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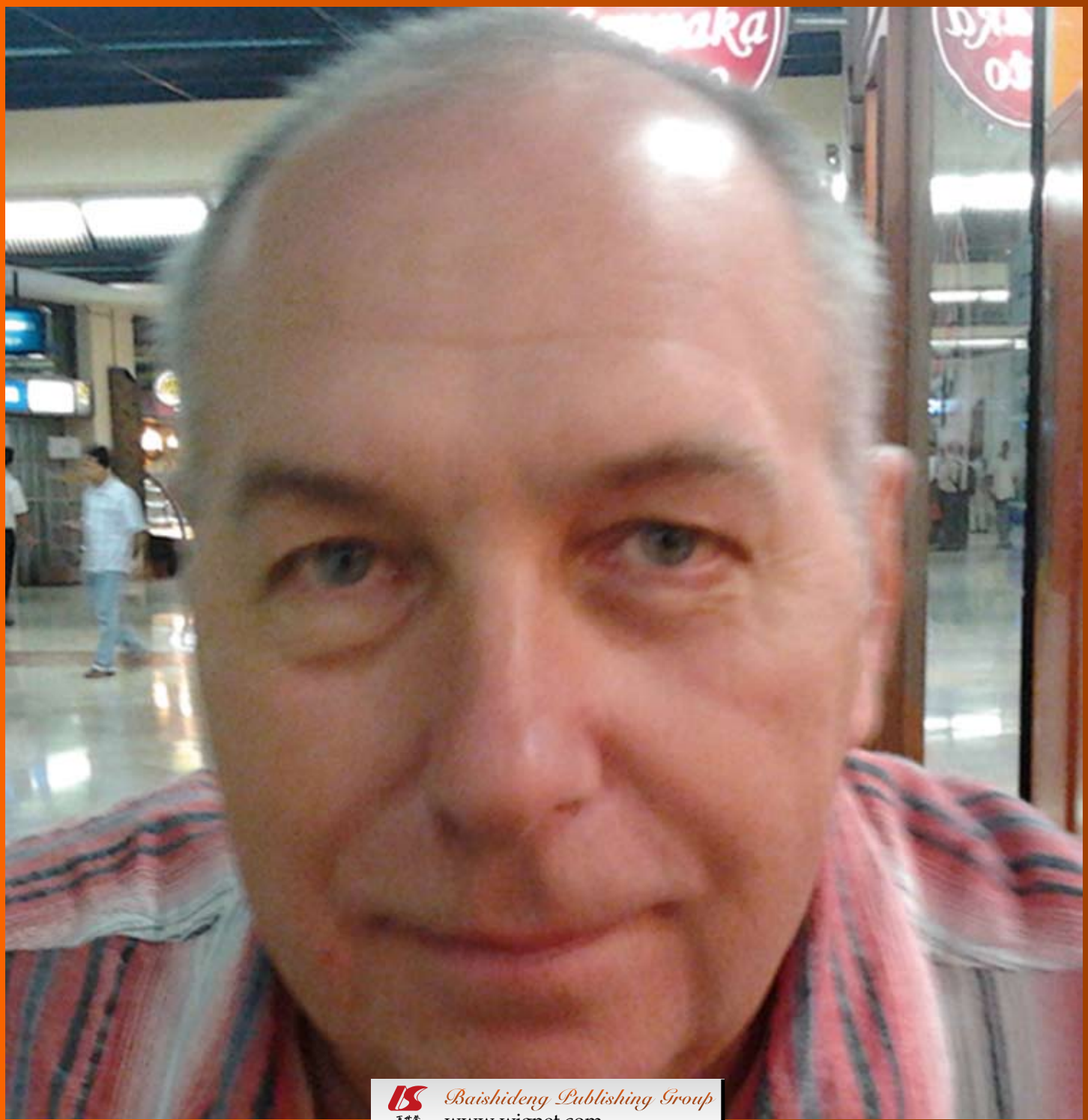

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Coronary artery calcium score: Re-evaluation of its predictive value for coronary artery disease

Mansour Almoudi, Zhonghua Sun

Mansour Almoudi, Zhonghua Sun, Discipline of Medical Imaging, Department of Imaging and Applied Physics, Curtin University, GPO Box U1987 Perth, Western Australia 6845, Australia
Author contributions: Both authors contributed equally to this paper.
Correspondence to: Zhonghua Sun, Associate Professor, Discipline of Medical Imaging, Department of Imaging and Applied Physics, Curtin University, GPO Box, U1987, Perth, Western Australia 6845, Australia. z.sun@curtin.edu.au
Telephone: +61-8-92667509 Fax: +61-8-92662377
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Abstract

Coronary artery disease is the leading cause of death in advanced countries and its prevalence is increasing among the developing countries. Cardiac computed tomography (CT) has been increasingly used in the diagnosis of coronary artery disease due to its rapid improvements in multislice CT scanners over the last decade, and this less-invasive technique has become a potentially effective alternative to invasive coronary angiography. Quantifying the amount of coronary artery calcium with cardiac CT has been widely accepted as a reliable non-invasive technique for predicting risk of future cardiovascular events. However, the main question that remains uncertain is whether routine, widespread coronary artery calcium scoring in an individual patient will result in an overall improvement in quality of care and clinical outcomes. In this commentary, we discuss a current issue of the clinical value of coronary artery calcium scoring with regard to its value of predicting adverse cardiac events. We also discuss the applications of coronary artery calcium scores in patients with different risk groups.

INVITED COMMENTARY ON HOT ARTICLES

We read Grayburn's[1] recent perspective on interpreting the coronary artery calcium score with great enthusiasm, and would like to discuss the potential value of using coronary artery calcium (CAC) scoring in patients with different risk factors of cardiovascular disease.

Grayburn[1] presented a case vignette with a patient having a low Framingham risk score (less than 10% risk of a coronary event over the next 10 years); then highlighted a common clinical problem of how to utilize CAC scoring in asymptomatic and symptomatic populations with suspected coronary artery disease (CAD). Evidence supporting different strategies was presented, followed by the author's clinical recommendations.

The first of the three comments in this commentary is that CAC scoring should be wisely used by physicians. CAC scoring is usually performed as a screening method with the use of low radiation dose scanning techniques. The purpose of the scan is to detect and calculate the calcium density, volume or mass. The total coronary calcium is used as a way of predicting and stratifying the risk

of CAD. The rationale behind it is that coronary artery calcification is part of the atherosclerotic degeneration of the arterial vessel wall, and coronary atherosclerosis is the only disease associated with calcium in the coronary arteries[2]. Thus, measurement of the amount of calcium allows accurate estimation of the amount of coronary atherosclerosis and therefore the risk of coronary artery disease. Quantifying the amount of CAC scoring has been widely accepted as a reliable non-invasive technique for screening risk of future cardiac events, and is usually quantified by using the Agatston score[3].

Clinical application of CAC has been supported by evidence showing that the absence of calcium reliably excludes obstructive coronary artery stenoses, and that the amount of CAC is a robust predictor for risk assessment of incident cardiovascular events, independent of conventional coronary risk factors. However, the prognostic value of CAC depends on the risk groups as to whether patient risk is reclassified and patient management can be changed based on CAC scores when compared to traditional risk assessments[4].

The Framingham risk score is one of the most commonly used risk-estimation systems, which enables clinicians to estimate cardiovascular risk in asymptomatic patients. It is calculated using traditional risk predictors, including age, gender, total cholesterol, high-density lipoprotein cholesterol, smoking status, and systolic blood pressure, and is represented as a 10-year risk score for the prediction of coronary artery disease events[5]. However, there is growing evidence to show that these traditional risk assessment methods, based on risk factor analysis, have significant limitations when used to guide individual patient therapy. CAC scoring by multislice CT has been increasingly used as an additional assessment tool to evaluate the risk of developing major cardiac events in asymptomatic and symptomatic patients. Guidelines vary on the question of whether CAC is indicated for screening asymptomatic patients at intermediate risk for CAD[6-8], however, CAC screening of symptomatic patients with known CAD is generally believed to be not helpful[9]. As Grayburn pointed out in this article, it is important for physicians to evaluate the CAC score within the clinical context before further tests are recommended for patients.

The second comment is that CAC is added to traditional risk factors, and it leads to a significant improvement in the classification of risk for the prediction of cardiac events in an asymptomatic population. Since the CAC score indicates the presence or absence and measures the extent of coronary atherosclerosis, it is not unexpected that a high CAC score is regarded as a marker for an increased risk of coronary events. Thus, a CAC score of zero is associated with a very low risk of subsequent cardiac events[10,11], whereas increasing CAC scores are associated with a step-wise increase in the risk of events.

The goal of CAC screening in asymptomatic persons is to refine the risk assessment with the aim of determining whether preventive strategies should be intensified, not identifying persons with asymptomatic coronary stenosis[12]. Polonsky *et al*[7] in their multi-ethnic cohort consisting of 5878 participants without known cardiovascular disease investigated the additional value of CAC score to traditional cardiovascular risk factors with regard to the potential role for risk stratification. Their results showed that when CAC score was added to traditional risk factors, it contributed to a significant improvement in the classification of risk for the prediction of cardiac events in an asymptomatic population.

In asymptomatic individuals, zero CAC is associated with a very low (< 1% per year) risk of major cardiac events over the next 3-5 years, whereas in asymptomatic patients with extensive coronary calcification, the major cardiac events have been reported to be increased by up to 11-fold[13]. The recent population-based multi-ethnic study of atherosclerosis, conducted in 6722 asymptomatic patients belonging to four racial ethnic groups and followed for 3.8 years, showed a significant difference in the prevalence of CAC among different ethnic groups. Nonetheless, CAC has demonstrated incremental prognostic value over traditional risk factors, with a sevenfold increase in the incidence of cardiac events for Agatston scores > 100 when compared with patients with zero CAC[14].

Other studies evaluating the prognostic value of the measurement of CAC have shown that coronary calcification is predictive of cardiac events in asymptomatic patients with different age groups[15-17]. In the Prospective Army Coronary Calcium Project among men and women 40 to 45 years of age, Taylor *et al*[15] concluded that the presence of coronary calcium was associated with an increase in the risk of coronary events by a factor of 12 during 3 years of follow-up. LaMonte *et al*[16] in their study consisting of nearly 11 000 patients ranging from 22 to 96 years of age who underwent a screening medical examination, reported increased cardiac events in patients with coronary calcium scores of 400 or more during a mean follow-up of 3.5 years. Similarly, higher calcium scores were found to be associated with the relative risks of coronary events in the population-based Rotterdam Study of elderly asymptomatic patients[17].

Although the association between CAC scores and cardiovascular events has been well documented, a clinical question arises regarding whether CAC scoring has a favourable effect on clinical outcomes, and there are concerns about the associated radiation exposure[18-21]. The radiation dose associated with CAC scoring is small but real, which ranges from 0.9 to 2.4 mSv with multislice CT[22], and some cardiac CT imaging protocols are associated with estimated radiation doses higher than 10 mSv[18,22,23]. This results in a small but measurable increase in the risk of radiation-induced cancer[18], thus, this should be considered if CAC scoring (and repeated testing) were used for widespread population screening.

The third comment is that CAC scoring is not recommended for screening of symptomatic patients. Coronary calcification is considered only marginally related

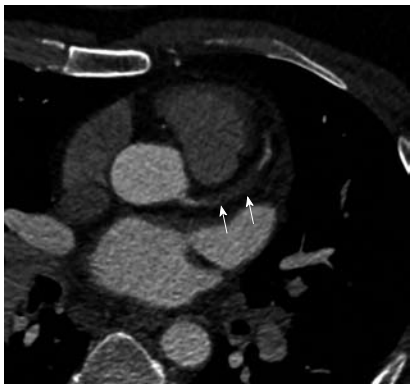

Figure 1 Coronary computed tomography angiography in a 43-year-old male presenting with chest pain and raised cardiac enzymes shows non-calcified plaque at the left main and left anterior descending arteries (arrows) causing a complete total occlusion of these vessels.

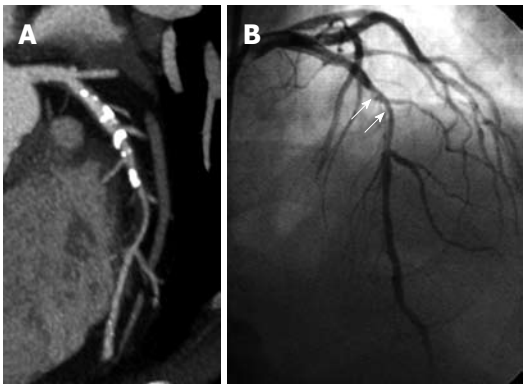

Figure 2 Calcified plaques and stenosis of left anterior descending. A: Extensive calcified plaques are noticed in the proximal and middle segments of left anterior descending (LAD) on curved planar reformatted image, resulting in significant stenosis or total lumen occlusion; B: A 50% stenosis of LAD is confirmed on invasive coronary angiography (arrows).

to the degree of coronary stenosis and it is well known that both obstructive and non-obstructive CAD can occur in the absence of calcification[24,25]. Significantly, coronary stenoses are frequently found to be non-calcified (Figure 1), and highly calcified plaques are frequently non-occlusive or obstructive (Figure 2). Thus, the value of a zero or low calcium score in symptomatic patients remains unclear.

Several studies have reported the presence of obstructive non-calcified plaque in up to 8.7% of symptomatic patients with zero or low calcium score[26,27]. The question has been raised as to whether only using CAC score is a reliable tool to determine the extent of CAD, since non-calcified coronary artery plaque may not be detected. Cheng *et al*[26] reported that low but detectable CAC scores are less reliable in predicting plaque burden due to their association with high overall non-calcified coronary artery plaque. They concluded that low CAC scores are significantly less predictive of prevalence or severity of underlying non-calcified coronary plaque.

Villines *et al*[9] in their recently published international multi-centre study, concluded that in symptomatic patients with a CAC score of 0, obstructive CAD is possible and is associated with increased cardiovascular events. Thus, low but detectable CAC scores are considered less reliable in predicting disease burden due to the association with high overall non-calcified coronary plaques. Symptomatic patients should be referred for coronary CT angiography to determine the extent of CAD and predict disease outcomes, as there is no significant incremental value of CAC scoring beyond the CCTA prognostic information in symptomatic patients[28].

In summary, the author has raised an important issue of whether CAC scoring should be widely used in cardiovascular prevention strategies. There are many limitations of applying CAC scoring as a screening tool to broad populations and this has been systematically reviewed[12]. There is a lack of prospective randomized controlled trials showing that an abnormal CAC score impacts treatment decisions or clinical outcomes. However, an abnormal CAC score may be helpful in encouraging some patients to take their prescribed medications and follow recommended lifestyle changes[8,29,30]. Until these data is available, CAC scoring should be judiciously used by physicians in patients with different risk factors of developing cardiovascular events.

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REVIEW

Next generation sequencing in cardiovascular diseases

Francesca Faita, Cecilia Vecoli, Ilenia Foffa, Maria Grazia Andreassi

Francesca Faita, Cecilia Vecoli, Ilenia Foffa, Maria Grazia Andreassi, CNR, Institute of Clinical Physiology, 54100 Massa, Italy
Author contributions: Faita F revised the literature, organized the different sections of the paper, and helped to draft the manuscript; Vecoli C revised the literature, organized the different sections of the paper and drafted the manuscript; Foffa I revised the manuscript; Andreassi MG revised the literature, organized the different sections of the paper and critically revised the manuscript; and all authors read and approved the final manuscript.
Correspondence to: Dr. Cecilia Vecoli, CNR, Institute of Clinical Physiology, via Aurelia Sud, 54100 Massa, Italy. vecoli@ifc.cnr.it
Telephone: +39-585-493693 Fax: +39-585-493501
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Abstract

In the last few years, the advent of next generation sequencing (NGS) has revolutionized the approach to genetic studies, making whole-genome sequencing a possible way of obtaining global genomic information. NGS has very recently been shown to be successful in identifying novel causative mutations of rare or common Mendelian disorders. At the present time, it is expected that NGS will be increasingly important in the study of inherited and complex cardiovascular diseases (CVDs). However, the NGS approach to the genetics of CVDs represents a territory which has not been widely investigated. The identification of rare and frequent genetic variants can be very important in clinical practice to detect pathogenic mutations or to establish a profile of risk for the development of pathology. The purpose of this paper is to discuss the recent application of NGS in the study of several CVDs such as inherited cardiomyopathies, channelopathies, coronary artery disease and aortic aneurysm. We also discuss the future utility and challenges related to NGS in studying the genetic basis of CVDs in order to improve diagnosis, prevention, and treatment.

Key words: Next generation sequencing; Genetics of cardiovascular diseases; Cardiomyopathies; Coronary artery disease; Complex disease

Peer reviewers: Cristina Vassalle, PhD, G. Monasterio Foundation and Institute of Clinical Physiology, Via Moruzzi 1, I-56124 Pisa, Italy; Yuri V Bobryshev, PhD, Associate Professor, School of Medical Sciences, Faculty of Medicine, University of New South Wales, Kensington, NSW 2052, Australia; Mohamed Chahine, PhD, Professeur Titulaire, Le Centre de Recherche Université Laval Robert-Giffard, Local F-6539, 2601 chemin de la Canardière, Québec G1J 2G3, Canada

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INTRODUCTION

In the years after 2000, the completion of the Human Genome Project (HGP) has completely changed the approach to many genetic research studies.

Indeed, the knowledge of the genome sequence has been increasingly important in order to define the basis of human biology and medicine, providing a single, essential reference for all genetic information. Currently, ten years after the HGP, a new technology, next-generation sequencing (NGS), has revolutionized the genomic and transcriptomic approaches to biology reducing the sequencing cost and significantly increasing the throughput[1]. Whole-genome sequencing has become a possible and efficient way to obtain global genomic information[2].

At present, Roche/454 (Roche), Solexa (Illumina) and AB SOLiD (Applied Biosystem) are the NGS technologies predominantly used in genetics. In all NGS platforms, a whole genome, or targeted regions of the genome, are randomly digested into small fragments (or

short reads) which are sequenced and are then, either aligned to a reference genome or assembled[3] (Figure 1). The unique combination of specific protocols distinguishes the NGS technology determining limits or advantages. This new strategy of sequencing producing many short reads (tens or hundreds of Gbp for each run) has made necessary the development of several bioinformatics tools to perform the correct alignment/assembly or to analyze large amounts of data. To date, many bioinformatics programs have been created for the different platforms of NGS[4].

For instance, Mapping and Assembly with Quality (MAQ) is a very popular NGS software program developed to efficiently map short reads to a reference genome and derive genotype calls to the consensus sequence with quality scores[5]. MAQ is one of the first reference guided assembly programs. It is accurate, efficient, versatile and has been successfully applied to several NGS projects[6]. Efficient Large-Scale Alignment of Nucleotide Databases (ELAND) is a different NGS program designed to search DNA files for short DNA reads allowing up to 2 errors per match[7,8]. Benchmarks comparing ELAND with other popular NGS software, such as MAQ, Basic Local Alignment Search Tool, Short Oligonucleotide Alignment Program[9] or SeqMap[7] generally place ELAND as one of the fastest available programs. Since many of the programs are open source, additional programming may be needed to modify the program to the needs of a specific NGS project. Finally, some online utility programs, such as EagleView[10] or LookSeq[11], provide some additional assistance on NGS data analysis and interpretation.

However, data management remains the biggest challenge in NGS and the major limiting factor in moving the sequencing to the clinical setting.

Indeed, the production of large numbers of low-cost reads could make the NGS platforms useful for many applications including variant discovery by re-sequencing targeted regions of interest or whole genomes, cataloguing the transcriptomes of cells, and genome-wide profiling of epigenetic marks[12].

NGS has very recently been shown to be successful in identifying novel causative mutations of rare or common Mendelian disorders, also from a very small number of affected individuals[1,12].

Furthermore, it is expected that NGS will be increasingly important in the studies of complex diseases, such as common cardiovascular diseases in which one or more variants in a single gene or more variants in different genes are involved. Currently, the NGS approach to the genetics of cardiovascular diseases (CVDs) represents a territory which has not been widely investigated.

The purpose of this paper is to discuss the recent results of NGS in monogenic classic and in complex genetic cardiovascular disorders, such as inherited cardiomyopathy, channelopathies and coronary artery disease (CAD). We also discuss the potential contribution of future NGS applications in order to significantly improve our understanding of the genetics of CVDs.

NGS AND CVDs

In the etiology of the most CVDs a clear hereditary component has been demonstrated.

The CVDs can be divided in two major categories: the monogenic (more rare) and the polygenic/multifactorial forms (Figure 2).

In the monogenic diseases, the mutation of a single gene causes the pathology. These diseases are rare Mendelian traits that show the classical inheritance patterns: autosomal dominant, autosomal recessive, X-linked, or mitochondrial (maternally inherited). Examples of these traits in cardiovascular medicine include structural cardiomyopathies (i.e., hypertrophic or dilated cardiomyopathy) and channelopathies (i.e., Brugada and long QT syndrome) as well as familial dyslipidemias[13].

In clinical practice, the most common CVDs (i.e., CAD) are complex traits that arise from elaborate gene-gene and gene-environmental interactions that confer risk for disease in a probabilistic manner[14]. In these cases, a series of polymorphic variants in several genes increases the risk of developing the disease.

NGS AND INHERITED CARDIOMYOPATHIES

The inherited cardiomyopathies are heterogeneous diseases caused by functional abnormality of cardiac muscle[15]. Hypertrophic cardiomyopathy (HCM) and dilated cardiomyopathy (DCM) are two major clinical forms of inherited cardiomyopathy. HCM, the major cause of sudden death in young people and of heart failure, is characterized by left ventricular hypertrophy, often asymmetric, accompanied by myofibrillar disarrays and reduced compliance (diastolic dysfunction) of cardiac ventricles. Conversely, DCM is characterized by dilated ventricular cavity with systolic dysfunction. The clinical symptom of DCM is heart failure, often associated with sudden death[16]. More than half of HCM patients have a family history of the disease consistent with an autosomal dominant genetic trait[17]. In the case of DCM, about 20%-35% of patients show a family history of the disease (consistent with autosomal dominant inheritance) although some familial cases can be explained by an autosomal recessive or X-linked recessive trait[18-22].

The inherited forms of cardiomyopathies can be caused by mutations in at least 30 different genes. A specific genetic test in patients with cardiomyopathy is of immense clinical importance since some genetic forms of heart muscle diseases are associated with disease manifestation at an early age, an overall poor prognosis, or a high incidence of sudden cardiac death.

Characterizing the genetics of DCM has been a challenging task due to incomplete knowledge of the genes involved in the disease. Unlike HCM, which is largely a disease of the sarcomere (more than 450 mutations in 16

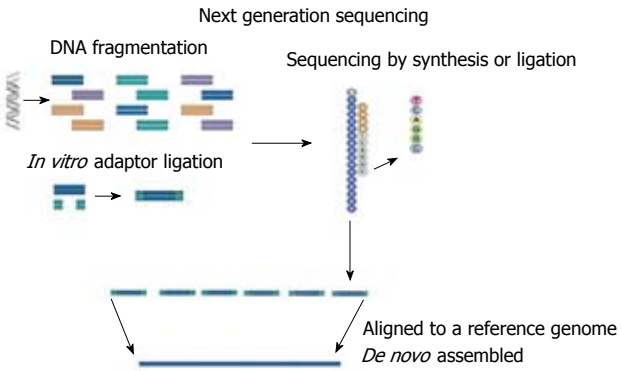

Figure 1 Basic principles of next generation sequencing. A whole genome or a targeted region of the genome are randomly digested into small fragments and then sequenced. The sequence obtained is subsequently aligned to a reference genome or *de novo* assembled.

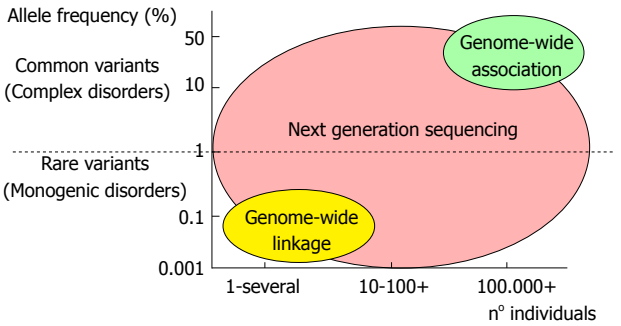

Figure 2 Genetic contribution to monogenic and multigenic cardiovascular diseases and their study approach. The monogenic diseases are caused by a rare mutation in a specific gene and are very rare (< 10 individuals). In the complex diseases, many common genetic variants occur and have a major frequency in the population (> 100 000). The study approach for monogenic diseases is a genome wide linkage study in which one single mutation is identified. Conversely, for complex diseases the genome wide association study is very important to identify a series of common variants contributing to the etiology of the disease. Target re-sequencing by next generation sequencing is an approach which permits the study of both monogenic and complex diseases.

genes have been identified codifying for the sarcomere proteins)[23], the pathways leading to DCM are considerably more diverse, involving genes encoding components of the sarcomere, Z-disk, nuclear lamina proteins, intermediate filaments, and the dystrophin-associated glycoprotein complex[22].

NGS offers a new approach in the diagnosis of cardiomyopathies, and it has recently been used to characterize both HCM and DCM patients. At the present time, despite its good cost-efficiency ratio, NGS is not suitable for clinical practice because of a lack of efficient reduction in genomic complexity and established protocols[24].

Using HCM as a diagnostic model, Dames *et al*[25] developed a NGS-based approach for multi-gene re-sequencing in a clinical laboratory. Sixteen genes implicated in HCM have been sequenced using an uncharacterized human DNA sample. A Long-Range polymerase chain reaction (PCR) (a PCR method used to amplify a long region of genome) was used for gene enrichment, followed by comparative sequencing on the Illumina Genome Analyzer and Roche 454 GS FLX platforms[25]. Both platforms detected several different variants, of which only 27 were common and have been confirmed by Sanger classical sequencing. According to these results the authors proposed a targeted re-sequencing by combining Long-Range PCR and NGS as a new approach for multigene analysis.

Conversely, Meder *et al*[24] established a microarray-based target enrichment followed by SOLiD NGS for a comprehensive and cost-efficient genetic diagnosis of cardiomyopathies. This approach increased the mean depth of coverage of cardiomyopathy genes analyzing 1092 disease exons and adjacent intronic regions in only one NGS run and identified 1891 sequence variants within these regions (of which 349 were nonsynonymous)[24].

NGS has also been used to study the maternally-inherited cardiomyopathy caused by mutations in mitochondrial DNA. Zaragoza *et al*[26] studied 18 patients with mitochondrial cardiomyopathy and two patients with suspected mitochondrial disease. Sequencing PCR-amplified mtDNA with a single run on Roche's 454 Genome Sequencer identified 427 variants.

Recently, Herman *et al*[27] analyzed the gene encoding the sarcomere protein, titin (TTN), in subjects with DCM, subjects with HCM, and in controls using NGS, and evaluated the deleterious variants for cosegregation in families and assessed clinical characteristics. Using this approach they found that TTN truncating mutations are a common cause of DCM, occurring in approximately 25% of familial cases of idiopathic DCM and in 18% of sporadic cases. The authors concluded that the incorporation of sequencing approaches which detect TTN truncations in genetic testing for DCM should substantially increase test sensitivity, thereby allowing earlier diagnosis and therapeutic intervention for many patients with this pathology[27].

Finally, the NGS tools may be used to develop RNA sequencing methodologies for high-throughput comprehensive analysis of individual transcriptomic profiles. In Gαq transgenic mice, a well-characterized model of cardiac hypertrophy/cardiomyopathy[28], the results of sequencing through NGS (Illumina platform) have been compared with an array-based transcriptional profiling.

The results of this study revealed superior dynamic range for mRNA expression and enhanced specificity for reporting low-abundance transcripts by RNA sequencing.

Together these studies suggest that the application of NGS tools in the inherited cardiomyopathies will be increasingly important to define the genetic component of these disorders and to detect cardiomyopathy-causing mutations with high accuracy in a fast and cost-efficient manner which will be suitable for daily clinical practice of genetic testing[24].

NGS AND CHANNELOPATHIES

A different group of CVDs included in inherited cardiomyopathies is the primary electrical diseases such as

Brugada syndrome (BrS) and long QT syndrome (LQTS). Each of these cardiac channelopathies is characterized by a unique genetic profile and clinical features[29]. Advances in molecular biology have allowed the identification of many disorders linked to specific genes previously ascribed as idiopathic. Genetic studies in families with LQTS have associated this disorder with gene mutations affecting cardiac ion channels - specifically the sodium and potassium channels. To date, hundreds of variants have been identified in 13 LQTS genes[30].

Conversely, BrS has been associated with more than 100 mutations in 7 genes. Loss-of-function mutations in the SCN5A gene, which encodes the alpha-subunit of the sodium ion channel, causes 18%-30% of BrS cases[31].

To date, NGS methodologies have not been applied to these disorders.

In addition, since more genes are involved in these diseases, the application of NGS will provide important advantages for identifying pathogenetic mutations in a fast and cost-efficient manner.

NGS AND CAD

CAD remains the leading cause of death in industrialized and developing countries.

It has been estimated that heritable factors contribute 30%-60% of the inter-individual variation in the risk of CAD[32].

Mendelian disorders such as familial dyslipidemia which lead to alterations in the lipid profile are heritable risk factors for CAD[33]. While these rare mutations are well-recognized and well-characterized, the identification of common genetic variants associated with CAD is more difficult despite strong evidence that disease susceptibility is heritable.

Recently, genome-wide association studies (GWAS) have identified several common variants (single nucleotide polymorphisms, SNPs) associated with the risk of CAD. Notably, these SNPs are not inherited independently, but as "bins" or "blocks"[34]. Furthermore, the genotype of 1 SNP may be sufficient to affect the genotype of all other SNPs within a given linkage disequilibrium block (haplotype), thereby "tagging" an entire region of interest[35].

GWAS are performed using the array technologies that in parallel captured hundreds of thousands and now over a million SNPs in independent haplotype blocks in large case/control samples[14,36-44].

Next generation sequencing has enabled targeted re-sequencing of genomic regions found to be involved in the disease. In particular, the re-sequencing of genomic regions identified by GWAS in healthy and diseased populations represents a powerful strategy for assessing the contribution of rare variants to disease etiology[45]. This is because NGS is able to identify rare genetic variants with a minor allele frequency (MAF) of < 5%, which complement the common susceptibility SNPs (MAF > 5%) established through the GWAS[46].

For instance, after GWAS several other studies confirmed the exceptional role of the chromosome 9p21.3 region on the risk of CAD[36,42].

Interestingly, a very recent study analyzed sequence data from a 240-kilobase (kb) region on chromosome 9p21 in 47 individuals using the Illumina GA platform[47]. The authors compared the results of targeted sequencing with NGS to pilot data from the 1000 Genomes Project[48] (characterized by a description of the location, allele frequency and local haplotype structure of approximately 15 million SNPs). The findings showed that the targeted sequencing provides high sensitivity for lower-frequency variants despite several gaps in sequence coverage which existed after the alignment to a reference genome.

Furthermore, NGS has been used to detect rare variants in the gene encoding adiponectin. In this study, a combination of family-based linkage, whole-exome sequencing (by NGS), direct sequencing and association methods has been developed in order to efficiently identify rare variants associated with large effects in families from the Insulin Resistance Atherosclerosis Family Study[49]. These results suggest that this approach could be advantageous in discovering novel genes influencing complex traits in a wide range of family studies.

NGS AND OTHER CVDs

The NGS technologies have also been applied in other cardiovascular diseases with complex traits, such as aortic aneurysm. Harakalova *et al*[50] performed a pilot experiment designed to find an efficient method for the detection of rare genetic variants in regions of interest in large sample groups with aortic aneurysm using SOLiD platform. They discussed the challenges and limitations connected with this approach and showed that the high number of novel variants detected per pool can be limiting factors for successful variant prioritization and confirmation. Indeed, they discovered 681 coding variants, however, the majority of the detected candidate novel variants were false positives.

Moreover, a very recent study using exome sequencing of 2 distantly affected relatives, efficiently and successfully identified a frameshift mutation in the *SMAD3* gene as the cause of vascular disease in a family with arterial aneurysms and dissections inherited in an autosomal dominant pattern. Subsequent sequencing of families involving multiple members with thoracic aortic aneurysms and acute aortic dissections identified *SMAD3* mutations in 2% of familial thoracic aortic aneurysms and dissections[51].

Additionally, Sakai *et al*[52] used two different technologies (re-sequencing array technology and NGS) to analyze eight genes associated with syndromic aortic aneurysms and/or dissections. They identified eighteen variants with both technologies and concluded that NGS was able to detect almost all types of mutations, but it requires improved informatics methods.

Finally, NGS with the Illumina platform has been

Table 1 Next generation sequencing approaches in cardiovascular diseases

	Disease	NGS approach	Ref.
Monogenic diseases	DCM	Target re-sequencing in 47 associated genes	Meder *et al*[24]
		Sequencing of *TTN* gene	Herman *et al*[27]
	HCM	Target re-sequencing in 47 associated genes	Meder *et al*[24]
		Target re-sequencing in 16 associated genes	Dames *et al*[25]
		RNA sequencing	Matkovich *et al*[28]
	Mitochondrial cardiomyopathies	Mitochondrial DNA sequencing	Zaragoza *et al*[26]
	Channelopathies	No approach	
Complex diseases	CAD	Target re-sequencing of 9p21.3 region	Shea *et al*[47]
		Sequencing of *ADIPOQ* gene	Bowden *et al*[49]
	AA	Target sequencing in associated genes	Harakalova *et al*[50], Sakai *et al*[52]
	Thrombophilia	Target sequencing in gene associated	Dewey *et al*[53]

DCM: Dilated Cardiomyopathies; HCM: Hypertrophic cardiomyopathies; CAD: Coronary artery disease; AA: Aortic aneurysm; NGS: Next generation sequencing; TTN: Titin; ADIPOQ: Adiponectin.

used to sequence genomic DNA in a nuclear family with a history of thrombophilia[53]. Two hundred variants have been identified and compared with different groups of HapMap populations. This method has allowed the identification of multigenic risk for inherited thrombophilia and appropriate pharmacological therapy.

FUTURE PERSPECTIVE

Table 1 summarizes the current application of NGS in cardiovascular disorders. NGS technology promises to improve our understanding of the genetic architecture and the missing heritability of CVDs.

In the near future, NGS will revolutionize the genetic study of cardiovascular disease allowing unprecedented opportunities to detect mutations in disease-genes with high accuracy in a fast and cost-efficient manner in daily clinical practice.

In particular, the targeted re-sequencing of the region of interest selected by GWAS, using NGS technologies, will allow identification of rare SNPs involved in the risk of CVD.

To date, data on the reproducibility of NGS results in the cardiovascular setting are limited by too few available studies. In addition, results in other more explored fields such as cancer showed that two independent groups can simultaneously arrive at different sets of gene alterations, without overlap between the two sets of mutations identified[54,55]. This finding suggests that the reproducibility of data could be one major limitation of these advanced techniques. Furthermore, as previously cited, Dames *et al*[25] demonstrated that different variants were found using two different NGS tools of which only a few were successively confirmed by the conventional Sanger approach of sequencing. Thus, new efforts are needed to improve sequencing accuracy and streamline technical processes as the next steps toward transitioning NGS into the clinical laboratory.

The major challenge in NGS is that although it produces an enormous volume of data cheaply, in most cases, the millions of reads generated do not cover the coding regions of disease genes[56]. Indeed, NGS provides only 50-500 continuous base-pair reads[4], making it difficult for both the assembly and the data analysis. Therefore, new methods should be developed to selectively capture DNA from the region of interest in order to sequence only targeted regions.

In addition to short DNA sequence reads, these technologies can generate terabyte-sized data files for each instrument run, greatly increasing the computer resource requirements. Given the vast amount of data produced by NGS, the creation of informatics tools for the storage and analysis of data will be essential to the successful application of NGS[4].

In the future, with the advent of NGS and the progressive increase in data sequences of the human genome from projects such as HapMap and the 1000 Genomes Project, investigators will have to choose between the multiple strategies to test a reference panel of polymorphic sites.

Moreover, parallel genome-wide studies are characterizing a large number of genes affecting the risk factors for CAD including dyslipidemia, hypertension, diabetes mellitus, and obesity. These findings are to be integrated with loci directly associated with CAD to obtain the fullest picture. Thus, in the next few years, the main focus of these studies will be to define a risk prediction as well as a preventive and individual therapy for CAD.

In the last few years, a technological revolution has taken place in the field of epigenomics.

The development of NGS devices are now providing researchers with tools to draw high-resolution maps of DNA methylation and histone modifications in normal tissues and diseases[57]. NGS technologies may be used to profile epigenetic alterations that influence gene expression and to study the genome-wide epigenetic changes that occur in the genome in cardiovascular disease.

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CASE REPORT

Low doses of intravenous epinephrine for refractory sustained monomorphic ventricular tachycardia

Aimé Bonny, Antonio De Sisti, Manlio F Márquez, Richard Megbemado, Françoise Hidden-Lucet, Guy Fontaine

Aimé Bonny, Antonio De Sisti, Françoise Hidden-Lucet, Guy Fontaine, Hôpital Pitié-Salpêtrière, Unité de Rythmologie, 47-83 Boulevard de l'Hôpital, 75651 Paris, France
Manlio F Márquez, Instituto Nacional de Cardiología Ignacio Chávez, Departamento de Electrofisiología, Juan Badiano 1, Col. Sección XVI, 14080 Mexico City, Mexico
Richard Megbemado, Hôpital Saint Camille, Service de Cardiologie, 2 Rue des Pères Camilliens, 94366 Bry-sur Marne, France
Author contributions: Bonny A and Megbemado R managed the patients and collected the data; Bonny A, De Sisti A, Márquez MF, Hidden-Lucet F and Fontaine G wrote the manuscript.
Correspondence to: Aimé Bonny, MD, Hôpital Pitié-Salpêtrière, Unité de Rythmologie. 47-83 Boulevard de l'Hôpital, 75651 Paris, France. aimebonny@yahoo.fr
Telephone: +33-608964024 Fax: +33-608964024
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Abstract

We report three cases of sustained monomorphic ventricular tachycardia (VT) in the setting of coronary artery disease, resistant to beta-blockers in two patients and to amiodarone in all, successfully terminated by low doses of intravenous (IV) epinephrine. VT was the first manifestation of coronary artery disease in one patient, whereas the other two patients had a previous history of myocardial infarction and were recipients of an implantable cardioverter-defibrillator (ICD). One of these two patients experienced an arrhythmic storm. All had hemodynamic instability at the time of epinephrine administration. A single slow administration of IV epinephrine (0.5 to 1 mg administered over 30 to 60 s) restored sinus rhythm after 30-90 s with only minor side effects. In the ICD patient with recurrent VT and several cardioversions due to transformation of VT to ventricular fibrillation, epinephrine injection led to the avoidance of further shocks. Although potentially harmful, low doses of IV epinephrine used alone or in combination with beta-blocker treatment and electrical cardioversion may be an alternative effective therapy for sustained monomorphic VT refractory to amiodarone. The role of epinephrine in the termination of VT should be studied further, especially in patients pre-treated with amiodarone in combination with beta-blockers.

Key words: Ventricular tachycardia; Epinephrine; Cardiopulmonary resuscitation; Ischemic heart disease; Coronary artery disease; Amiodarone

Peer reviewer: Klaus Kettering, MD, Department of Cardiology, University of Mainz, Langenbeckstrasse 1, D-55131 Mainz, Germany

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INTRODUCTION

Sustained ventricular tachycardia (VT) is common in coronary heart disease, usually occurring in the setting of acute myocardial ischemia/infarction or later as scar-related arrhythmia.

Catecholamines have been described in the treatment of VT in the past in both animal and human models[1-5]. In clinical practice, however, their use is restricted to a few indications. Epinephrine is indicated in the setting of cardiopulmonary resuscitation for shock-resistant ventricular fibrillation (VF), pulseless electrical activity or asystole[6,7]. Currently, isoproterenol is recommended

as an antiarrhythmic treatment for arrhythmic storm in Brugada syndrome[8].

Acute therapy for sustained VT depends on hemodynamic tolerance. In the case of VT with hemodynamic instability, electrical cardioversion is the standard of care. In cases of recurrence, intravenous (IV) amiodarone is the drug of choice. However, resistance to this drug, even in association with a beta-blocker, has been described[9-11].

We report three cases of coronary heart disease presenting with hemodynamically unstable sustained monomorphic VT, resistant to beta-blockers and amiodarone, in which arrhythmias were rapidly terminated by single-bolus low doses of IV epinephrine.

CASE REPORT

Patient 1

A 47-year-old man, a smoker without past medical history, was admitted to the Cardiac Care Unit (CCU) due to severe fatigue and palpitations. Physical examination was normal with the exception of a heart rate (HR) of 170 beats/min. Blood pressure (BP) was 125/86 mmHg. A VT was documented on a standard 12-lead electrocardiogram (ECG) (Figure 1). An IV dose of 300 mg amiodarone administered over 5 min failed to stop VT, and the patient then developed hemodynamic instability with a drop in BP to 89/46 mmHg, and profuse sweating without loss of consciousness. Because of rapid hemodynamic alteration and no available anesthesiologist to perform sedation for electrical cardioversion according to the protocol of this remote medical hospital, a single slow infusion of epinephrine (1/10 000, 1 mg within 1 min) was administered. VT stopped within 30 s. Termination was preceded by an increase in BP up to 130/84 mmHg and a slight increase in VT rate from 170 to 180 beats/min (Figure 2). The side effects of epinephrine were chest discomfort, nausea, and anxiousness. ECG in sinus rhythm (SR) after cessation of VT showed a pathologic Q wave in inferior leads (Figure 3). Echocardiography performed in SR showed a dilated left ventricle (62 mm), and left ventricular ejection fraction (LVEF) of 40% with inferior wall akinesia. Troponin I was slightly elevated at 0.82 ng/mL. No electrolyte disturbances were observed. Coronarography showed occlusion of the middle segment of the right coronary artery and a bare-metal stent was implanted. Continuous ECG monitoring did not record any ectopic ventricular beat in the following days. The patient was discharged seven days later.

Patient 2

A 64-year-old male was admitted to the CCU due to sustained VT with a HR of 140 beats/min (Figure 4A). Past medical history showed an inferior myocardial infarction (MI) with low ejection fraction and an implantable cardioverter-defibrillator (ICD) for secondary prevention. He was on bisoprolol 10 mg daily associated with amiodarone 200 mg daily added three months prior for recurrent non-sustained VT. On arrival, he was conscious and had a palpable pulse although BP was 85/50 mmHg;

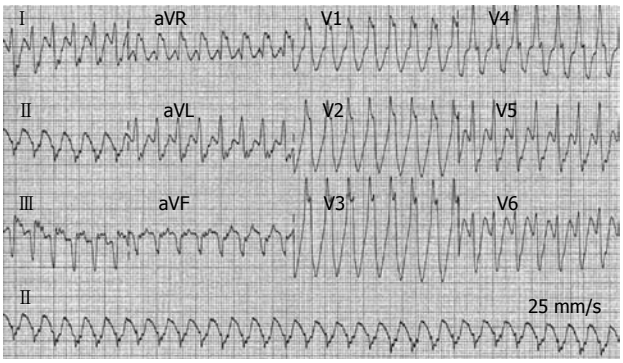

Figure 1 Twelve-lead electrocardiogram from a 47-year-old man (patient 1) with acute inferior myocardial infarction. Ventricular tachycardia at a heart rate of 170 beats/min with right bundle branch block morphology and left axis deviation is present.

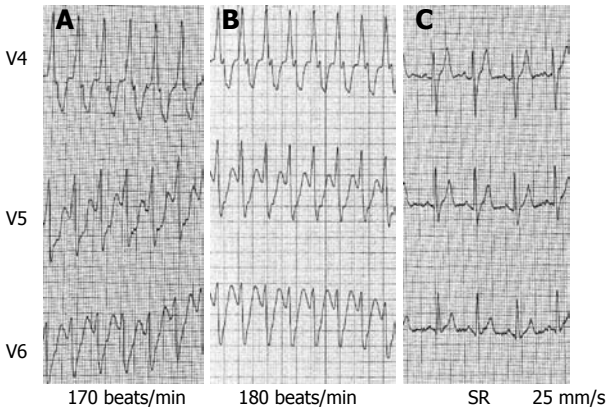

Figure 2 V4 to V6 leads also from patient 1. A: Ventricular tachycardia (VT) at admission with a heart rate (HR) of 170 beats/min, blood pressure (BP) 125/86 mmHg. Amiodarone (300 mg intravenous) did not interrupt VT, no substantial change was observed in VT cycle length, BP dropped to 89/46 mmHg; B: Immediately after a bolus of epinephrine (1 mg over 30 s), an increase in HR up to 180 beats/min was observed; C: Electrocardiogram 30 s after epinephrine bolus: sinus tachycardia (HR 110 beats/min) after VT termination.

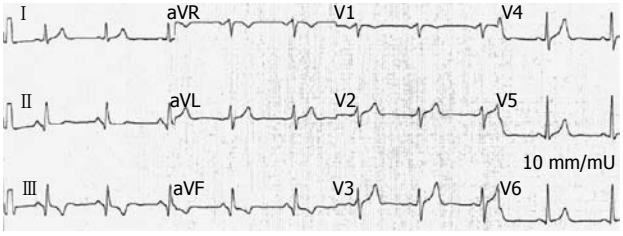

Figure 3 In patient 1, electrocardiogram performed 2 d after the reduction of ventricular tachycardia showed pathologic Q wave displaying a sequelae of a transmural myocardial infarction in inferior leads.

ICD interrogation was not attempted due to unavailability of this expertise at the time of patient management. One slow-rate infusion of IV epinephrine (1/10 000; 0.5 mg in 30 s) was then administered. VT termination occurred approximately 30 s after the end of the epinephrine infusion. Cessation was preceded by a slight increase in the VT rate (from 140 to 148 beats/min). During epinephrine administration the patient experienced brief

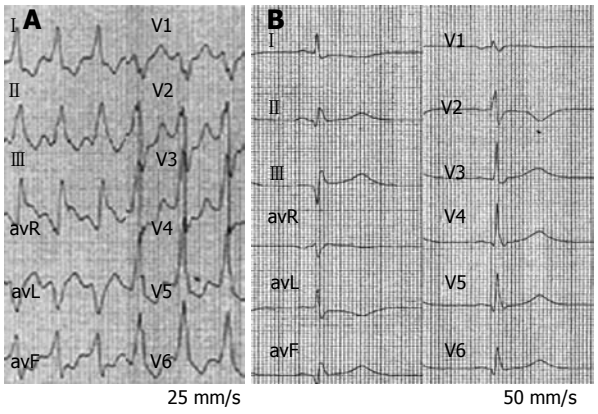

Figure 4 Electrocardiogram tracings from a 64-year-old man with ischemic dilated cardiomyopathy with an implanted cardioverter-defibrillator, on chronic treatment with bisoprolol and amiodarone (patient 2). A: Ten-lead electrocardiogram (ECG) at admission with sustained ventricular tachycardia (VT) at 140 beats/min with a left bundle branch block morphology and right axis deviation, blood pressure was 85/50 mmHg; B: Twelve-lead ECG 30 s after intravenous epinephrine bolus (0.5 mg over 30 s) shows sinus rhythm with pathologic Q waves in inferior leads. VT termination was preceded by a slight increase in VT rate from 140 to 148 beats/min (not shown).

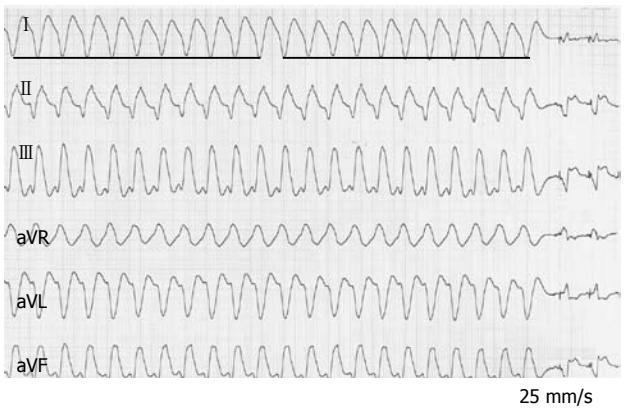

Figure 5 Six-lead electrocardiogram from a 47-year-old man in arrhythmic storm with ischemic dilated cardiomyopathy and an implanted cardioverter-defibrillator (patient 3). A sustained ventricular tachycardia (VT) with a heart rate of 140 beats/min is observed, blood pressure (BP) was 90/45 mmHg. intravenous amiodarone failed to terminate VT and decreased BP to 70/40 mmHg. Epinephrine (0.5 mg over 30 s) increased BP up to 125/85 mmHg, and VT terminated within 90 s, preceded by a small shortening of VT cycle length. At the end of the tracing, VT stopped. The bold line on the left side of the picture includes ten VT cycles; the bold line on the right side includes the last ten VT cycles before interruption.

chest discomfort and headache. Twelve-lead ECG in SR displayed pathologic Q waves in inferior and lateral leads (Figure 4B). Troponin I was slightly elevated (0.57 ng/mL). Serum electrolytes were within normal limits. Two-D echocardiography indicated a LVEF of 35%. Coronary angiography revealed a restenosis of the right coronary artery and a sub-occlusion of the ostium of a marginal artery. A drug-eluting stent was implanted in both lesions. An ICD interrogation before discharge did not register any arrhythmic event as the programming VT zone (150 beats/min) was higher than the patient's VT (140 beats/min). No further episodes of VT were observed during hospitalization, and the patient was discharged seven days later.

Patient 3

A 67-year-old man was admitted to the CCU due to an arrhythmic storm. The patient had a previous history of two MI (inferior and anterior) with a large akinetic area in the anterior wall and a low ejection fraction (LVEF < 20%). He was on chronic treatment with carvedilol (12.5 mg twice daily) and amiodarone (200 mg daily). An ICD had been implanted for primary prevention. As the patient developed permanent atrial fibrillation and remained symptomatic despite an optimal pharmacological regimen, the ICD was upgraded to CRT-D because of the need for chronic ventricular pacing. Four months previously, the patient had been admitted for syncope. At that time, ICD interrogation confirmed an arrhythmic storm. Coronary angiography showed an occlusion of the first marginal artery, but no intervention was performed due to the absence of viability. He underwent successful radiofrequency catheter ablation of two inducible monomorphic VTs. Four months later, the patient was re-admitted to the CCU due to another arrhythmic storm.

On admission to the CCU, a tachycardia with a VT pattern was recorded (Figure 5). BP was 90/45 mmHg. No electrolyte disturbance was observed. Troponin I was within normal limits. Once in the CCU, he experienced six more appropriate ICD shocks within 5 min. The underlying rhythm was VT with a HR of 140-150 beats/min. The ICD was programmed with one VT zone from 130 to 200 and the VF zone > 200 beats/min. Appropriate ICD shocks were delivered, but were followed by recurrence of the VT. Amiodarone (150 mg IV over 15 min) failed to terminate VT and decreased BP to 70/40 mmHg. Different protocols of direct manual overdrive pacing also failed to interrupt VT. One "aggressive" attempt transformed VT to VF managed by the ICD. Less than 60 s later, the VT re-appeared. BP steadily continued to decrease down to 65/30 mmHg. A bolus IV injection of epinephrine (1/10 000, 0.5 mg in 30 s) was administered while his BP was continuously monitored. Following a BP increase up to 125/85 mmHg, VT terminated within 90 s, preceded by a small shortening of VT cycle length (Figure 6). Treatment was supplemented by amiodarone IV (300 mg over 20 min) and atenolol IV (25 mg over 15 min). The next day, he underwent coronary angioplasty of the first marginal artery. The patient remained free of arrhythmias.

DISCUSSION

We illustrated three cases of drug-resistant sustained monomorphic VT with hemodynamic instability, in which the arrhythmia was terminated with IV epinephrine. All patients had coronary heart disease, but whether acute coronary syndrome was concerned is difficult to state. Indeed, a moderate troponin increase is also seen after tachyarrhythmias and post-VT ECGs did not show either ST-segment elevation or depression. Amiodarone

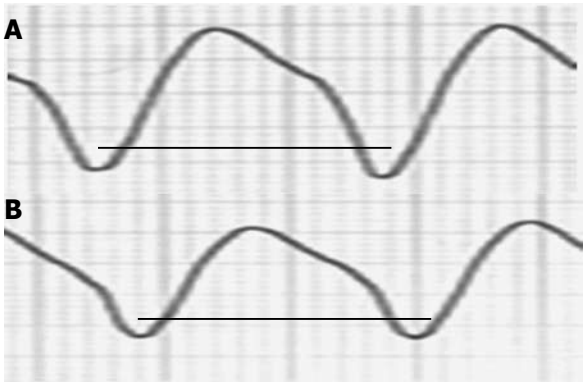

Figure 6 A ventricular tachycardia of 140 beats/min from a 47-year-old man in arrhythmic storm with ischemic dilated cardiomyopathy and an implanted cardioverter-defibrillator (patient 3) was interrupted by Epinephrine (0.5 mg over 30 s) after a small cycle length shortening. A: First ventricular tachycardia (VT) cycle of the patient in Figure 5; B: Last VT cycle before VT interruption. The two bold lines have the same length. A small VT cycle shortening before interruption is noted.

was ineffective in all patients. Two of the patients had an ICD. One ICD carrier experienced an arrhythmic storm. Termination of long-duration (> 2 h) VT was observed within 30-90 s after the administration of epinephrine, an alpha- and beta-sympathomimetic agent. An arrhythmic storm was stopped after epinephrine infusion. No short-term VT recurrence was observed, or in the patient with an arrhythmic storm.

VT is a common arrhythmia in coronary heart disease[12,13]. The standard of care for sustained VT with a pulse is to use either anti-arrhythmic drugs or external electrical shock. Advanced Cardiac Life Support (ACLS) protocol recommends the use of amiodarone, procainamide, mexiletine, sotalol or lidocaine as anti-arrhythmic drugs[7,8]. In clinical practice, amiodarone is the most commonly used anti-arrhythmic before electrical cardioversion[9,10].

When to use epinephrine in sustained VT

In cases of drug-resistant poorly tolerated VT, immediate external electrical cardioversion must be attempted. However, there are cases in which VT recurs immediately after the shock, and cardioversion involves the need for anesthesia when the patient is still conscious. Although shocks delivered by ICD often effectively terminate VT, in some patients VT can restart quickly, as the ICD is unable to prevent arrhythmia. In this context of arrhythmic storm, sedation and anti-arrhythmic drugs are of great interest[14]. However, in the setting of coronary disease, either acute phase or scar-related VT, effective drug therapy is limited to lidocaine, mexiletine, procainamide, amiodarone and beta-blockers[15,16]. However, the first three cited drugs are not commonly available or used in remote medical centers or in low-income countries. Moreover, in some cases, ICD-carriers live or are travelling in a part of the world where ACLS is not provided at all, and only amiodarone is available. In the past, some case reports described interruption of sustained VT using intravenous sympathomimetic amines[2-4]. Based on the cases reported herein, low doses of IV epinephrine may be able to terminate sustained monomorphic VT, when the arrhythmia is refractory to amiodarone used alone or in combination with beta-blockers and electrical cardioversion.

Possible mechanisms of VT reduction by epinephrine

Two possible mechanisms may underlie the interruption of a monomorphic sustained VT by low doses of intravenous epinephrine.

Effects on BP, baroreceptor stimulation and coronary perfusion: Epinephrine has an alpha-sympathomimetic effect by which systemic BP is known to increase. This increase in BP could have two different effects. The rise in BP could result in better coronary artery perfusion, thus inhibiting ischemia-induced VT (patient 1). Alternatively, a baroreceptor-mediated increase in parasympathetic tone *via* the carotid body could lead to VT termination *via* vagal stimulation[17]. The latter hypothesis is difficult to demonstrate because vagal myocardial innervation is not completely defined in humans[18]. Finally, a direct effect of epinephrine on coronary perfusion, through coronary vasodilatation, is a possible mechanism in ischemia-related VT[19].

Modification of conduction properties: Conduction velocity and refractoriness of the myocardium are modified in opposite ways by epinephrine and beta-blockers and/or amiodarone. Adrenaline-dependent increased conduction velocity with shortening of VT cycle length could be responsible for extinction of the circuit by a simple mechanism of head-tail conjunction. Considering a circus movement reentry as the mechanism of the VT, the acceleration of the VT by epinephrine will translate into progressive shortening of the excitable gap until it will be so short that the "head" meets the "tail", and then the circuit becomes extinct. This being the case, termination of the VT will be preceded by a faster epinephrine-induced, small transient VT acceleration in all 3 patients. A change in myocardial fiber stretch by epinephrine could also modify the circuit components favoring VT interruption[20].

On the other hand, amiodarone could limit the refractoriness shortening of epinephrine which, combined with an increased conduction velocity determined by epinephrine itself, might be responsible for tachycardia interruption because of a residual refractoriness prolongation of part of the circuit[21-23]. Tonet *et al*[24,25] have shown that beta-receptor blockade by beta-blocker agents and chronic amiodarone therapy converts VT to SR *via* prolongation of the refractory period and cycle length. Thus, pre-treatment with amiodarone might enhance the effectiveness of IV epinephrine which, reducing repolarization dispersion, interrupts reentry and could be useful in cases of electrical storm.

Detailed analysis of the cases reported herein can support both hypotheses. In all patients, elevation of BP preceded VT termination, suggesting a mechanism of better coronary artery perfusion or parasympathetic

stimulation[26,27]. Otherwise, in all patients, the cycle length of the VT shortened just before the return to SR, suggesting a modification in myocardial conduction properties with an increase in conduction velocity and a subsequent shortening of the VT cycle length able to induce reentry interruption by a head-tail conjunction mechanism. All patients were previously treated with amiodarone, whose antiarrhythmic properties mainly concern refractoriness prolongation, especially when taken on a chronic basis[28]. A possible intricate electrophysiological mechanism could also be conjectured, that is, a residual prolonged refractoriness by beta-blocker and amiodarone pre-treatment combined with increased velocity when epinephrine is administered[24].

Precautions on the use of epinephrine for VT

The most frequent and clinically significant side effects of IV epinephrine were not observed in our patients, which may be explained by the lower doses (≤ 1 mg) used[29]. The potential complications of epinephrine while performing cardiopulmonary resuscitation were described for doses greater than those that are the standard[8,9]. VF induction in subjects treated with bolus or continuous infusion of epinephrine for severe hypotension or cardiogenic shock have been reported, and the conversion of VT to VF is a matter of real concern[6,7]. It is of interest to note that in experimental models of chronically infarcted canine hearts, selective beta1-adrenergic stimulation alone does not cause dispersion of myocardial refractoriness and does not cause significant proarrhythmia[30]. Also, in dogs with experimentally induced myocardial infarction, infusion of isoproterenol increases the incidence of inducible VT, but does not facilitate the induction of VF[31]. In any case, given the potentially harmful intervention in the setting of coronary heart disease, further research to explore the mechanism of action, its effectiveness and its safety is mandatory. In our patients, evidence of acute ischemia was modest with small increases in troponin level; this factor is to be considered when deciding whether to use epinephrine.

Clinical implications

It is well known that spontaneous episodes of VT can accelerate, slow, or even terminate spontaneously. However, in all cases, the termination of a long-duration (> 2 h) VT was rapidly observed after 90 s or less following epinephrine administration, preceded by a shortening of the VT cycle length before SR restoration, strongly suggesting an epinephrine-related effect. Although the use of sympathomimetic amines to treat sustained monomorphic ischemic VT could be questionable, mainly because its rationale is against the current paradigm of the pathophysiology of ischemic VT as well as the wide availability of appropriate drugs and external cardioversion, the effect observed in our cases merits further investigation. Remote medical centers and under-resourced countries with difficulties providing ACLS management may be concerned. We might also speculate on a possible protective effect of amiodarone and beta-blocking treatment for the prevention of the degenerative arrhythmic effects of epinephrine itself. In fact, VT cycle length was slightly shortened in all patients, without any change in VT morphology or VF degeneration.

Study limitations

Some limitations merit consideration. First, as these are only three observational cases, this remains a non-conclusive assessment of the efficacy and safety of this unconventional "heroic" intervention. Second, although all subjects had beta-blocker and/or amiodarone resistant VT, another antiarrhythmic drugs such as lidocaine or mexiletine were not attempted before the epinephrine regimen. Third, the treatment of patients 1 and 2 is not a conventional one and should not, therefore, be advocated at the first intention. Fourth, one of the questions raised by our observations is whether VT was actually self-limiting or truly stopped by the epinephrine injection; however, the rapid onset of action in a longstanding sustained monomorphic VT serves to support considering it a direct effect of epinephrine.

Conclusion

Low dose IV epinephrine may terminate sustained monomorphic VT refractory to amiodarone used alone or in combination with beta-blockers and electrical cardioversion. Although potentially harmful, epinephrine may be an alternative effective therapy for refractory VT, particularly in remote medical centers and low income countries where advanced cardiac life support is under-provided. This treatment requires a clinical study.

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Muscat, Oman

January 27, 2012
ESC Global Scientific Activities at the 23rd Annual Conference of the Saudi Heart Association
Riyadh, Saudi Arabia

January 29–31, 2012
Integrated management of acute and chronic coronary artery disease
Innsbruck, Austria

January 30, 2012
Webinar on "Best of Euroecho 2011"
Sophia Antipolis, France

February 1-3, 2012
American Heart Association and American Stroke Association International Stroke Conference 2012
New Orleans, Louisiana, United States

February 3-5, 2012
6th Asian-Pacific Congress Of Heart Failure 2012
Chiang Mai, Thailand

February 9, 2012
4th British Society for Heart Failure Medical Training Meeting
London, United Kingdom

February 23–25, 2012
Advanced Invasive Cardiac Electrophysiology
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February 24–26, 2012
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March 8–10, 2012
Cardiac Pacing, ICD and Cardiac Resynchronisation
Vienna, Austria

March 8–10, 2012
24th Colombian Congress of Cardiology and Cardiovascular Surgery
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March 10-11, 2012
23rd International Meeting "Cardiology Today"
Limassol, Cyprus

March 14-18, 2012
Ninth Mediterranean Meeting on Hypertension and Atherosclerosis
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March 28–31, 2012
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INSTRUCTIONS TO AUTHORS

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Telephone: +86-10-59080039
Fax: +86-10-85381893
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Acknowledgments

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2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 285-287

In press

3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

Both personal authors and an organization as author

5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

No author given

6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

Volume with supplement

7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

Issue with no volume

8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; (**401**): 230-238 [PMID: 12151900 DOI:10.1097/00003086-200208000-00026]

No volume or issue

9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

Books

Personal author(s)

10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)

11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

Author(s) and editor(s)

12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings

13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

Conference paper

14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

Electronic journal (list all authors)

15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

Statistical data

Write as mean ± SD or mean ± SE.

Statistical expression

Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as υ (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

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