The hunt for fatal myocardial infarction biomarkers: predictive circulating microRNAs

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Introduction

The concept of a hype cycle is a well-established business concept, in which novel ideas are said to have an initial wave of hype followed by disillusionment. Only after that, the novel concept takes off and become truly useful entering a so-called plateau of productivity. In biomedical science, the field of microRNAs (miRNAs) certainly had a peak of interest in the end of the last decade. This led by high impact publications (1) and characterization of both novel miRNA-entities as well as their associations to a broad range of diseases. Nonetheless, no clear pharmaceutical successes emerged: miRNA targets are being pursued as therapeutic targets, but none have as of yet successfully made it through clinical trials (2). Likewise the use of miRNA-based treatment strategies targeting regular mRNA is an area of interest (3). In this editorial we focus on a third aspect of miRNAs: the use of miRNAs as prognostic biomarkers in disease, asking the question if miRNAs are now entering this plateau of productivity in which actual benefit will be seen.

We focus on the recent paper by Bye *et al.*: "*Circulating microRNAs predict future fatal myocardial infarction in healthy individuals - The HUNT study*" (4). This paper is of particular interest because it presents strong evidence for prognostic benefit of miRNAs. The study was based on the HUNT cohort, a Norwegian biobank-initiative in which an impressive 88% of the adult population of the Nord-Trøndelag County participated, giving blood samples and questionnaire information in 1984, 1995, and 2006. With the unique advantage of having both frozen serum and decades of follow-up information, the study was designed as a prospective nested case-control design with fatal acute myocardial infarct (AMI) as endpoint and controls matched on risk factors such as body mass index (BMI), total cholesterol and high-density lipoprotein cholesterol (HDL-C) (4). The main discovery phase results yielded 12 miRNAs that were associated with future AMI. This editorial will discuss the perspectives of these findings as well as considerations for similar future miRNA studies.

The methodology of miRNA normalization

A key consideration in biomarker studies is the existence of similar studies. For miRNA biomarkers predicting AMI, several studies already exist that address AMI risk. Typically the main focus is the discovery of biomarkers for immediate use, such as distinguishing patients with ST-elevated AMI from patients with stable ischemic heart disease (5), or between ongoing AMI and healthy controls (6). The most comparable study to the interest of this editorial is the one Zampetaki *et al*. In this study, the main focus was the prediction of future AMI and the authors did find association of miR-126, miR-223 and miR-197 to the disease (7). It is noteworthy that none of these miRNAs were identified by Bye *et al*. (4). Hence, for the overall purpose of using miRNAs as predictive biomarkers it prompts an important discussion on methodological choice.

Bye *et al*. suggested that one main discrepancy reason could be the choice of data normalization method and the platform for miRNA analysis. In fact, while Bye *et al*. used a panel of seven reference genes for normalization

and quality control by means of the RNA Spike-in kit including cel-miR-39-3p, UniSp2, UniSp4, UniSp5 and UniSp6, Zampetaki *et al*. (7) solely used U6, which is not a suitable endogenous control for the quantification of circulating miRNAs based on previous works (8,9). It has been shown that the Spike-in system improves the quality of the normalization step (10). The normalization method for circulating miRNA quantification is one of the critical aspects in this field and from this point of view the normalization procedure used in the work of Bye *et al*. is the most robust to date.

Sample collection and processing in miRNA analysis

Another crucial aspect for the analysis of circulating miRNAs is the collection and processing of blood samples. In fact, it has been suggested that blood must be processed within a few hours after collection in order to prevent cell-derived miRNA contamination from red blood cells or platelets (11-13). Unfortunately, many studies do not follow this suggestion or do not report this important information. Moreover, it has been shown that the difference between serum and plasma strongly affects the spectrum of circulating miRNA in blood (11) demonstrating higher miRNA concentrations in serum samples compared to the corresponding plasma samples. Considering that Bye *et al*. and Zampetaki *et al*. extracted miRNAs from serum and plasma, respectively, and that they used two different RNA isolation kits (miRCURY RNA isolation and miRNeasy kit, respectively), it is plausible that the two studies did not find the same miRNAs. All these considerations point the attention on the fact that, given the numerous factors that generate variability in circulating miRNA studies, it is now mandatory to develop standard protocols for blood specimen collection and processing to allow the comparison across studies.

Using small RNA-seq to improve the quality of the results

Circulating miRNAs are considered novel non-invasive biomarkers. Yet, the mechanism of action at the molecular level both in healthy and disease is still largely unknown. Since there is a great opportunity to establish a new paradigm of intercellular communication, the National Institutes of Health (NIH) funded a novel Common Fund's Extracellular RNA Communication (ERC) program "(I) to discover fundamental biological principles about the mechanisms of extracellular RNA (exRNA) generation, secretion, and transport; (II) to identify and develop a catalogue of exRNA in normal human body fluids; (III) and to investigate the potential for using exRNAs as therapeutic molecules or biomarkers of disease". In order to disseminate the knowledge derived from this program, the results are shared through the exRNA research portal, a community-wide resource for exRNA standards, protocols and data. These efforts have already generated new small RNA-seq data for several conditions (including cardiovascular diseases and cancer), biofluids (e.g., plasma and serum) and RNA sources (e.g., exosomes and other extracellular vesicles). Since the quality, the amount and the specific body fluid are important factors (as discussed above), RNA-seq is likely to be the future standard technique in this field. Still, small RNA-seq is not the common method used as shown in the miRandola database, the circulating RNAs database (14). In the work of Bye *et al*. and in many other published studies, qRT-PCR has been used as golden standard for miRNA quantification. Since in this context the normalization step is crucial and there is no clear agreement in the scientific society, using small RNA-seq could solve this crucial problem, increasing the quality of the results. Overall, rigorous standardized methods and analyses are needed in this field. It has been reported that many confounding factors exist in the phases of processing, extraction and quantification of exRNAs.

Statistical considerations in search for predictive biomarkers

In understanding biomarker discovery studies it is important to be very aware of the statistical pitfalls associated with them (15). The archetypical discovery study pitfall is that of the winner's curse; that testing hundreds of metrics will inevitable yield significant findings by chance and that their effect estimates will be inflated (16). In the Bye *et al*. study (4) the study design was built around a discovery phase as well as a validation phase. In the discovery phase, 76 miRNAs were tested, of which 12 were selected at an uncorrected $P<0.05$ significance threshold. This alone is of course a clear example of test metric inflation and winner's curse, and it follows that the $\Delta\Delta$Cq values should decrease in the validation phase, which in fact they do. However, the study also included a validation phase within which it was shown that ten of the 12 miRNAs were significantly associated with future AMI at $P<0.05$. No metric was provided

reporting with a formal multiple testing correction of 12 miRNAs, but it is reported that four of the 12 miRNAs also were significant at P<0.01. Further, Bye *et al*. presents a combination signature of five other miRNAs. These resulted from the testing of 4,095 different combinations with no independent replication. It is from this signature that the 0.91 AUC is concluded.

The replication and validation setup is a good strategy to amend winners curse problems. Ultimately, however, the proper question to debate here is of course whether these statistics will hold up in a general case. The combination signature score of 0.91 AUC finding was based on a large un-replicated multiple burden and so is highly likely to decrease on independent validation. However, the individual miRNA scores were independently replicated suggesting that they are true in a general case. And that is a novelty in a field burdened by underpowered discovery studies without genuine follow up, so we believe that at least the four strongly replicating miRNAs, let-7g-5p, miR-26a-5p, miR-106a-5p, and miR-151a-5p, could play important future roles in the field of AMI prognostics.

Conclusions

Current prediction algorithms in clinical use include the European Society of Cardiology's Systematic Coronary Risk Evaluation (SCORE) and the Framingham risk score (17,18). These algorithms have some impact in a clinical setting; by accurately assigning patients to risk-groups they can prompt important discussions of smoking patterns, LDL-cholesterol levels and overall healthy lifestyle. However, the scores are still too inaccurate to clearly pinpoint the individuals who will in fact become future patients. This is the reason why predictive biomarkers are of such interest. Having the ability to identify individuals who has a high risk of adverse events with only low chance of false positives; that is a hallmark of precision medicine, and one that is not possible only with the current life-style based clinical scores.

While the work with miRNA biomarkers for AMI is still in its infancy, studies like Bye *et al*. pave a way for a future in which life-style scores could be supplemented with simple and cheap blood-test-based biomarker predictions, and resultant in early and accurate intervention. More accurate intervention, importantly, also means less wasteful and non-required treatment of individuals who are in fact not at risk. And this optimization of the health care system really is the grand perspective to have in mind when considering precision medicine in general, and studies like Bye *et al*. in particular.

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Footnote

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