MicroRNA profiling unveils hyperglycaemic memory in the diabetic heart

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Aims
Recent randomized trials suggest that intensive glycaemic control fails to reduce heart failure-related events in patients with diabetes. The molecular cues underlying persistent myocardial damage despite normoglycaemia restoration remain elusive. MicroRNAs (miRNAs), a class of small non-coding RNAs, orchestrate transcriptional programs implicated in adverse cardiac remodelling. The present study investigates whether miRNAs participate to hyperglycaemic memory in the diabetic heart.

Methods and results
miRNA landscape was assessed by Mouse miRNome miRNA PCR Arrays in left ventricular specimens collected from 4-month-old streptozotocin-induced diabetic mice, with or without intensive glycaemic control by slow-release insulin implants. A dysregulation of 316 out of 1008 total miRNAs was observed in the diabetic hearts when compared with controls. Of these, 209 were up-regulated and 107 were down-regulated by >2.0-fold. Interestingly enough, the expression of 268 of those miRNAs remained significantly altered in diabetic mice even after subsequent normoglycaemia. Ingenuity pathway analysis revealed that dysregulated miRNAs were implicated in myocardial signalling networks triggering apoptosis (miR-320b, miR-378, miR-34a), fibrosis (miR-125b, miR-150, miR-199a, miR-29b, miR30a), hypertrophic growth (miR-1, miR-150, miR-199a, miR-133a, miR-214, miR-29a, miR-125b, miR-221, miR-212), autophagy (miR-133a, miR-221, miR-212, miR30a), oxidative stress (miR-221, miR-146a, miR-34a, miR-210, miR-19b, miR-125b, miR27a, miR-155), and heart failure (miR-423, miR-499, miR-199a), respectively.

Conclusions
Glycaemic control is not able to rescue hyperglycaemia-induced alterations of miRNA landscape in the diabetic heart. These findings may provide novel insights to understand why diabetic cardiomyopathy progresses despite normalization of blood glucose levels.

Keywords
Diabetic cardiomyopathy • MicroRNAs • Epigenetics • Glycemic control • Cardiovascular disease

Introduction
Patients with diabetes display a high risk of developing heart failure, even after adjusting for coronary artery disease or hypertension.1 This has led to the increased recognition of a distinct disease process defined as diabetic cardiomyopathy.2 Such condition is characterized by myocardial hypertrophy, fibrosis, and impairment of left ventricular performance that occur independently of a recognized cause, namely myocardial ischaemia.3,4 Although the pathogenesis of diabetic cardiomyopathy is multifactorial, hyperglycaemia is still considered the main driver of myocardial damage in this setting.

High glucose levels foster accumulation of reactive oxygen species (ROS) by altering the balance between ROS generating and scavenging pathways. Oxidative burst, in turn, is capable of amplifying mal-adaptive signalling, including protein kinase C, mitogen-activated protein kinases, advanced glycation end products (AGES), and aldose reductase leading to apoptosis, hypertrophy, fibrosis, impaired calcium homeostasis and contractile dysfunction.2 Of note, the detrimental effects of hyperglycaemia may persist even after restoration of normal glucose levels, a phenomenon known as ‘hyperglycaemic’ or ‘metabolic’ memory.4,5 In line with these observations, long-term randomized trials comparing the effects...
of intensive vs. standard glycemic control in high-risk diabetic patients failed to reduce macrovascular complications and mortality. Moreover, a recent meta-analysis including 8 randomized controlled trials with a total of 37,229 diabetic patients showed that the risk of heart failure-related events was not significantly affected by intensive glycemic control. The molecular mechanisms implicated in the progression of myocardial damage despite normoglycemia restoration are largely unknown. MicroRNAs (miRNAs) represent a class of small non-coding RNAs that control the expression of entire networks of complementary transcripts. In the heart, miRNAs are critically involved in the maintenance of tissue homeostasis. While dysregulation of miRNAs has been linked to cardiac embryonic development, ischemia, and age-related changes, the miRNA landscape of the diabetic heart and its relation with glycemic control remains poorly elucidated. The present study was designed to address whether miRNAs may represent putative molecular drivers of hyperglycemic memory in the diabetic myocardium. Here, we show that hyperglycemia significantly affects miRNA expression in the heart of diabetic mice and these detrimental signatures are retained despite intensive glycemic control. These findings show the existence of hyperglycemic memory in the heart and set the stage for mechanism-based therapeutic strategies to combat diabetic cardiomyopathy and heart failure in patients with diabetes.

**Methods**

An expanded methods section is available in Supplementary material online.

**Induction of diabetes and glycemic control in mice**

Diabetes was induced in 4-month-old male C57/B6 mice by streptozotocin. Three experimental groups were studied: (i) control mice; (ii) diabetic mice; and (iii) diabetic mice undergoing intensive glycemic control by slow-release insulin implants. Treatment with insulin started 3 weeks after the induction of diabetes and was maintained for the following 3 weeks. All mice were euthanized after 6-week follow-up (see Supplementary material online, Figure S1).

**miRNA expression profiling workflow**

Profiling of 1008 total miRNAs in heart samples from the three experimental groups (n = 3 in each group) was performed by Mouse miRNome miRNA PCR Arrays (SABioscience, USA). Raw data and detailed information about array analysis are available in Supplementary material online. The miRNAs, differentially expressed in each experimental group, were uploaded in the ingenuity pathway analysis (IPA) tool (Ingenuity Systems, www.ingenuity.com) and analysed on the basis of IPA library of canonical pathways.

**Results**

**Intensive glycemic control does not revert miRNA signatures in the diabetic heart**

Blood glucose levels were significantly higher in streptozotocin-induced diabetic mice when compared with controls. After 3 weeks of diabetes, optimal glycemic control was obtained by means of slow-release insulin implants and maintained for 3 additional weeks (see Supplementary material online, Figures S1–S2). After 6 weeks, mice were euthanized and miRNA profiling was assessed in left ventricular specimens collected from the different experimental groups. A total of 316 miRNAs were differentially expressed in diabetic and control hearts, of which 209 were up-regulated and 107 were down-regulated by >2.0-fold (Figure 1A, Supplementary material online, Tables S1–S2). Interestingly, 268 out of 316 miRNAs remained dysregulated despite 3 weeks of intensive glycemic control by insulin (Figure 1B). Venn diagrams showed that 178 out of 209 miRNAs were persistently up-regulated, whereas 90 out of 107 remained down-regulated despite normoglycemia restoration (Figure 1C). Moreover, glycaemic control with insulin was associated with dysregulation of 26 additional miRNAs (Figure 1C).

**miRNAs and myocardial damage**

Ingenuity pathway analysis was employed to assess the functional impact of the observed miRNA variations, clustering miRNA signatures into relevant pathways of myocardial damage. Ingenuity pathway analysis revealed that a large proportion of dysregulated miRNAs orchestrate transcriptional programs implicated in apoptosis, fibrosis, hypertrophy, autophagy, oxidative stress, and heart failure (Figure 1D, Supplementary material online, Table S3). Specifically, miR-320b, miR-378, and miR-34a, key initiators of apoptosis, were significantly overexpressed in the diabetic heart and intensive glycemic control was unable to revert these changes (Figure 1D). We found that five miRNAs (miR-125b, miR-150, miR-199a, miR-29b, miR30a) were involved in the regulation of pathways underlying myocardial fibrosis. Of note, such signatures persisted even after normoglycemia restoration (Figure 1D). miRNAs implicated in hypertrophic growth of cardiomyocytes (miR-1, miR150, miR-199a, miR-133a, miR-214, miR-29a, miR-125b, miR-221, miR-212) were also strongly impaired by hyperglycemia and remained unchanged after insulin treatment. Furthermore, miRNAs regulating redox signalling pathways (miR-221, miR-146a, miR-34a, miR-210, miR-19b, miR-125b, miR27a, miR-155), autophagy (miR-133a, miR-221, miR-212, miR30a), and heart failure (miR-423, miR-499, miR-199a) were also persistently dysregulated after normalization of blood glucose levels (Figure 1D).

**Discussion**

In the present study, we have shown, for the first time, that diabetes induces a profound alteration of miRNAs expression in the heart and, most importantly, these detrimental signatures are not reverted by glycemic control. Specifically, a persistent alteration of several miRNAs orchestrating apoptosis, myocardial fibrosis, hypertrophy, autophagic response, redox signalling, and heart failure suggests the existence of hyperglycemic memory in the heart.

Long-lasting hyperglycemic stress after normalization of glucose level has been reported in previous studies, but its molecular mechanisms remain incompletely understood. This phenomenon, defined as ‘hyperglycemic’ or ‘metabolic’ memory, may explain why diabetic cardiovascular complications progress even in the presence of glycemic control. We and others have recently demonstrated that hyperglycemia triggers maladaptive signatures in the vascular endothelium, which persist after the restoration of...
near-normal glucose levels. While emerging evidence has provided key insights to understand the molecular basis of vascular hyperglycaemic memory, no previous work has investigated whether this phenomenon occurs in the heart.

This issue deserves particular attention in light of the results of a recent meta-analysis including 37,229 diabetic patients from 8 randomized trials, showing that intensive glycaemic control has no impact on the risk of heart failure. Overall, the risk of heart failure-related events did not differ significantly between intensive glycaemic control and standard treatment (OR 1.20, 95% CI 0.96–1.48). These results prompted us to investigate whether molecular cues might be involved in the progression of myocardial damage despite restoration of normoglycaemia. Over the last years epigenetic changes—including modifications of DNA/histone complexes and miRNAs—have been advocated as key determinants of adverse cardiovascular phenotypes.

MiRNAs are short, non-coding RNAs that modify gene expression by targeting the 3’ untranslated region of mRNA. Although
miRNA functionality is well studied in different cardiac diseases, their role in the pathogenesis and progression of diabetic cardiomyopathy remains elusive. Here, we show that 316 out of 1008 total miRNAs were dysregulated in the diabetic heart. Notably, 268 of those miRNAs remained significantly altered after 3 weeks of intensive glycaemic control with insulin. Inguinity pathway analysis revealed that most of miRNAs showing a memory effect regulate myocardial pathways associated with apoptosis, fibrosis, hypertrophy, autophagy, oxidative stress, and heart failure. Among dysregulated miRNAs, we found that miR-221 and miR-212 were massively overexpressed in the diabetic heart and glycemic control failed to normalize their levels. MiR-221 and miR-212 are pivotal mediators of myocyte hypertrophy and autophagy by targeting p27/mTOR and calcineurin/NFAT signalling, respectively. Since hypertrophy and impaired autophagic response are main features of diabetic cardiomyopathy, our findings suggest that those miRNAs may significantly contribute to the progression of diabetic myocardial damage during subsequent normoglycaemia. Similarly, miR-199a was persistently up-regulated, regardless of intensive glucose control. Overexpression of miR-199a in cardiomyocytes has been associated with myocardial hypertrophy and reduced mRNA expression of alpha-myosin heavy chain. Of note, knockdown of endogenous miR-199a in cardiomyocytes attenuates phenylephrine-induced increase in cell size. Therefore, the persistent increase of miR-199a levels observed in the present study might help to explain why adverse cardiac remodelling is not affected by glycaemic control. Seminal work has recently demonstrated that miR-34a is induced in the ageing heart and in vivo silencing or genetic deletion of miR-34a reduces age-associated cardiomyocyte cell death. Interestingly, we found that miR-34a was strongly increased in the diabetic heart and its expression was unaffected by normoglycaemia restoration. This latter result suggests that miR-34a can be regarded as a putative mediator linking diabetes and cardiac senescence. Furthermore, we found that glycaemic control was unable to restore the expression of relevant downregulated miRNAs such as the anti-fibrotic miR-29b as well as miR30a and miR-1, potent inhibitors of mitochondrial fission, apoptosis, and hypertrophy. Reduced levels of miR-1 may play a pivotal role in our setting since miR-1 replacement therapy has recently shown to revert cardiac hypertrophy and fibrosis by targeting Fibulin-1, a secreted protein implicated in extracellular matrix remodelling. Our findings have potential clinical relevance. Indeed, growing evidence is supporting the notion that miRNA expression and function can be reprogrammed in patients through systemic or local delivery of miRNA modulators (anti-miRs or miRNA mimics). In this regard, pharmacological modulation of miRNA activity has demonstrated a therapeutic benefit for the treatment of cancer, heart failure, atherosclerosis, and HCV infection, respectively.

Taken together, our results may help to explain why diabetes-related myocardial damage progresses also in the presence of improved glycaemic control and may assist in defining novel therapeutic targets to reduce the deleterious effect of hyperglycaemic memory in the heart.

Supplementary material
Supplementary material is available at European Heart Journal online.

Authors’ contributions
S.C. and F.P. conceived the study, analysed and interpreted data, and drafted the manuscript; T.F.L. revised the manuscript for important intellectual content; F.C. also is a part of conception, interpretation of data, manuscript drafting, final approval of the manuscript.

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References


Fully integrated whole-body [18F]-fludeoxyglucose positron emission tomography/magnetic resonance imaging in therapy monitoring of giant cell arteritis

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A 68-year-old man with giant cell arteritis (GCA) was admitted to our hospital 1 year after diagnosis for reevaluation of disease status. Initially increased inflammatory biomarkers C-reactive protein (7.2 mg/dL; norm,<0.5 mg/dL) and blood sedimentation rate (80 mm/h; norm, <30 mm/h) disappeared within a few months during glucocorticoid treatment, which has been reduced to 5 mg/day as maintenance therapy. Due to slightly elevated C-reactive protein (0.8 mg/dL) suggesting low activity of GCA, [18F]-fludeoxyglucose ([18F]-FDG) positron emission tomography/magnetic resonance imaging (PET/MRI) was performed to identify activity and extent of suspected recurrent vasculitis. Positron emission tomography revealed extensive large vessel involvement with marked FDG vessel wall uptake in the aorta and its branches, indicative for active inflammation (Panel A: coronal PET, Panel D: fusion of PET and T2 STIR). Magnetic resonance imaging, however, did not show any vessel changes suggestive for active vasculitis (Panel E: axial T1 VIBE, Panel F: angiography). After changing the therapeutic regimen by adding methotrexate to ongoing glucocorticoid therapy, FDG vessel wall uptake decreased significantly and remained normal in 12- and 24-month follow-up PET/MRI studies (Panels B and C). Simultaneously, C-reactive protein disappeared and remained undetectable in further laboratory tests suggesting remission of GCA. This case emphasizes the important role of molecular imaging by PET in therapy monitoring of GCA, since biochemical evaluation of disease activity by C-reactive protein completely underestimated disease flare. Hybrid PET/MRI might be a useful approach for aiding in the management of GCA, given its high sensitivity based on PET and the reduced radiation exposure compared with well-established PET/CT.