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REVIEW

The association of micronucleus frequency with obesity, diabetes and cardiovascular disease

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15 **Obesity and metabolic syndrome (MetS) are serious and**
growing health care problems worldwide, leading an
increased risk for type 2 diabetes (T2D) and cardiovascu-
lar disease (CVD). Over the past decade, emerging
evidence has shown that an increased chromosomal
damage, as determined by the cytokinesis-block micronu-
cleus (CBMN) assay, is correlated to the pathogenesis of
metabolic and CVD. An increased micronuclei (MN)
frequency has been demonstrated in peripheral blood
lymphocytes of patients with polycystic ovary syndrome,
a common condition in reproductive-aged women associ-
ated with impaired glucose tolerance, T2D mellitus and the
MetS. High levels of MN have been detected to be
significantly correlated with T2D as well as with the
occurrence and the severity of coronary artery disease
(CAD). Long-term follow-up studies have shown that an
increased MN frequency is a predictive biomarker of
cardiovascular mortality within a population of healthy
subjects as well as of major adverse cardiovascular events
in patients with known CAD. Overall, these findings
support the hypothesis that CBMN assay may provide an
useful tool for screening of the MetS and its progression to
diabetes and CVD in adults as well in children. Large
population-based cohorts are needed in order to compare
the MN frequencies as well as to better define whether MN
is a biomarker or a mediator of cardiometabolic diseases.

Introduction

Cardiovascular disease (CVD) and type 2 diabetes (T2D) are
 leading causes of morbidity and death in the Western society,
 accounting for a great proportion of health care costs (1,2).
 45 These diseases share common risk factors, including obesity,
 insulin resistance, blood glucose, lipid oxidation toxicity and
 low-grade inflammation, and they coexist in a great number of
 patients.

[AQ5] The incidence of CVD, especially coronary heart disease
 50 (CAD), and diabetes is increased in persons with the metabolic
 syndrome (MetS), a cluster of metabolic abnormalities
 including central obesity, hypertension and insulin resistance
 [AQ6] (3). According to clinical criteria from a recent joint interim
 statement of the Scientific Societies of diabetes and cardiology

(3), any three of the five criteria reported in Table I are
 diagnostic for the MetS. The MetS is a growing clinical and
 public health problem worldwide, and it is reaching pandemic
 proportions from the United States to Europe as well as in
 developing countries, such as India and China (4). In these
 countries, an increasing number of adolescents and young
 60 adults show signs of the MetS that may markedly affect the
 incidence and prevalence of diabetes mellitus and CVD (4,5).
 Indeed, it has been estimated that people with the MetS are at
 twice the risk of developing CVD as compared with those
 without the syndrome and experience a 5-fold increased risk of
 65 T2D (5).

T2D is the most common form of diabetes in humans,
 affecting 95% of all diagnosed cases. The global number of
 people with diabetes was 151 million in 2000, and it is
 projected to increase to 221 million in 2010 (an increase of
 70 46%) both in developed and developing countries (6)

Accordingly, the epidemic of diabetes and its complications
 confers major burdens of mortality, morbidity and health care
 costs. T2D is characterised by an increased risk for the
 development of microvascular complications (neuropathy,
 75 renal disease and retinopathy) and macrovascular disease
 (coronary heart disease, cerebrovascular disease and peripheral
 vascular disease). Coronary artery disease is the most common
 complication and cause of morbidity and mortality in patients
 with T2D (6). 80

Furthermore, it is also important to note that there is
 considerable evidence of an increased cancer risk in diabetic
 patients, being more evident for primary liver cancer and
 pancreatic cancer (7).

Primary prevention is, thus, a fundamental strategy for
 85 diminishing the overall burden of MetS, and the identification
 of biomarkers for early detection and prognosis is of the utmost
 importance in order to reduce the progression to T2D and CVD.

In recent years, the role of chromosomal instability,
 mediated by oxidative DNA damage and shortened dysfunc-
 90 tional telomeres, in the pathogenesis of CVD and diabetes has
 attracted a continuously growing research interest (8).

The cytokinesis-block micronucleus (CBMN) assay is the
 most frequently used chromosomal biomarker in human
 lymphocytes in order to study genotoxicity and cytotoxicity
 both *in vitro* and *in vivo* (9,10). 95

It is now well established that the CBMN assay in its
 [AQ7] comprehensive 'cytome' mode provides simultaneous infor-
 mation on chromosomal breakage, chromosome rearrange-
 ments and gene amplification as well as other critical events,
 100 such as cell death (both apoptosis and necrosis) and cell
 cytotoxicity (10).

Therefore, the CBMN assay may prove to be very useful in
 the prediction and possibly the clinical management of chronic
 degenerative diseases, including diabetes and CVD. 105

The purpose of the present article is to review the current
 knowledge concerning the application of CBMN test in the

Table 1. Diagnostic criteria for MetS defined by the presence of ≥ 3 risk factors

Abdominal obesity (waist circumference)	Men > 94 cm (European); >102 cm (USA) Women > 80 cm (European); ≥ 88 cm (USA)
Elevated triglycerides	≥ 150 mg/dl (1.7 mmol/l)
Reduced HDL-C	Men < 40 mg/dl (0.9 mmol/l) Women < 50 mg/dl (1.1 mmol/l)
Elevated blood pressure	≥ 130 systolic blood pressure ≥ 85 mmHg diastolic blood pressure
High fasting glucose	≥ 100 mg/dl (5.6 mmol/l)

HDL-C, high-density lipoprotein-cholesterol.

pathogenesis of cardiometabolic diseases as well as to discuss the future research perspectives in order to elucidate the prognostic power of micronuclei (MN) as biomarker for the detection and the progression from MetS to T2D mellitus and CVD.

Significant achievements

MN, obesity and MetS

Obesity is a main causative factor in the development of MetS and is a growing worldwide problem. Recent estimates show that in the USA, about a third of adults 20–74 years of age are obese with body mass index (BMI) ≥ 30 and another third are overweight (BMI 25.0–29.9) (11). Importantly, pediatric obesity is also increasing at an alarming rate, highlighting the importance of accurate, timely identification of metabolic complications of obesity (12).

Obesity is a natural consequence of over nutrition and sedentary lifestyle that dysregulates metabolic processes, including action of insulin on glucose-lipid-free fatty acid metabolism, blood glucose, blood pressure and lipids.

Obesity, particularly abdominal obesity, is a major risk factor for MetS and plays a central role in the 'insulin resistance', often leading to T2D mellitus and the development of atherosclerotic CVD. The mechanisms responsible for developing the MetS are not well known, but it is likely that ectopic fat accumulation (such as visceral and hepatic fat accumulation) and the pro-inflammatory state are central to the development of insulin resistance and hyperinsulinemia.

In addition, chronic oxidative stress might be a major mechanism underlying obesity-related co-morbidities (13). Specifically, the inability to store fatty acids in subcutaneous adipose tissue (14), and the expansion of abdominal fat determine the exposure of body organs to an excessive flux of free fatty acids (FFAs), which are known to affect insulin signal transduction pathways, and induce endothelial dysfunction, due to increased reactive oxygen species (ROS) generation and oxidative stress.

Prolonged release of FFAs is implicated in the development of diabetes since it promotes insulin resistance and the associated loss of pancreatic beta-cell function (Figure 1).

Insulin resistance is also by itself a risk factor for CVD. It blunts vascular production of nitric oxide, a factor that is crucial to the normal vasodilatory response and endothelial function.

Furthermore, visceral obesity is strongly associated with high plasma triglycerides and low plasma high-density lipoprotein-cholesterol, and with high plasma concentrations of apolipoprotein B (apoB)-containing lipoproteins. The generation of ROS, as the result of an imbalance between

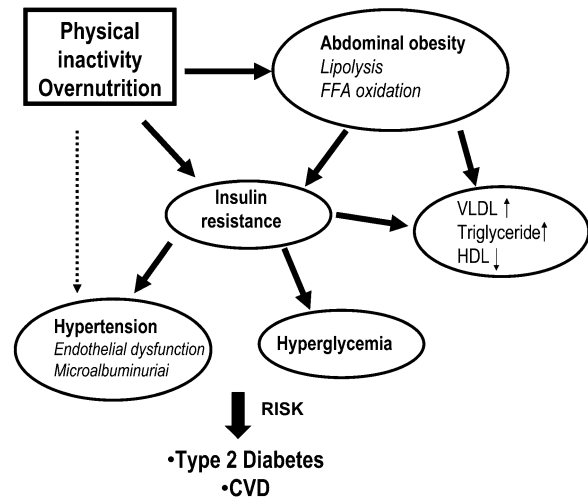


Fig. 1. Schematic representation of progression and outcomes of the MetS. HDL, high-density lipoprotein; VLDL, very low-density lipoprotein.

tissue free radicals and antioxidants as well as of multiple by-products of glucose and (pro)inflammatory molecules, may also exert adverse effects on the DNA, contributing to the initiation and progression of CVD and diabetes (8).

Indeed, some studies have evidenced the association of overweight and obesity with enhanced levels of different indices of oxidants (13,15). In these studies, DNA damage levels were significantly higher in both subjects with obesity (16) and with MetS (17) than in normal subjects. Importantly, subjects showed a significant decreasing trend in total antioxidant capacity levels and a significant increasing trend in DNA damage values with the increase in the number of metabolic disturbances (17).

However, there is yet very little information available on the association between MN levels, obesity and MetS. Recently, several papers have demonstrated an association between increased MN frequency and polycystic ovary syndrome (PCOS) (18–20). PCOS is a complex endocrine condition affecting 4–8% of women of reproductive age. Its primary manifestations are the reproductive complications of menstrual dysfunction, infertility and hyperandrogenism and the metabolic complications of insulin resistance, an increase in cardiovascular risk factors and an increased risk of T2D mellitus in conjunction with a high incidence of generalised and abdominal obesity (21).

It is noteworthy that elevated levels of genomic instability (greater number MN and chromosome malsegregation) present in women with PCOS was positively correlated with the BMI and insulin resistance levels (17,18).

MN and diabetes mellitus

Diabetes, termed 'diabetes mellitus' in clinical terminology, is a serious and growing health care problem worldwide and is associated with severe acute and chronic complications. Type 1 insulin-dependent diabetes accounts for only 5–10% of all diabetic patients and results from an absolute deficiency of insulin caused by the destruction of insulin-secreting pancreatic β -cells. T2D, which is the most prevalent form, explaining 90–95% of cases of diabetes mellitus, is characterised by insulin resistance and relative insulin deficiency. In both type 1 and T2D, chronic hyperglycaemia is the primary cause of the clinical complications of the disease.

Diabetic complications in target organs arise from chronic elevations of glucose via increased production of ROS and reactive nitrogen species and subsequent oxidative stress. Excessive production of oxygen-free radicals through glucose auto-oxidation and non-enzymatic glycation, especially in diabetic patients with poor glycemic control, can accelerate oxidative damage to the macromolecules, including DNA damage.

During the last years, increased oxidative DNA damage in diabetes has been well documented, and it has been suggested that it may be a useful clinical marker in order to predict diabetic complications, such as microangiopathy and macroangiopathy (22–26). Interestingly, it has also been found that oxidative DNA damage as observed in the comet assay was higher in individuals with T2D mellitus as compared to those with type-1 diabetes (25). As indicated in Figure 2, it is also noteworthy that levels of oxidative DNA damage were significantly more elevated in patients with CAD and dyslipidemia or T2D (25).

Furthermore, DM is a strong determinant of chromosomal DNA damage in both in type-1 diabetes mellitus and T2D (28–31,27). Patients with type-1 diabetes showed a significantly higher sister chromatid exchange frequency than healthy subjects (28,30). In women of the Ludwigshafen Risk and Cardiovascular Health study a high level of stable chromosomal aberrations in peripheral lymphocytes is associated with T2D and directly correlated with the risk of early diabetes-related death (29).

As regards MN assay, no significant difference was found in MN frequency in type-1 diabetic patients as compared with controls (30). On the contrary, significantly high levels of MN

frequency were found in patients with type 2 with no microvascular or macrovascular complications (31). In a large population of patients undergoing coronary angiography, we found that T2D was the major independent determinant of an increased MN frequency in circulating lymphocytes of patients with ischemic heart disease (27). We have also shown that diabetes was a significant determinant of MN levels, even when the patients were stratified according to the presence of CAD (Figure 2).

Recently, Zúñiga-González *et al.* (32) demonstrated that either controlled (glycosylated haemoglobin levels <7%) or uncontrolled diabetic patients (glycosylated haemoglobin levels >7%) had ~2-fold higher frequency of MNs in buccal mucosa samples than healthy subjects. There was also evidence of an increased MN frequency among patients with uncontrolled type 1 diabetes as compared with patients with a good metabolic control. Furthermore, a significant reduction in MN was observed after folate supplementation for 30 days (32).

MN and CVD

The American Heart Association reports that CVD is the dominant health problem in the Western Society (1) and is expected to be the number one cause of death worldwide in 2020 (2).

Major clinical manifestations of CVD include myocardial infarction, coronary artery disease, stroke, peripheral artery disease and congestive heart failure. In most cases, these clinical conditions result from atherosclerosis, a progressive and inflammatory disease of the arterial wall, characterised by focal thickening and luminal obstruction (33).

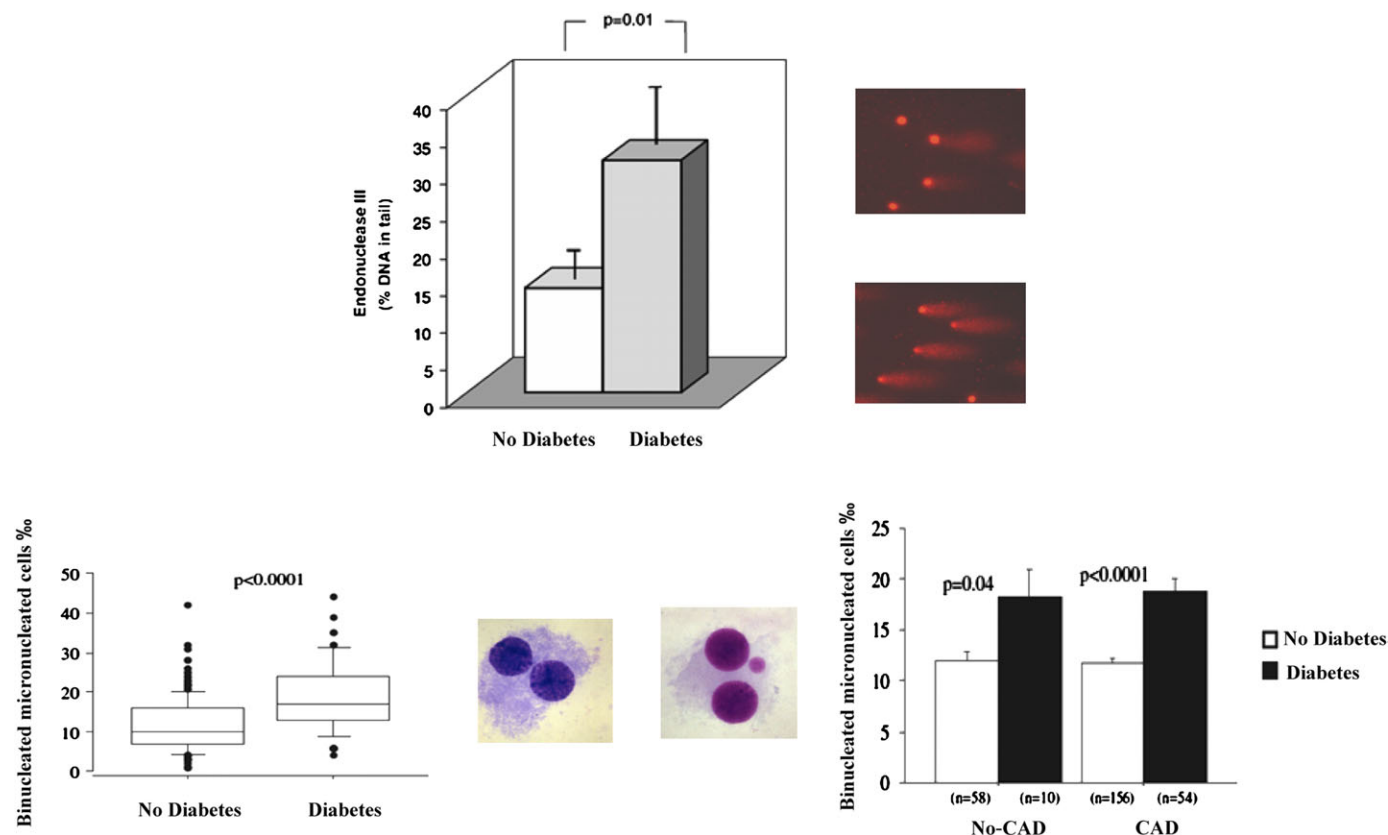


Fig. 2. Diabetes is a strong determinant of chromosomal and oxidative DNA damage in patients with ischemic heart disease as measured by comet assay and CBMN assay. [Figure adapted from references (25) and (27); statistical differences were evaluated by Student's *t*-test after the logarithmic transformation of data].

At the present time, there is consistent evidence supporting the notion that oxidative stress-induced-genetic instability is a relevant contributor of atherosclerotic plaque development and its acute complications (34–36).

Indeed, DNA damage is present in human atherosclerotic plaque ranging from ‘macro’ damage, including deletions or additions of whole chromosomes or parts of chromosomes to ‘micro’ damage which includes loss of heterozygosity and microsatellite instability (mutations in DNA regions that may affect gene expression), DNA strand breaks and modifications of DNA (including oxidation) or DNA adducts (37–46).

Specifically, the existence of chromosomal aneuploidy in multinucleated endothelial cells may be important in atherogenesis by strongly expressing low-density lipoproteins (LDL) receptors and increasing LDL uptake to the subendothelial intima (38). Chromosomal aberrations can also occur in vascular smooth muscle cells of human atherosclerotic plaques, especially in unstable plaques (37,39).

Human plaques show markers of oxidative damage, including DNA strand breaks and oxidative modification of guanine residues in DNA (45). Interestingly, DNA strand breaks, oxidised pyrimidines and altered purines are also significantly higher in leukocytes of patients with CAD, and DNA strand breaks levels are higher in patients with acute coronary syndrome as compared with patients with stable CAD (47).

During the last years, our group and others have demonstrated the presence of chromosomal damage in circulating cells of patients with CAD using the CBMN assay (48,49). In these studies, an elevated frequency of MNs was significantly correlated with both the occurrence (48,49) and the severity of coronary artery disease (48). Furthermore, it is also important to note that further chromosomal DNA damage is produced in the peripheral lymphocytes of patients undergoing repeated X-rays medical investigations and chronically administered drugs, such nitrate therapy (27,50,51).

Finally, recent evidences have emphasised that both micro-nutrient deficiencies and genetic variations in enzymes involved in homocysteine (Hcy) metabolism may contribute to an increased risk for CAD (34). In fact, hyperhomocysteinemia has been identified as an independent risk factor for cerebral, coronary and peripheral atherosclerosis, although the pathological mechanism of this risk is not fully understood (34). High levels of Hcy have been also reported to be an increased risk factor for neural defects, Alzheimer’s disease and loss of cognitive functions (52).

Accordingly, it is interesting to note that C677T polymorphism within the 5,10-methylenetetrahydrofolate reductase gene and the levels of plasma Hcy and vitamin B12 are important determinants of MN frequency in patients with CAD, supporting the hypothesis that pathways of folate metabolism may be a risk factor for atherosclerosis by influencing genomic stability (53).

MN and clinical follow-up

Recently, we also validated the predictive value of MN in peripheral blood lymphocytes for clinical outcome of CVDs. The cytogenetic monitoring, carried out in 1991–93 on 1650 healthy subjects, and aimed to define some reference biological parameters of a ‘normal population’ to be used as a control for further analysis, gave us the opportunity to carry out a nested case–control study (54). The surviving status, or the cause of death, was monitored for all subjects until January 2005. At the end of the follow-up, 111 deaths were recorded and 39 deaths for CVD cases were observed. A significantly higher MN

frequency observed at the time of the recruitment in the CVD group was found in comparison with the control group (54).

The Kaplan–Meier analysis demonstrated a significantly shorter survival for CVD for subjects with a higher MN frequency as compared to subjects with a lower frequency (Figure 3).

Finally, our findings indicate that MN levels are the significant predictors of future cardiovascular events in patients with known CAD.

In a long-term follow-up prospective study of 178 consecutive patients, we assessed the relationship between tertiles of MN and the risk of major adverse cardiovascular events (cardiac death, myocardial infarction, stroke, congestive heart failure, unstable angina, coronary and peripheral revascularization). After a mean follow-up of four years, we found that the overall event-free survival was 77.5, 70.4 and 49.0% in patients with the low, medium and upper tertiles of MN, respectively (Figure 3). We found that patients in the upper tertile had a 2.2-fold increased risk of developing adverse cardiac events (55).

Important knowledge gaps

Overall, these studies indicate that an increased MN frequency is correlated to the pathogenesis of metabolic and CVD (Table II). The results support the hypothesis that the CBMN assay may expand the prognostic power of established biomarkers for the detection and the progression from MetS to T2D mellitus and CVD.

Despite the findings from prior studies, many gaps in knowledge currently exist in our understanding of the association between elevated MN frequency and adverse outcomes in patients with cardiometabolic disorders.

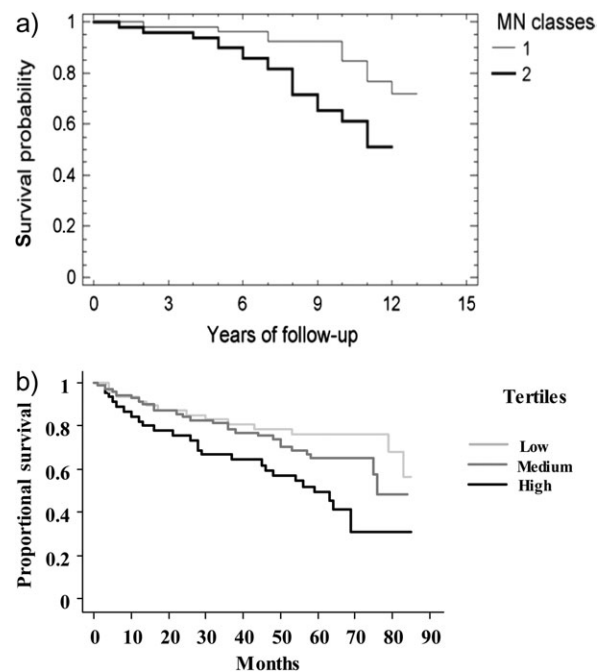


Fig. 3. Kaplan–Meier curves showing cumulative rates of survival for cardiovascular mortality within a population of healthy subjects (a) and for major adverse cardiovascular events in patients with known CAD (b) according to the levels of MN. [Figure adapted from references (54) and (55)].

Table II. Human studies showing associations between MN and metabolic/CVDs

Study groups number of patients	Results	References
Polycystic ovary syndrome		
n = 14	+	(18)
n = 19	+	(19)
n = 36	+	(20)
Type 1 diabetes		
n = 35	–	(30)
n = 55 ^a	+	(32)
T2D		
n = 15	+	(31)
n = 64	+	(27)
n = 23 ^a	+	(32)
Coronary artery disease		
n = 147	+	(49)
n = 210	+	(27)

^aMN in buccal mucosa samples.

One of the most important gaps is the question as to whether MN is an independent cardiometabolic risk factor or only a surrogate of the chronic oxidative stress and/or inflammation.

Indeed, more and more evidence suggests that telomere shortening associated with chromosome instability may be key determinants to the pathogenesis of cardiometabolic diseases by inducing premature vascular senescence (8). Telomeres are, indeed, necessary for the genomic stability and integrity, preventing chromosomal fusion by cellular DNA repair processes. Progressive telomere shortening has been demonstrated in peripheral blood cells from patients with MetS, diabetes mellitus, CAD and premature myocardial infarction (8).

It is interesting to note in this context that a recent report reported a significant correlation between telomere shortening and MN frequency (56). These data support the hypothesis that the levels of genetic instability (blood telomere length and MN) may be useful biological markers for cardiometabolic diseases.

However, the role of both telomere shortening and chromosomal damage remains a poorly understood process in the pathogenesis of cardiometabolic disease. Thus, establishing conclusively whether genetic instability plays a causative role in vascular dysfunction still remains to be established.

Particularly, an important knowledge gap is clarifying the relationship in humans between MN frequency in lymphocytes and that in cells of the vascular wall, such as endothelial cells.

Moreover, a better understanding of the association between MN and cardiometabolic disease need to carefully consider a series of clinical and confounding factors such as the quality of the metabolic control, the type of diabetes, the duration of the disease, the influence of genetic polymorphisms, the drugs and radiation imaging treatment used for therapy. To gain more insights in the role of MN in cardiometabolic disease, we also need large population-based cohorts with MN and the associated nucleoplasmic bridge and nuclear bud biomarker measurement at multiple time-points.

A coordinated effort involving multiple centers is warranted in order to compare the MN frequencies as well as to better define whether MN is a biomarker or a mediator of cardiometabolic diseases.

Concluding remarks and future work

Increasing burdens of obesity, MetS and T2D require preventive and therapeutic interventions against these conditions

and the accompanying CVD risk. In the clinical practice, there is the need for easy, not expensive and safe biomarkers for the early detection of diseases and also to identify high-risk subjects who might benefit from increased screening surveillance.

The simplicity, rapidity and sensitivity of the CBMN assay may provide an useful tool for screening of the MetS in adults as well as in children. In order to explore this potential, further studies are needed to investigate in more depth what is the role of MN in predicting the risk of these diseases, especially of diabetes, since there is no previous record. Specifically, there is a need for prospective studies to establish whether increased MN frequency appears to be related to ‘lifestyle diseases’, which are associated with hypernutrition and lack of exercise, and are characterised by metabolic abnormalities, i.e. primarily insulin resistance and obesity.

Clinical research must seek to define whether MN frequency can be prevented by tight control of blood glucose, pressure and lipids and/or by caloric restriction, improvement in physical fitness and antioxidant supplementation.

This point is of particular interest in relation to evidence showing that a range of healthy lifestyle factors, including exercise, are associated significantly with reduced MN frequency (57).

According to recent evidence, supporting the fascinating concept that the MetS may originate *in utero* (58,59), further studies should also determine the impact of maternal over nutrition and diet composition on MN frequency of the foetus and newborns. The evaluation of chromosomal damage can be an important pathogenetic factor and an additional prognostic predictor—evaluating a biologic dimension presently ignored by the current stratification of risk—and a potential target of therapeutic intervention.

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