10. CIRCULATING NUCLEIC ACIDS AS DIAGNOSTIC TOOL

Maurizio Ferrari, Laura Cremonesi, Silvia Galbiati

Università Vita-Salute San Raffaele, ²Genomic Unit for the Diagnosis of Human Pathologies, San Raffaele Scientific Institute, Milan, Italy and ³Diagnostica e Ricerca S.Raffaele SpA, Milan, Italy

10.1 Introduction

Circulating nucleic acids are present in small amounts in the plasma of healthy individuals. However, the discovery of circulating nucleic acids has long been explored for the non-invasive diagnosis of a variety of clinical conditions. The first studies concerning the detection of circulating DNA were investigated for finding various forms of cancer. Metastasis and recurrence in certain tumor types have been associated with the presence of high levels of cancer-derived DNA in circulation. The detection of fetal DNA in maternal plasma in pregnant women is useful in detecting and monitoring fetal diseases and pregnancy-associated complications. Similarly, levels of circulating DNA in acute medical emergencies including trauma and stroke, have been reported to be increased and have been explored as indicators of clinical severity. In the last few years, other than circulating DNA, much attention and effort has been put into the study of circulating RNA, starting from the detection of tumor-derived RNA in the plasma of cancer patients. Soon after that, detection of circulating fetal RNA in maternal plasma was described. Plasma fetal RNA detection looks to be a promising approach for the development of gender- and polymorphism-independent fetal markers for prenatal diagnosis and monitoring complications during pregnancy. This development also opens up the possibility of non-invasive prenatal gene expression profiling by maternal blood analysis.

10.2 Noninvasive prenatal diagnosis

The discovery of cell-free fetal DNA in maternal plasma by Dennis Lo in 1997, outlined new scenarios for non-invasive prenatal diagnosis. The quantitative analysis of the free fetal DNA showed that this can be made up of as much as 6.2% of the total DNA present in the maternal plasma (1). A deep and extensive search for non-invasive techniques of fetal DNA sampling has been carried out to substitute invasive prenatal diagnosis that carry a significant risk of miscarriage.

Fetal DNA release into maternal plasma has been shown to be a very early physiological phenomenon increasing progressively throughout pregnancy (2-3). Circulating fetal DNA molecules (SRY gene) have been detected in maternal plasma in the first trimester (starting from the 5th gestational week) onwards with an accuracy approximately of the 100% (2-3). This approach has been used for the prenatal investigation of sex-linked diseases and fetal RhD status condition in which the fetus presented a gene absent in the mother.

A majority of research groups use sequences of chromosome Y in male embryos as a marker of fetal DNA and standardization of the assays, due to the fact that a woman (46,XX) does not possess this chromosome in her genome. Sexing analysis is also important, mainly for diseases with a recessive X-linked pattern of inheritance, with female being normal or being carriers of the mutation, but healthy, while male are normal or affected by the disease. An
application of fetal sexing is Congenital Adrenal Hyperplasia (CAH) an autosomal recessive genetic disease which carries a defect in 21- hydroxylase deficiency. Homozygous girls for this pathology are born with masculinization of the external genitalia and often require surgical operations as opposed to affected boys whom present normal external genitalia. Prenatal treatment of CAH with dexamethasone to prevent genital ambiguity has been successfully used (4). However, to minimize the side effects, the interruption of therapy has been indicated in the case of affected or normal male embryos and normal female embryos. For this reason, fetal sexing is necessary during pregnancy and is usually carried out by invasive methods. Noninvasive fetal sexing based on free fetal DNA in maternal plasma would bring the additional advantage of early discontinuation of medication in the case of male embryos (5).

Moreover, many genetic diseases are caused by mutations that result in subtle differences between the sequences of maternal and fetal DNA, such as achondroplasia (6), ã and ß thalassemia (7- 9).

Rh alloimmunization is a crucial problem in medical and obstetrical clinical practice, potentially leading to hemolytic disease in the newborn. For pregnant negative Rh women (15% of the population), a positive Rh embryo involves a 16% risk of sensitization to the Rh antigen. Diagnostic procedures and invasive therapy may be necessary to reduce perinatal mortality of positive Rh embryos (10). Thus, the early detection of fetal RhD status through fetal DNA in the plasma of negative Rh mothers is of great importance in defining the need for interventions, with known risks of gestational loss, or of gestational immunoprophylaxis.

10.3 Preeclampsia/Intrauterine Growth Restriction (IUGR)

Preeclampsia is a hypertensive disorder affecting approximately 5% of pregnancies and is still one of the main causes of mortality of both the fetus and the mother. Typical symptoms are maternal hypertension and proteinuria, which usually develop in the late second or third trimester of pregnancy (11).

Increased fetal DNA release can be a marker of pathological conditions affecting both the fetus and the placenta (12-19). Abnormal placentation has often been found to be involved in the pathogenesis of intrauterine growth restriction (IUGR) and preeclampsia, which can occur either isolated or in combination. IUGR is defined as the presence of an ultrasonographically estimated fetal weight below the fifth percentile confirmed post-natally, in the absence of chromosomal and structural abnormalities. Preeclampsia and IUGR have been linked to abnormalities in trophoblast invasion into the placental bed. During normal pregnancy, trophoblastic invasion of uterine spiral arteries takes place reducing the vascular resistance and allowing adequate fetoplacental blood supply. In IUGR and preeclampsia this adaptive phenomenon is often insufficient, resulting in a diminished infiltration and modification of the spiral arteries, which lead to the maintenance of a high-resistance uterine circulation (20-21). Several studies have addressed the issue of quantifying fetal DNA in maternal plasma in pregnancies complicated by preeclampsia, and there is a general agreement of up to fivefold increased fetal DNA levels in the presence of this pathology (19, 22).

Since IUGR is mostly caused by impaired placental perfusion, similar to what is found in preeclampsia, it might be also associated with high levels of fetal DNA in maternal circulation.
The increase in the rates of circulating fetal and maternal DNA would correspond to the degree of severity of the illness and, therefore, the level of fetal DNA may serve as a marker of the prognosis and severity of the clinical picture (23-24).

Although most researchers use the Y-chromosome in this specific application, other non-gender markers have been studied, including epigenetic markers, to improve the number of pregnant women that could be submitted to quantitative investigation (25).

Recently, fetal RNA has also been found in maternal plasma. Such fetal RNA has been shown to originate from the placenta and to be remarkably stable. The use of microarray-based approaches has made it feasible to rapidly generate new circulating RNA markers. It is hoped that further developments in this field will make the routine and widespread practice of noninvasive nucleic acid–based prenatal diagnosis for common pregnancy-associated disorders feasible in the near future.

10.4 Cancer

Cancer is a common malignant disease in industrialised countries. Early diagnosis of tumours and accurate identification of haematogenic metastases can improve the success of treatment (26–29). Therefore, the detection of single tumour cell released in the blood in early stages could help physicians choose the most advantageous therapy for patients. The presence of small amounts of cell free tumor DNA (cfDNA) circulating in the plasma or serum of cancer patients was first demonstrated 30 years ago and provides another possibility of examining tumour derived genetic material in the circulation and to detect haematogenic spread of tumour cell DNA (30). Qualitative alterations in circulating DNA, such as microsatellite alterations (31), oncogene mutations (32), mitochondrial DNA, tumour-specific methylated DNA (33) and viral DNA (34), have been found in patients with different types of cancer. Quantitative alterations of circulating cfDNA have also been observed in several tumours, such as prostate cancer (35), lung cancer (36), pancreatic cancer (37), leukaemia and lymphoma (38). High levels of circulating cfDNA were correlated with tumour metastasis, response to therapy and recurrence (36–39). Therefore, tumour-derived circulating nucleic acids in the plasma or serum of cancer patients were introduced as a tool for detection and surveillance of cancers (40). The proportion of patients with altered cfDNA varies with the pathology and the nature of the marker. However, several studies have reported the presence of altered cfDNA in over 50% of cancer patients (41), suggesting that this marker may be common and amenable for a variety of clinical and epidemiological studies. Because the mechanisms and timing of cfDNA release in the blood stream are poorly understood, only few studies have addressed the use of cfDNA for early cancer detection or as a biomarker for mutagenesis and tumourigenesis in molecular epidemiology. (41). In some circumstances, cfDNA alterations are detectable ahead of cancer diagnosis, raising the possibility of exploiting them as biomarkers for monitoring cancer occurrence.

10.5 Trauma

The mechanisms by which cell-free DNA is freed into the circulation of human subjects are unknown; one possibility is that DNA is released following cell death (42-43).

It is also possible that direct damage or hemodynamic compromise of the organ systems responsible for circulating DNA clearance may also lead to increased plasma DNA. Candidate
organ systems include the liver, spleen, and kidneys, which may have a role in both liberating and clearing circulating DNA.

Along this line of reasoning, Lo’s group hypothesized that DNA may be liberated from body tissues into the plasma after trauma and that plasma DNA may be a potentially useful prognostic tool (44).

In this study Lo shows that circulating plasma DNA in the peripheral blood of trauma patients increases early after injury and that these increases are related to the development of posttraumatic complications, suggesting that plasma DNA may be a potentially useful marker for monitoring patients after trauma.

10.6 Stroke

Stroke ranked as the second leading cause of all deaths worldwide in 1990, accounting for 4.4 million victims (45), and is also currently the leading cause of brain injury in adults (46). Preventive strategies have led to a decrease in the rate of stroke attacks and deaths. Increased concentrations of several neurobiochemical protein markers have been detected in the peripheral blood of patients with stroke, but at present there is no simple and accurate blood test that may be used to determine the severity of a stroke or to predict mortality and morbidity in stroke patients on arrival in emergency wards in clinical practice.

In Lo’s paper, the authors concluded that plasma DNA concentrations correlate with stroke severity and may be used to predict mortality and morbidity in the emergency room (47).

As both hemorrhagic and ischemic strokes (48) involve cell death and disruption of the blood–brain barrier, they hypothesized that DNA would be liberated into the plasma early after the onset of stroke and that it might be useful for assessing disease severity and for predicting mortality.

They have also shown that plasma DNA measurements may be useful for early risk stratification and for predicting in-hospital and 6-month disability and mortality. The greatest differences in plasma DNA concentrations between patients with good and poor outcomes occurred within 3 h of the onset of symptoms.

The mechanisms by which circulating cell-free DNA increases after stroke require further study but are likely to be a result of increased liberation from damaged cells. Strokes involve a complicated cascade of events involving cerebral ischemia, altered cerebral blood flow, inflammation, the production of reactive oxygen radicals, neuronal necrosis and apoptosis, and neurologic dysfunction (49-53). DNA may be liberated from cells undergoing apoptosis or necrosis.
Recommended literature:

1. Lo YM, Tein MS, Lau TK, Haines CJ, Leung TN, Poon PM et al. Quantitative analysis of fetal DNA in maternal plasma and serum: implications for noninvasive prenatal diagnosis. Am J Hum Genet 1998; 62:768-75.
2. Birch L, English CA, O’Donoghue K, Barigye O, Fisk NM, Keer JT. Accurate and robust quantification of circulating fetal and total DNA in maternal plasma from 5 to 41 weeks of gestation. Clin Chem 2005;51:312-20.
3. Galbiati S, Smid M, Gambini D, Ferrari A, Restagno G, Viora E et al. Fetal DNA detection in maternal plasma throughout gestation. Hum Genet. 2005;117:243-8.
4. New MI. An Update of Congenital Adrenal Hyperplasia. Ann N Y Acad Sci. 2004;1038:14-43.
5. Rijnders RJ, van der Schoot CE, Bossers B, de Vroede MA, Christiaens GC. Fetal sex determination from maternal plasma in pregnancies at risk for congenital adrenal hyperplasia. Obstet Gynecol 2001;98:374-8.
6. Saito H, Sekizawa A, Morimoto T, Suzuki M, Yanaihara T. Prenatal DNA diagnosis of a single gene disorder from maternal plasma. Lancet 2000;356:1170.
7. Chiu RW, Lau TK, Leung TN, Chow KC, Chui DH, Lo YM. Prenatal exclusion of â-thalassaemia major by examination of maternal plasma. Lancet 2002;360:998-1000.
8. Ding C, Chiu RW, Lau TK, Leung TN, Chan LC, Chan AY et al. MS analysis of single-nucleotide differences in circulating nucleic acids: Application to noninvasive prenatal diagnosis. 2004;101:10762-7.
9. Li Y, Di Naro E, Vitucci A, Zimmermann B, Holzgreve W, Hahn S. Detection of paternally inherited fetal point mutations for beta-thalassemia using size-fractionated cell-free DNA in maternal plasma. JAMA 2005;293:843-9.
10. Lo YM. Fetal RhD genotyping from maternal plasma. Ann Med 1999;31:308-12.
11. Younis JS, Samueloff A. Gestational vascular complications. Best Pract Res Clin Haematol 2003;16:135–51.
12. Leung TN, Zhang J, Lau TK, Hjelm NM, Lo YM. Maternal plasma fetal DNA as a marker for preterm labour. Lancet 1998;352:1904–5.
13. Lo YM, Lau TK, Zhang J, Leung TN, Chang AM, Hjelm NM et al. Increased fetal DNA concentrations in the plasma of pregnant women carrying fetuses with trisomy 21. Clin Chem 1999a;45:1747–51.
14. Lo YM, Leung TN, Tein MS, Sargent IL, Zhang J, Lau TK et al. Quantitative abnormalities of fetal DNA in maternal serum in preeclampsia. Clin Chem 1999b;45:184–8.
15. Sekizawa A, Sugito Y, Iwasaki M, Watanabe A, Jimbo M, Hoshi S et al. Cell-free fetal DNA is increased in plasma of women with hyperemesis gravidarum. Clin Chem 2001;47:2164–5.
16. Smid M, Vassallo A, Lagana F, Valsecchi L, Maniscalco L, Danti L et al. Quantitative analysis of fetal DNA in maternal plasma in pathological conditions associated with placental abnormalities. Ann N Y Acad Sci 2001;945:132–7.
17. Zhong XY, Laivuori H, Livingston JC, Ylikorkala O, Sibai BM, Holzgreve W, et al. Elevation of both maternal and fetal extracellular circulating deoxyribonucleic acid concentrations in the plasma of pregnant women with preeclampsia. Am J Obstet Gynecol 2001;184:414–9.
18. Sekizawa A, Jimbo M, Saito H, Iwasaki M, Sugito Y, Yukimoto Y et al. Increased cell-free fetal DNA in plasma of two women with invasive placenta. Clin Chem 2002;48:353–4.
19. Smid M, Galbiati S, Lojacono A, Valsecchi L, Platco C, Cavoretto P et al. Correlation of fetal DNA levels in maternal plasma with Doppler status in pathological pregnancies. Prenat Diagn. 2006;26:785-90.
20. Brosens IA, Robertson WB, Dixon HG. The role of the spiral arteries in the pathogenesis of preeclampsia. Obstet Gynecol Annu 1972;1:177–91.
21. Lyall F. The human placental bed revisited. Placenta 2002;23:555–62.
22. Sekizawa A, Farina A, Sugito Y. Proteinuria and hypertension are independent factors affecting fetal DNA values: a retrospective analysis of affected and unaffected patients. Clin Chem 2004;50:221–4.
23. Zhong XY, Holzgreve W, Hahn S. Circulatory fetal and maternal DNA in pregnancies at risk and those affected by preeclampsia. Ann N Y Acad Sci 2001;945:138-40.
24. Bischoff FZ, Lewis DE, Simpson JL. Cell-free fetal DNA in maternal blood: kinetics, source and structure. Hum Reprod Update 2005;11:59-67.
25. Poon LL, Leung TN, Lau TK, Chow KC, Lo YM. Differential DNA methylation between fetus and mother as a strategy for detecting fetal DNA in maternal plasma. Clin Chem 2002;48:35-41.
26. Pantel K, Muller V, Auer M, Nusser N, Harbeck N, Braun S. Detection and clinical implications of early systemic tumour cell dissemination in breast cancer. Clin Cancer Res 2003;9:6326–34.
27. Diel IJ, Solomayer EF, Bastert G. Bisphosphonates and the prevention of metastasis: First evidences from preclinical and clinical studies. Cancer 2000;88:3080–8.
28. Diel IJ. Antitumour effects of bisphosphonates: First evidence and possible mechanisms. Drugs 2000;59:391–9.
29. Diel IJ, Mundy GR. Bisphosphonates in the adjuvant treatment of cancer: experimental evidence and first clinical results. International Bone and Cancer Study Group (IBCG). Br J Cancer 2000;82:1381–6.
30. Anker P, Stroun M. Circulating DNA in plasma or serum. Medicina (B Aires)2000;60:699–702.
31. Nawroz-Danish H, Eisenberger CF, Yoo GH, Wu L, Koch W, Black C et al. Microsatellite analysis of serum DNA in patients with head and neck cancer. Int J Cancer 2004;11:96–100.
32. Diel I, Li M, Dressman D, He Y, Shen D, Szabo S, et al. Detection and quantification of mutations in the plasma of patients with colorectal tumours. Proc Natl Acad Sci USA 2005;102:16368–73.
33. Wong TS, Kwong DL, Sham JS, Wei WI, Kwong YL, Yuen AP. Quantitative plasma hypermethylated DNA markers of undifferentiated nasopharyngeal carcinoma. Clin Cancer Res 2004;10:2401–6.
34. Leung SF, Chan AT, Zee B, Ma B, Chan LY, Johnson PJ et al. Pretherapy quantitative measurement of circulating Epstein–Barr virus DNA is predictive of posttherapy distant failure in patients with early-stage nasopharyngeal carcinoma of undifferentiated type. Cancer 2003;98:288–91.
35. Boddy JL, Gal S, Malone PR, Harris AL, Wainscoat JS. Prospective study of quantitation of plasma DNA levels in the diagnosis of malignant versus benign prostate disease. Clin Cancer Res 2005;11:1394–9.
36. Gautschi O, Bigosch C, Huegli B, Jermann M, Marx A, Chasse E, et al. Circulating deoxyribonucleic acid as prognostic marker in non-small-cell lung cancer patients undergoing chemotherapy. J Clin Oncol 2004;22:4157–64.
37. Giacoma MB, Ruben GC, Iczkowski KA, Roos TB, Porter DM, Sorenson GD. Cell-free DNA in human blood plasma: length measurements in patients with pancreatic cancer and healthy controls. Pancreas 1998;17:89–97.
38. Wu TL, Zhang D, Chia JH, Tsao KH, Sun CF, Wu JT. Cell-free DNA: measurement in various carcinomas and establishment of normal reference range. Clin Chim Acta 2002;321:77–87.
39. Thijssen MA, Swinkels DW, Ruers TJ, de Kok JB. Difference between free circulating plasma and serum DNA in patients with colorectal liver metastases. Anticancer Res 2002;22:421–5.
40. Goebel G, Zitt M, Zitt M, Muller HM. Circulating nucleic acids in plasma or serum (CNAPS) as prognostic and predictive markers in patients with solid neoplasias. Dis Markers 2005;21:105–20.
41. Gormally E, Caboux E, Vineis P, Hainaut P. Circulating free DNA in plasma or serum as biomarker of carcinogenesis: Practical aspects and biological significance. Mutation Research 2007;635:105–17.
42. Fournie GJ, Martres F, Pourrat JP, Alary C, Rumeau M. Plasma DNA as cell death marker in elderly patients. Gerontology 1993;39:215–21.
43. Fournie GJ, Courtin JP, Laval F, Chale JJ, Pourrat JP, Puazon MC, et al. Plasma DNA as a marker of cancerous cell death. Investigations in patients suffering from lung cancer and in nude mice bearing human tumours. Cancer Lett 1995;91:221–7.
44. Lo YM, Rainer TH, Chan LY, Hjelm NM, Cocks RA. Plasma DNA as a prognostic marker in trauma patients. Clin Chem. 2000;46:319-23.
45. Murray CJL, Lopez AD. Alternative projections of mortality and disability by cause 1990–2020: Global Burden of Disease Study. Lancet 1997;349:1498–505.
46. Broderick JP, Brott T, Tomsick T, Huster G, Miller R. The risk of subarachnoid and intracerebral hemorrhages in blacks as compared with whites. N Engl J Med 1992;326:733–6.
47. Rainer TH, Wong LK, Lam W, Yuen E, Lam NY, Metreweli C et al. Prognostic Use of Circulating Plasma Nucleic Acid Concentrations in Patients with Acute Stroke. Clin Chem. 2003;49:562-9.
48. Williams GR, Jiang JG, Matchar DB, Samsa GP. Incidence and occurrence of total (first-ever and recurrent) stroke. Stroke 1999;30:2523–8.
49. Yang GY, Pang L, Ge HL, Tan M, Ye W, Liu XH, et al. Attenuation of ischemia-induced mouse brain injury by SAG, a redoxinducible antioxidant protein. J Cereb Blood Flow Metab 2001;21:722–33.
50. Sairanen T, Carpen O, Karjalainen-Lindsberg ML, Paetau A, Turpeinen U, Kaste M, et al. Evolution of cerebral tumor necrosis factor-alpha production during human ischemic stroke. Stroke 2001;32:1750–8.
51. Graham SH, Chen J. Programmed cell death in cerebral ischemia. J Cereb Blood Flow Metab 2001;21:99–109.
52. Reed JC. Mechanisms of apoptosis. Am J Pathol 2000;157:1415–30.
53. MacManus JP, Buchan AM. Apoptosis after experimental stroke: fact or fashion? J Neurotrauma 2000;17:899–914.