

# Circulating Nucleic Acids in Plasma and Serum

## Recent Developments

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DNA and RNA fractions have been isolated from the whole blood, serum, plasma, the surface of blood cells, and urine of both healthy individuals and patients. The ability to isolate, quantify, and analyze these molecules has led to the identification of specific nucleic acid fragments related to particular disorders such as diabetes, cancer, myocardial infarction, and stroke, thereby permitting their early diagnosis. Currently, a number of methods for isolating the nucleic acids are employed and although a start has been made to compare the efficiencies of these methods, there is still a way to go before there are precise protocols for nucleic acid extraction. The older chemical methods of extraction still outperform some of the available kits. Some progress is being made to determine the origin of the circulating nucleic acids, although there are still many questions to be answered, including whether the source is through the spontaneous release of newly synthesized nucleic acid or whether it just derived from necrotic and apoptotic cells. In addition, it can be demonstrated that the nucleic acids can enter cells and exhibit a biological activity in the recipient cells. Hence, the question remains: Are the circulating nucleic acids freely entering tissues and cells from the blood and inducing changes in those tissues and cells? Further work is needed to elucidate these areas, and the various protocols must be standardized if the new methodology is to be widely and accurately applied in the diagnosis of disease and the monitoring of therapy. This chapter summarizes the work reported in this volume.

**Key words:** circulating nucleic acids; CNAPS in oncology; CNAPS in fetal medicine; biology of CNAPS; CNAPS in general medicine

## Introduction

The fifth international conference on circulating nucleic acids in plasma and serum was held in Moscow, Russia on August 23-26, 2007, and this volume reports that meeting. Its aim was to bring together clinicians and scientists working in this subject to present their latest findings in the basic biology, methodology, and clinical applications of circulating nucleic acids in blood and urine.

The conference celebrated the 60th anniversary of the first report of circulating DNA by Mendel and Métais in 1947.<sup>1</sup> After a long break, evidence accumulated in the 1960s and 1970s for the spontaneous release of newly synthesized DNA by living cells (reviewed in Stroun<sup>2</sup>). In parallel, increased DNA in the blood of patients with systemic lupus erythematosus was demonstrated,<sup>3</sup> followed in the 1970s by studies on increased DNA levels in blood from patients with other disorders such as pancreatitis and inflammatory bowel disease.<sup>4</sup> Higher levels of DNA were also found in blood from cancer patients,<sup>5</sup> especially in those with metastases compared to patients with

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localized disease. These high values decreased after radiotherapy.<sup>5</sup> In the 1980s, Stroun and Anker demonstrated that the circulating DNA originated from cancer cells.<sup>6</sup> The field expanded rapidly upon the finding of fetal DNA in maternal plasma<sup>7</sup> and the demonstration of organ-specific RNA in the blood of patients with various disorders, such as diabetes.<sup>8</sup>

## Biology of Circulating Nucleic Acids

A major question remaining over circulating nucleic acids in plasma and serum, and in urine, concerns the origin of this DNA. A range of studies have indicated that they originate from apoptotic and necrotic cells,<sup>9,10</sup> especially in the case of cancer-related DNA release and, in particular, the release of nucleosomal fragments (reviewed in this volume by Holdenreider *et al.*). Cells releasing these forms of DNAs could include tissue-based cells related to the particular disorder concerned or to tumors as well as to circulating blood cells.

More recent studies have shown that blood cells have DNA on the cell surface,<sup>11</sup> and it has been assumed that this DNA is in the process of entering the cells. Indeed, analysis of the entry of DNA into HeLa and transformed cells by Skvortsova *et al.* has demonstrated that methylated DNA fractions can enter twice as readily as nonmethylated DNA. Furthermore, Ermakova *et al.* have shown that if lymphocytes are irradiated *in vitro* there is a transposition of the pericentromeric loci from the perimembrane sites to perinucleolar sites, where homologous recombination is thought to occur. If nonirradiated lymphocytes are cultured in the medium from the irradiated lymphocytes, then they also behave similarly. When DNA released from the irradiated cells is isolated and given to the nonirradiated cells, they show similar cytological changes, implying that DNA has passed to the nonirradiated cells from the culture medium of the irradiated cells. In addition, Kulagina *et al.* have shown that ribosomal 5.8S RNA

can enter the cytoplasm of human adenocarcinoma MCF7 cells by 1 hour after commencing feeding and into the nucleus after 2 hours. Adenosines were edited by adenosine deaminases, especially at stack-closing A-U pairs A53 and A96 as well as unpaired A44 and A65, indicating that these enzymes participated in capturing and intracellular trafficking of internalized RNAs.

A range of studies have shown that newly synthesized DNA is released spontaneously from human,<sup>12</sup> mammalian,<sup>13</sup> avian,<sup>14</sup> amphibian,<sup>15,16</sup> and plant<sup>17</sup> cells *in vitro*, and from bacteria both *in vitro*<sup>2</sup> and *in vivo*.<sup>18</sup>

In consequence, the cell-surface DNA could represent DNA leaving as well as entering the cells. The mechanism by which DNA leaves the cell has been considered here by Morozkin *et al.*, who used molecules that block protein secretion on the grounds that the DNA has been shown to be associated with proteins. They demonstrated that the surface-bound DNA of both HeLa and HUUEV cells is reduced by 30%, 35%, and 19% by monensin, glyburide, and methylamine, respectively.

Furthermore, there is reason to believe that in addition to—or as an alternative to—the release of DNA through either necrosis or apoptosis, cells could also be releasing newly synthesized DNA fractions into the circulation. The point of origin of such DNA is not clear, although studies on chick embryo fibroblasts have shown that DNA labeled with <sup>3</sup>H-thymidine in nondividing cells appears first in the nucleus, passes to the cytosol after a 3-h delay and then to the environment after a further 2-h delay (for passage across the cytosol?).<sup>15</sup> Gahan *et al.* have suggested the possibility that this newly synthesised, spontaneously released DNA is actually the earlier-described metabolic DNA (This volume).

RNA is also found in plasma and serum, though its source of origin is less clear than that for the DNA. It is possible that it is released from either necrotic or apoptotic cells as has been postulated for the DNA. Nevertheless, it is also possible that the RNA could be released

from living cells. It has been shown that the newly synthesized DNA released spontaneously is often associated with a glycolipoprotein complex,<sup>2,18</sup> and also with RNA.<sup>2,18,19</sup> Furthermore, the DNA complex released from bacteria carries a DNA-dependent RNA polymerase that permits RNA to be synthesized outside of the bacterial cells.<sup>20</sup> Since the complex released from eukaryote cells has also been shown to contain a DNA-dependent RNA polymerase,<sup>21</sup> it is possible also that eukaryote RNA is synthesized outside of the cells.

## Methodology

Many studies have shown the successful application of CNAPS in the diagnosis and monitoring of cancer and in prenatal diagnosis, as well as in other diseases. However the methods used for the extraction and quantification of nucleic acids vary widely. Thus, variation in the strategies of nucleic acid isolation will result in varied amounts and types of both RNA and DNA isolated, with perhaps important molecules being missed in some cases. Nucleospin Plasma XL was shown by Kirsch *et al.* to produce all fragment sizes as opposed to the QIAamp DNA Blood Mini Kit (QIAGEN, Valencia, CA), which failed to produce 50-bp fragments and also yielded lower amounts of the other sized fragments. A comparison of four different kits by van der Vaart and Pretorius showed the QIAamp DNA Blood Mini Kit and the Magna Pure Compact System (Roche Diagnostics, Basel, Switzerland) to give lower yields than by either salting-out or extracting with phenol-chloroform. Board *et al.* showed that QIAamp Viral Spin Kit was the most effective of four commercially available kits tested. Other methods include the Short NA Isolation Kit used by Mala'shakova *et al.* and solid phase carboxylated magnetic beads and QIAvac24 used by Su *et al.* for urine nucleic acids, whereas Dovc-Drnovsek used the QIAamp Ultrasens Virus Kit. Adsorption of nucleic acids to Q-sepharose has been used by Melkonyan *et al.*

to isolate ultrashort fragments of nucleic acids from urine. This has resulted in the identification of DNA and RNA fragments of 10–150 bases (or base pairs) in addition to 150–290-bp DNA fragments and high-molecular-weight DNA. While some workers have made an attempt to determine the kit with the greatest yield and the largest range of fragment sizes for both RNA and DNA, there is still a lack of clarity concerning the use of the broad range of kits and their applicability.

It is also clear, as Cherepanova *et al.* reported, that any DNAase present in the blood will have an impact upon the levels of DNA to be isolated.

Hence, the methodology is far from consistent, and if there are to be reliable protocols for diagnostic purposes, there needs to be a form of quality assurance for these procedures.

## Nucleic Acids in Fetal Medicine

The early studies of Li *et al.* showing that fetal DNA is smaller than the comparable maternal circulating DNA (fetal DNA fragments are <300 bp and maternal DNA fragments are >300 bp) has opened up a range of studies on fetal and maternal circulating DNA.<sup>22</sup> The analysis of maternal plasma for DNA has been exploited in the early identification of a range of maternal complications such as pre-eclampsia<sup>23</sup> and intrauterine growth retardation, as well as in fetal disorders including Rhesus incompatibility,<sup>24</sup> and  $\beta$ -thalassemia.<sup>25</sup>

In order to improve diagnosis, Lo has considered the need to enrich the fetal DNA fraction that represents only about 3% of the total maternal blood DNA. One approach exploits the use of fetus-specific methylated markers such as SERPIN35. The other detects fetal chromosome aneuploidies using digital PCR in a discriminatory quantitative method for detecting small excesses of nucleic acids.

Several studies have shown that the sex of the fetus could be determined using fetal DNA in maternal plasma, many of which have used the presence or absence of Y chromosome

markers. If the markers are absent from the maternal plasma, then it is assumed that the fetus is female. Vainer *et al.* have used Y chromosome-specific multicopy DAZ sequence for the detection of males, with the absence of this marker being taken as indicative of a female.

On the other hand, Vecchione *et al.* have shown that during the first trimester of pregnancy, reliable sex determination can be achieved using ten short tandem repeat sequences and the amelogenin gene sequence from the paternal X chromosome. These have been shown to be very specific markers for the female fetus, with their absence being a reliable indication a male fetus.

Papasavva *et al.* have shown that the paternal allele of thalassemia could be detected in the maternal blood employing paternally inherited single nucleotide polymorphisms using the array primer extension methodology.

## Nucleic Acids in Oncology

The development of malignancy is usually associated with a number of genetic and epigenetic alterations, some of which have been detected in circulating DNA. A number of studies have shown that the identification of certain fragments of circulating DNA can be used not only as an early diagnostic marker (e.g., in breast, lung, head and neck, and gastrointestinal tumors), but also as a means of following changes during therapy (as in the case of melanoma).<sup>26</sup> There are two forms in which the blood DNA may be found—free in the blood, as either free DNA or protein-associated DNA, and as DNA bound to the surface of blood cells.

Thus, although there were no measurable differences between the amounts of cell-surface DNA in controls and patients with gastric cancer, Kolesnikova *et al.* showed that methylation-specific PCR of three genes—MGMT, p15 and hMLH1—from the cell-surface-bound DNA enabled the gastric cancer patients to be identified with a 75% sensitivity and a 54% specificity.

Holdenreider *et al.* also found that plasma and serum concentrations of DNA were little different between patients with benign diseases and cancer patients. It was also shown that elevated nucleosome levels in blood did not permit a distinction between cancer and other diseases, although, in general, a reduction of nucleosome levels accompanied remission, while there was an increased nucleosome concentration during progressive disease.

As previously mentioned, breast cancer diagnosis has benefited from analysis of circulating DNA. A further step has been reported by Sunami *et al.* involving LINE 1, of which there are about 520,000 copies per human genome. A preliminary study has shown LINE 1 to be present in high levels in the plasma of patients with breast tumor, but at limited levels in normal females. LINE 1 appears to be a useful early marker for breast cancer, with the copy number correlating with tumor size. The use of circulating DNA in lung cancer patients has been less successful for distinguishing between patients with lung cancer and those with benign lung disease (Schmidt *et al.*) and between lung cancer patients and healthy donors (Tamkovich *et al.*). Elevated DNA levels have been found in the blood from colorectal cancer patients compared with healthy donors (Schwarzenbach *et al.*), although Su *et al.* suggest that K-ras DNA is an important marker of adenomatous polyps as a precursor to colon cancer. However, urine proved to be a better source of K-ras DNA than did blood, the K-ras gene mutation being rare in urinary tract cancers and hence a likely colon cancer marker.

## Nucleic Acids and Other Diseases

Circulating DNA and RNA have been useful as markers of nononcogenic disorders such as trauma, stroke, autoimmune disease, and diabetes mellitus. In the latter disorder, neuron-specific enolase mRNAs were found to be higher in the blood in the diabetic patients without complications as compared to

healthy donors, but lower levels were found in diabetic retinopathy patients compared to the two other groups (Butt and Swaminathan). Furthermore, patients with either preproliferative retinopathy or proliferative retinopathy had significantly higher levels than did patients with background retinopathy, indicating that this could be a useful marker for differentiating early from advanced eye disease. In addition, an earlier retinopathy marker, rhodopsin mRNA, was found to be increased in retinopathy patients suffering from sleep apnea when compared to retinopathy patients without sleep apnea (Wong *et al.*). Similarly, mRNA for RPE65 was greater in diabetics and patients with all forms of retinopathy as compared to healthy individuals, and higher in both preproliferative and proliferative retinopathy patients compared to diabetics with no retinopathy (Shalchi *et al.*).

Data concerning the DNA from the blood of patients after acute myocardial infarction showed that, *in vitro*, 0.5 ng mL<sup>-1</sup> of the GC-rich fragments of rDNA reduced the contraction of cardiomyocytes by a factor of 1.5–2.0, whereas the AT-rich fragments of the human satellite 3 repeat tandem (1q12 region) increased contraction by a factor of 2.0–2.5 at a concentration of 1 ng mL<sup>-1</sup>. This implies that these DNA fragments in the patients' blood could influence myocyte contractile function (Bulicheva *et al.*).

Circulating mRNA concentration for GH was found to be higher and mRNA for GHRH lower in acromegalic patients than in controls. Both markers were found to be lower in the >50-year-old cohort as compared to the <34-year-old cohort. It is proposed that ratios between the two mRNAs could be exploited to determine whether an individual had been using recombinant human growth hormone (Thakkar *et al.*).

### Conflicts of Interest

The authors declare no conflicts of interest.

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