exercise capacity and reduces angina. The stage is set for the development of large, long-term clinical trials on the effects of trimetazidine on clinical outcomes in patients with heart failure and in patients with stable angina.

References
Assay what? The troponin masqueraders—a case report

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Abstract
Guidelines defining the universal diagnosis of myocardial infarction recommend the rise and/or fall of a cardiac biomarker, preferably troponin, above the 99th percentile of a healthy population in patients presenting to hospital in conjunction with chest pain and/or electrocardiographic changes. This report considers the case of a 44-year-old woman with ongoing chest pain and chronic troponin elevation. Highlighting the importance of a delta change of troponin, with a rise and fall in blood level, in conjunction with clinical symptoms; without which interpretation of laboratory tests in the assessment of patients presenting with possible cardiac ischemic injury can be fraught with uncertainty. We describe sources of potential assay interference, as demonstrated in this case, and how such cases can be approached.

Keywords: acute myocardial infarction; cardiac troponin; assay interference; heterophile antibodies; delta change.

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Case report
A 44-year-old female with a history of ongoing chest pain and cardiac troponin T (cTnT) elevation presented to the outpatient department of St Thomas’ hospital, London, United Kingdom. She had a past medical history of antiphospholipid syndrome, but no other risk factors for coronary disease. Her medication regime was Warfarin, Plaquenil and Mycophenolate Mofetil. Her presenting complaint was that of ongoing atypical chest pains, occurring intermittently, not always associated with exertion. Her index admission was to another local hospital three years earlier, after complaining of left sided chest and arm pain. A laboratory measured cTnT was elevated at 0.5ng/ml (cut-off for diagnosis of myocardial infarction (MI) >0.03ng/ml, cTnT Elecsys E170, Roche). Interestingly, an ECG showed normal sinus rhythm at 85 beats per minute with T wave inversion in the chest leads V4-V6, with no ST-segment abnormalities; with these changes appearing static. A chest x-ray showed a normal cardiothoracic ratio with clear lung fields. Other blood tests performed included a full blood count, renal and liver function, glucose, CK, CK-MB and myoglobin, which were all within normal limits.

In view of the presenting symptoms and elevated cTnT treatment for a possible Acute Coronary Syndrome (ACS) was commenced, and a diagnostic coronary angiogram was subsequently performed. A delta change of cTnT was not considered as the initial result was high above the threshold for diagnosis of MI. Coronary angiography revealed a possible ostial
circumflex artery lesion of 50% diameter stenosis, but otherwise angiographically normal epicardial coronary arteries. However, the cardiologist performing the catheterization procedure noted severe chest pain upon each injection of intra-coronary contrast. The patient was transferred to the local interventional center for consideration of percutaneous coronary intervention (PCI) to the circumflex artery stenosis. Repeat angiography at the tertiary center, including intravascular ultrasound (IVUS) assessment of the ostial circumflex lesion, revealed normal mean luminal areas with no suggestion of a significant flow limiting stenosis, in this, or any of the other, coronary vessels. Therefore revascularization with PCI was not performed. Despite the reassuring nature of the angiogram, the patient continued to be troubled with chest pain, and repeated cTnT measurements continued to be elevated. Thus an echocardiogram and CT-pulmonary angiogram (CTPA) were performed to further investigate potential alternative causes of her symptoms. The echocardiogram, however, revealed normal left and right ventricular size and function and normal valve function and the CTPA excluded the possibility of a pulmonary embolus. The patient was discharged on aspirin, ramipril long-acting diltiazem and a GTN spray in addition to her prior medications. The cTnT concentrations were 0.5ng/ml at presentation and remained elevated, peaking at 0.9ng/ml (Fig. 1).

Unfortunately the patient continued to experience chest pains and returned again to the Emergency Department (ED) with left sided arm and chest pain, with her accompanying ECG being identical to her previous admission. A cTnT 12-hours after symptom onset was elevated at 0.7ng/ml and therefore a further coronary angiogram was performed. This did not demonstrate any new lesions and the suspicion of assay interference was raised. However, no confirmatory tests on the serum were performed and thus a diagnosis was not established for the patient.

Following further specialist reviews she presented to the outpatient department here with a cTnT measured on the high-sensitivity Roche assay of 52ng/ml (Roche Elecsys, high-sensitivity cTnT, 99th percentile 14ng/ml). Examination was normal and her ECG remained unchanged. A cardiac MRI showed no evidence of wall motion abnormality, myocardial scarring or fibrosis. Other bloods remained normal, but an NT-pro BNP was mildly elevated at 422ng/L (cut-off for interval outpatient echocardiogram is 400). As a first line of testing, serial dilutions of the samples were made which demonstrated a non-linear response. Furthermore, samples were incubated with blocking reagents (HBR) containing a mixture of animal immunoglobulins (Igs) and the concentrations of cTnT were normal after this treatment. This further emphasizes the likely presence of interfering heterophile antibodies (HAS). Also, samples were tested for cTnl and CK-MB, which did not show elevated concentrations. The patient had a chronic autoimmune condition and rheumatoid factors (RhF) were present in her blood. This finding further supported the presence of interfering antibodies in the serum. However, RhF antisera was not used in this case.

Comment

The cardiac troponins (cTnI), I (cTnI) and T (cTnT), by virtue of their biologically mediated high cardiac specificity have become the preferred biomarkers for myocardial cell death. They are incorporated into the 2007 “Universal definition of Myocardial Infarction” where a rise and/or fall above a threshold is part of the diagnosis of MI [1]. However, the clinical context is paramount to interpretation, a fact stressed in the Universal definition of MI, but often forgotten in the vim of the ED environment. Clinical evidence of myocardial ischemia is necessary because serum cTn elevations are not necessarily due to an ACS – cTn does not convey etiology [2]. Among patients with a high pretest probability of athero-thrombotic coronary heart disease (CHD), the diagnostic and prognostic value of cTn is clear [3, 4]. On the contrary, in patients with a low pretest probability of CHD, cTn elevations can be nonspecific and may divert attention from the true underlying clinical problem.
This leads to unnecessary cardiac evaluation, invasive testing and inappropriate medication. cTn elevation, but absence of significant coronary disease on coronary angiography may be termed a false positive [5]. Several differential diagnoses must be entertained in such instances [6]. Among this list of differentials are analytical issues pertaining to the specific assay being used to measure cTn concentration: interference can produce a false positive result [Table 1]. Such cases have been reported previously [7] and there are certain recommendations for laboratory staff to follow in order to confirm such false positive results [8]. Awareness of this possibility may assist the physician in the management of the patient and may spare the patient additional, potentially invasive, diagnostic procedures. It is also noted that these concentrations often are at the limits of analytic sensitivity where their presence can blur the separation between clinically important and interference.

Interference can result from a variety of sources but HAs are one common source [9, 10]. The incidence of interference from HAs alters depending on assay and study group varying from 0.2% to 40% [10, 11] and the magnitude of the interference varies from sample to sample, as well as within a patient over time. HAs are endogenous antibodies that cross-react with immunoglobulins of different species causing cross-linking of capture and detection antibodies in immunoassays (Fig. 2a, 2b). Their main interfering effects are seen in two-site immunometric assays. This cross-linking by the HAs can thus lead to positive or negative interference with cTn measurement [12]. Mouse monoclonal antibodies are often used in these assays (to aid specificity) and development of human anti-mouse antibodies (HAMAs) can follow, for instance, the use of murine monoclonal antibodies for therapeutic or imaging purposes [13]. A variety of methods from transfusions, vaccinations or autoimmune diseases can induce broadly reactive HAs that bind Igs from other species [7]. These antibodies are multispecific antibodies, with weak affinity usually of IgG class directed toward the Fc portion of the antigen.

The pertinent element of this case is the lack of rise and fall in the cTn concentrations that would be characteristic (and required) for MI [14] but given the initial high level treatment was commenced on the first positive sample, as it should be according to guidelines. This case also describes a patient with co-existent antiphospholipid syndrome (APS): which typically represents a syndrome of recurrent venous or arterial thrombosis and/or fetal losses in which persistently elevated concentrations of antibodies directed against membrane phospholipids are noted [15]. APS may

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**Table 1** Causes of assay interference (potential false positive results).

| Human anti-mouse antibodies (HAMAs) |
| Heterophile antibodies |
| Autoantibodies |
| Rheumatoid factor |
| Haemolysed samples |
| Clots |
| Macrocomplexes of troponin with IgG |

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**Fig. 2 A**: Usual method of binding with analyte across capture and detection antibodies to generate a fluorescent or chemiluminescent signal for detection and **B**: Interference from a heterophile antibody or HAMA to cross-link the sandwich antibodies and cause signal generation even in the absence of analyte (adapted with permission from Scantibodies, USA).
continue to be important in accurately aligning the clinical and electrocardiographic markers of ischemia, con-
diagnostic modalities, including a careful clinical history of ACS in contemporary clinical practice, other although the assays are an integral part in the diagno-
sitive cutoff for MI but none had indication of MI on invasive tests. By using polyclonal anti-
sera against RhF, cTnI concentrations were normal-
ized, interestingly, none of these specimens had detectable cTnT.

Conclusion
This case highlights the importance of communicating with our clinical chemistry colleagues. If suspicion of interference is raised it may be necessary to repeat the test, dilute the sample to check linearity of results or re-assay on another manufacturers platform. The use of blocking antibodies to pre-treat the sample (Heterophile blocking reagents, HBRs, Scantibodies, California, USA) can also be tried. When HBR binds to the human heterophilic antibody, the blocking may be accomplished by steric hindrance. Parallel analysis of CK-MB to observe a rise and fall may also help establish a cause. The laboratory may need hetero-
phile blocking tubes, protein A columns or be able to send to another lab to measure cTnT or I as appropri-
ate [18]. Many of the modern assays will now incorpo-
rate steps or reagents to minimize the cross-reactivity and interference but they are not always infallible and it remains the clinicians’ responsibility to alert the labora-
tory to any potential discrepancy between the assay measurement and the clinical picture. However, when clinical decisions are sometimes forced to be made at the technical limits of assays misinterpretation is inevitable and may well increase in the era of high-
sensitivity assays. Therefore, as in this case, a false-
positive cTn result should serve as a reminder that, although the assays are an integral part in the diagno-
sis of ACS in contemporary clinical practice, other diagnostic modalities, including a careful clinical history and electrocardiographic markers of ischemia, con-
tinue to be important in accurately aligning the clinical scenario with elevated cTn results in confirming the diagnosis of an ACS.

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Fundamentals of genome-wide association studies

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Abstract
During the past few years genome-wide association (GWA) studies have identified a large number of loci across a wide spectrum of traits. These findings provide valuable information of the genetic architecture of the complex trait in general. The aim of this review is to provide an overview of the new challenges and the approaches used in the GWA studies.

Keywords: genome-wide association; complex disease; single nucleotide polymorphisms (SNP); linkage disequilibrium.

Before 2007, two main strategies were used by geneticists hunting complex disease genes: association analysis of candidate genes and positional cloning based on linkage mapping.

In the candidate gene approach, the targets were selected based on their function or their potential involvement in the disease. For genome-wide linkage mapping, extended or nuclear pedigrees were analyzed using 300/400 markers evenly distributed (at ~10cM intervals) across the whole genome. The result of a genome-wide linkage study was the identification of one locus, which was the first step towards the identification of the disease gene. This assumption-free approach had proved to be very successful in the localization of Mendelian disease genes, but was not as effective in the hunt for complex disease genes.

In 2007, a series of papers [1–8] opened the era of genome-wide association studies which was later described as “the genomic gold rush” [9].

Two fundamental breakthroughs paved the way for this “revolution.” First, the international HapMap project [10] achieved the characterization of rare and common single nucleotide polymorphisms (SNPs), in a number of small datasets originating from different ethnic backgrounds. This task was undertaken with a view to elucidate patterns of linkage disequilibrium (LD) (a genetic phenomenon defined as the non-random association of alleles at different loci) conservation in different populations. From the first haplotype map release [10], it became evident that genotyping 250,000 carefully selected “tagging” SNPs (tag SNPs) would have captured the vast majority of the common variation (~7 million of SNPs) in the European and Asian populations. Indeed, based on the local pattern of LD each tag SNP predicted the genotype of closely linked SNPs. Second, this knowledge of LD patterns was quickly exploited to design genotyping chips containing a relatively small number of tag SNPs, which would...
capture a substantial proportion of common variation in genome sequence. Affymetrix (Affymetrix, Inc., Santa Clara, CA) and Illumina (Illumina Inc, San Diego, CA), the two competing companies providing the arrays, based their products on the same basic technology allowing the simultaneous genotyping of hundreds of thousands (millions, in the latest versions) SNPs.

With this new powerful tool in their hands, geneticists faced new challenges. The first problem was to select an appropriate sample size. The variants associated with complex traits are likely to have a modest effect [11]. Moreover, because GWA scans generate a large number of tests, the p-values must be corrected to reflect multiple hypothesis testing. The threshold for a statistically significant GWA p-value was set at 5x10^{-8} (0.05 divided by one million hypothetical tests) [12]. For these reasons it was important to analyze a large sample set. At the same time, although the price per genotype was very low (~0.50£ per genotype), the final cost per individual was still quite high (each chip generates hundred of thousand genotypes per individual). The result was a huge pressure to genotype the minimum number samples without losing power to detect the association. But power was not the only issue. The second problem faced by the analysts was to minimize the risk of reporting false positive associations. There are three main sources of false positives: 1) the enormous number of test; 2) population biases; 3) technical artifacts. The solution to both problems (sample size and false positive reports) was to use a multistage approach [13]. In the first stage, a relatively small number of samples were included in a GWA analysis (the first GWA studies included ~2,000 cases and 2,000 controls). All the SNPs showing a suggestive degree of association (using a liberal p-value threshold of p<10^{-6}) were re-tested in a second and, eventually, a third stage. Because those SNPs may be false positive, these validation steps were performed in independently ascertained, larger (or at least equivalent) datasets, using different genotyping techniques and more stringent p-value thresholds. In this way both statistical and technical issues were addressed.

Although different approaches have been used to bypass the cost problem (for example DNA pooling [14]), the multistage strategy was the most used in the first wave of GWA studies.

The first wave of GWA studies also highlighted a number of quality controls (QC) that should be performed throughout the analysis in order to obtain robust association results. For example, taking in account the scale of the data that was generate, the genotype calling was generated automatically. For this reason, the original raw data would be re-examined by visual inspection, for all SNPs showing suggestive evidence for association [6]. A number of tools were used to help the QC processes. The quantile-quantile (Q-Q) plot, for example, provides a good visual summary of the distribution of the observed statistical tests generated by a GWA. The inspection of the Q-Q plot provides information about population stratification or excess of strong associations (Fig. 1). A number of additional exclusion criteria were also applied, both at the sample and the SNP levels. For instance, the quality of the DNA sample would be assessed by examining the following parameters: overall SNP call rate (a low rate could indicate poor DNA quality), heterozygosity and gender check (an excess of heterozygous SNP calls or a discrepancy between the reported sex and the one revealed by genotyping are indicators of DNA contamination). Two major QCs were applied at the SNP level: Call rate (a low rate for a specific marker could be evidence of a technical problem) and departure from Hardy-Weinberg equilibrium (HWE, a formula that allows prediction of genotype frequencies, based on a marker allele frequencies) (deviations from HWE, in particular in the controls, may highlight a number of problems, from DNA contamination to inaccurate genotyping).

Finally, the Manhattan plot (Fig. 2), which takes its name from the similarity with the Manhattan skyscraper skyline, was developed and used as standard tool to display the whole GWA result in a single graphic.

The first wave of GWA studies was very successful, but only detected the “low hanging fruits”, i.e., the loci with higher effect sizes. In order to extract the maximum information from these expensive studies, most competitor groups joined their efforts in large GWA meta-analyses (in which different GWA results were pooled together) with the intent to detect those loci with lower effects, which necessitate larger datasets.

The problem was that the Affymetrix and Illumina genotyping chips did not include the same SNPs. While Illumina based the SNP selection almost entirely on tagSNPs identified by the HapMap project,
Fig. 1 Quantile-quantile (Q-Q) plot provides a graphical representation of the distribution of the p-values of GWA indicating whether the study has generated more significant results than expected by chance. The red dots represent the –log10 of the observed P values. The black line shows the expected distribution of the P values (–log10). In panel A the Q-Q plot generated by the raw data (with no quality control applied) of the CAD/MI GWA [6]. The analysis of this plot indicates a clear excess of strong associations due to a number of factors (i.e., low SNP call rate, departure from Hardy-Weinberg equilibrium). In panel B, the Q-Q plot of the same GWA results obtained after applying a standard set of quality controls. In this case there is a minimal inflation of the distribution of the observed p values towards the end of the graphic, indicating the presence of a number of positive findings in a clean dataset.

Fig. 2 The Manhattan plot of the CAD GWA results [6] (after QC). The plot displays the –log p values (y-axis) of the analyzed SNP across the genome (x-axis, chromosomes are represented in different colors). The plot show the genome wide significant (the red line represents the threshold for a statistically significant GWA p-value) SNPs associated with CAD/MI at chromosome 9p21.3 locus.
Affymetrix included only SNPs located in a subset of genomic regions showing specific sequence features, which they claimed to cover the majority of the common haplotypes in Caucasian populations [9].

GWA results generated with different chips, therefore, could not be directly compared or meta-analyzed. This issue was resolved, once again, using information from the HapMap project. The HapMap haplotype maps were used as reference panel to predict (impute) non-genotyped SNPs based on the local patterns of LD with the genotyped SNP [15]. Using this approach, not only was it possible to analyze new untyped markers, but also to combine data from genome-wide scans that used different SNP sets.

In the last five years this approach has led to the discovery of an enormous number of robust associations.

Since 2007, when three seminal papers reported the identification of a chromosome 9p21.3 locus [4–6], thirty loci have been associated with coronary artery disease (CAD) and/or myocardial infarction (MI) [16]. Since the first report, the chromosome 9p21.3 locus has been confirmed several times, in a variety of ethnic groups [16]. The 9p21.3 locus was also associated with a number of related disease phenotypes, including type 2 diabetes, ischemic stroke and aortic aneurysm [17].

Although GWA studies led to a boost of risk genes discoveries, in some cases, they highlighted genomic regions with no annotated genes (gene desert), as in the case of 9p21.3 locus [4–6].

These results might seem clinically irrelevant, but encouraged a number of further studies that led to the discovery of novel regulatory mechanisms and pathways.

SNPs discovered by GWAS studies typically account only for a small fraction of the genetic component of complex traits. So far, a number of explanations have been proposed to account for the phenomenon known as the “missing heritability” [18]. They include: the possibility that heritability of certain conditions may have been over-estimated [18,19]; the possibility that the variance explained by associated SNPs may have been under-estimated [18,20]; the effect of rare low frequency variants and structural variation such as copy number variants (CNV); the occurrence of gene-gene interaction and epigenetic modifications [18].

Conclusion
The solution to this “mystery” will be the next challenge for the geneticists. Some of the answers will be provided by the new technological “revolution” of next generation sequencing. With this technique, that allows the rapid sequencing of whole human genomes at relatively low costs, it will be possible to generate a comprehensive map of novel rare/low-frequency variants and CNVs which may explain part of the “missing heritability.”

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Reference


The struggles of translational medicine in ischemia-reperfusion injury

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The almost 50 years of research on ischemia-reperfusion (IR) injury have been rewarded by the discovery of numerous pathways that have permitted a better understanding of the phenomenon. In fact, it is now universally accepted that IR is the result of precisely orchestrated biological events, ultimately resulting in additional harm to the ischemic myocardium. However, as is the case in other research areas, modulation of the individual “player” often does not result in expected benefits. Given the enormous research efforts in this area, such an aspect is becoming increasingly worrisome and deserves some critical appraisal.

When findings are transferred from bench to bedside there are 3 main levels where failure may take potential origin: a) applying molecular findings to biological systems, b) applying animal model findings to humans, and c) applying the findings in the wrong timing.

Applying molecular findings to biological systems
Intracellular transduction pathways form a fit net of network that may interact at different levels so that, modulation of a single pathway can be compensated to a variable degree by activation of alternative pathways. Alternatively, some signal transduction systems may share common initial pathways that do not necessarily result in the same final target.

For example, intracellular calcium overload, is a key intracellular event for IR injury [1]. Inhibition of L-type calcium channels [2], which regulate intracellular accumulation of calcium, has proven to benefit recovery from IR injury. However, not all L-type calcium channels blockers exert beneficial effects on infarct size. In fact, while verapamil has extensively been shown to reduce infarct size [2], nicardipine, another calcium antagonist that similar to verapamil inhibits L-type calcium channels, has not been shown to induce similar benefits [3].

Applying animal-model findings to humans
There are several factors to be considered when experimental findings are transferred from experimental models to humans. First of all, the infarction model in experimental settings, constituted from artery ligation, is profoundly different from the “naturally occurring” myocardial infarction (MI). In fact, the latter occurs secondary to thrombotic occlusion and encompasses the three components of Virchow’s model for thrombosis (vascular, hemodynamic and coagulatory), which themselves may alter the response to treatment. The “naturally occurring” MI can also be preceded by angina, a recognized natural preconditioning mechanism, thereby biasing the results of a given treatment among patients with or without pre-infarct angina. In addition, pre-infarct events can be important stimuli for development/activation of collateral vessels in human models. On the other hand, collateralization in animal models is significantly different (i.e., pig models are known to not develop collateral vessels) and can subsequently affect the results among different species.
Importantly, patients experiencing MI, may also be carriers of cardiomyopathies (i.e., myocardial hypertrophy) or other comorbidities (diabetes, hypertension, etc.) that can negatively affect the expected benefit of a given treatment. This is obviously not the case in animal models, where one is able to control for all these factors and produce a “purely experimental” MI.

Pharmacodynamic and pharmacokinetic differences between humans and other species should also be considered. For example, different pharmacodynamic and pharmacokinetic properties among different species have been described for cyclosporine, and are considered to be the basis for the inconsistent results among clinical trials [4] and animal models [5].

Outcome measures may as well significantly differ among different settings. In fact, human studies have measured outcomes based principally on the rise in myocardial enzymes [6]. On the other hand, experimental models have traditionally used histopathological analysis as outcome measure.

Another practical issue is that, while easily performed in experimental models, delivery of cardio-protective agents before MI onset is difficult to achieve in clinical practice.

Applying the findings in the wrong timing

Following an acute MI, early and successful flow restoration is the most effective strategy to reduce infarct size and improve outcome [7]. Importantly, while progressively decreasing in magnitude thereafter, the largest achievable benefit is concentrated in the first 2–3 h after symptom onset [8].

Reperfusion injury is closely related to the preceding ischemic injury. In particular, the two phenomena display a peculiar time-dependent relationship. Myocytes experiencing an ischemic time that is long enough to cause irreversible damage will not benefit from restoration of blood flow, and hence, protection from IR injury. On the other hand, reperfusion therapy may benefit the myocardium presenting in the time window in which cells are still viable ( salvageable myocardium) [9]. However, it is exactly at this stage that reperfusion can turn out to be the cause of relevant additional harm, so that, the greater the salvageable myocardium, the greater the potential harm from reperfusion [10], but also the benefits that can be observed from targeted therapy. It is also important to note that different cells (i.e., endothelial, inflammatory and myocardial) display different time-related response to ischemia. For instance, following reperfusion, the microcirculation undergoes a profound degree of endothelial dysfunction within minutes (i.e., 2.5 to 5 min) [11].

Conclusions

A consistent part of the enthusiasm achieved from research in IR injury gets lost in translation when findings are applied into the clinical setting. For this reason, research efforts should be centered to treatment agents that have been shown to have a reliable cause-effect property, to be reproducibly effective in experimental models, and ultimately, to test the specific hypotheses in carefully designed clinical trials. Besides the proper selection of the optimal treatment, the design of an effective strategy to prevent IR injury must also consider the timing and the site of the intervention that is, using the right agent at the right spot!

References

Chromosomal “locus” and “focus”
The chromosomal “locus” refers to the specific location of a DNA sequence/gene on a chromosome, whereas the “focus” refers to the 3-dimensional architecture of mutually exclusive domains of a chromosome.

Matrix metalloproteinases (MMPs)
Matrix metalloproteinases (MMPs) are a family of extracellular matrix degrading enzymes that are involved in tissue remodeling. Alteration in MMP activity has been shown to contribute to ventricular remodeling in heart failure or following an acute myocardial infarction.

micro RNA (miRNA)
miRNAs are a class of highly conserved, endogenous, non-coding RNA molecules of approximately 22 nucleotides that silence gene expression at the post-transcriptional level by either promoting the degradation of messenger RNA (mRNA), or inhibiting the translation of protein from miRNA by translational repression.

Randle Cycle
The Randle Cycle is a metabolic phenomenon characterized via substrate competition between carbohydrate (glucose) and fatty acids for entry into oxidative pathways (the Krebs Cycle) for subsequent energy metabolism. As the oxidation of one substrate increases, it results in decreased oxidation of the competing substrate.

Renin angiotensin system (RAS)
RAS is the physiological hormone system responsible for the regulation of blood pressure and fluid balance. Renin (originating from the kidneys) stimulates the production of angiotensin, which induces blood vessel constriction and increases blood pressure. Angiotensin also stimulates the production of aldosterone, which acts on the kidneys to increase sodium and water reabsorption into the blood, also contributing to an increase in blood pressure.

Rheumatoid factor
Rheumatoid factor is an autoantibody (antibody that targets the host organism’s own tissues) against the Fc portion of immunoglobulin, and this rheumatoid factor/immunoglobulin immune complex is the most relevant autoantibody complex in the development of rheumatoid arthritis.

Transforming growth factor β (TGF-β)
Transforming growth factor (TGF)-β is a growth factor that has an important role in controlling fibroblast growth and accumulation of extracellular matrix proteins. Increases in TGF-β have been implicated in the maladaptive response to cardiac hypertrophy.

Troponins
Troponins are a complex of 3 regulatory proteins (Troponin C/I/T) essential for muscle contraction in skeletal and cardiac muscle. Troponins are not involved in smooth muscle contraction.