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Jarkko Romppanen

Serum Sialic Acid in Clinical Diagnostics

Doctoral dissertation

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Department of Clinical Chemistry
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Faculty of Medicine
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Author's address: Department of Clinical Chemistry
Kuopio University Hospital
P.O. Box 1777
FIN-70211 KUOPIO
FINLAND
Tel. +358 44 711 3180

Supervisors: Professor Kari Punnonen, M.D.
Department of Clinical Chemistry
University of Kuopio

Professor Matti Eskelinen, M.D.
Department of Surgery
University of Kuopio

Reviewers: Docent C.J. Peter Eriksson, Ph.D.
Department of Mental Health and Alcohol Research
National Public Health Institute
Helsinki, Finland

Associate Professor Kari Pulkki, M.D.
Department of Clinical Chemistry
University of Turku

Opponent: Docent Erkki Seppälä, M.D.
University of Tampere
Faculty of Medicine
Tampere, Finland

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ABSTRACT

N-acetylneuraminic acid (referred to as sialic acid) is a negatively charged nine-carbon monosaccharide commonly attached to the carbohydrate chains of glycoproteins and glycolipids. An elevation in the serum sialic acid (SA) concentration has been observed in a number of cancer types and recently also in alcohol abusers.

In the present study, serum SA concentrations in patients with breast cancer and benign breast disease, prostate cancer and benign prostate disease, children with different malignancies, alcohol abusers, and healthy controls were determined using high performance liquid chromatography (HPLC). Based on the SA levels in different patient groups and healthy controls, the diagnostic utility of SA was studied using receiver operating characteristic (ROC) analysis. Furthermore, serum total sialic acid (TSA) reference values for healthy controls were determined using a high performance anion-exchange chromatography with pulsed amperometric detection method (HPAE-PAD). HPAE-PAD is a simple and effective way to determine sialic acid without the need for derivatization. The HPAE-PAD system was clearly sensitive enough to allow the detection of the SA concentrations present in human serum.

On its own serum sialic acid was not found to provide reliable classification of undefined breast tumours. The logistic regression model combining TSA, prostate-specific antigen (PSA) and free to total PSA ratio with digital rectal examination results possessed good diagnostic accuracy in discriminating patients with prostate cancer from patients with benign prostate hyperplasia. Serum TSA and TSA to total protein ratio could provide some assistance in exclusion and follow-up of children with malignancies, but infection must be taken into account when interpreting increased sialic acid values. Finally, serum sialic acid measurements may be useful in the assessment of drinking problems especially in conditions where the secondary effects of liver disease hamper the use of the traditional biomarkers.

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Medical Subject Headings: N-acetylneuraminic acid/blood; N-acetylneuraminic acid/diagnostic use; liver diseases, alcoholic/blood; alcoholism/blood; alcohol drinking/blood; prostatic hyperplasia/diagnosis; prostatic neoplasms/diagnosis; infection/blood; breast neoplasms/blood; breast diseases/blood; adult; child; tumor markers, biological/blood; biological markers/blood; chromatography, high pressure liquid; chromatography, ion exchange; calibration; reference values

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Kuopio, July 2003

Jarkko Romppanen

ABBREVIATIONS

ALD	alcoholic liver disease
ALL	acute lymphoblastic leukemia
ALT	alanine aminotransferase
AML	acute myeloid leukemia
AST	aspartate aminotransferase
AUC	area under the curve
BPH	benign prostatic hyperplasia
CA	carbohydrate antigen
CDT	carbohydrate deficient transferrin
CML	chronic myeloid leukemia
CMP	cytidine-monophosphate
DRE	digital rectal examination
GT	γ-glutamyltransferase
HPAE-PAD	high performance anion-exchange chromatography with pulsed amperometric detection
HPLC	high performance liquid chromatography
LASA	lipid-associated sialic acid
ManNAc	N-acetylmannosamine
ManNAc-6-P	N-acetylmannosamine-6-phosphate
ManNAc-9-P	N-acetylmannosamine-9-phosphate
MCV	mean corpuscular volume
PSA	prostate-specific antigen
ROC	receiver operating characteristic
SA	sialic acid
Se	sensitivity
Sialyl-OGS	sialylated oligosacharides
Sp	specificity
TP	total protein
TPA	tissue polypeptide antigen
TPS	tissue polypeptide-specific antigen
TRUS	transrectal ultrasonography
TSA	total sialic acid
UDP-GluNAc	uridine-diphosphate-N-acetyl-D-glucosamine

LIST OF ORIGINAL PUBLICATIONS

This dissertation is based on the following articles referred to in the text by their Roman numerals:

I Romppanen J, Eskelinen M, Tikanoja S, Mononen I. Total and lipid-bound serum sialic acid in benign and malignant breast disease. Anticancer Res 1997; 17: 1249-1253.

II Romppanen J, Petäjä J, Heikinheimo M, Mononen I. Total and lipid-bound serum sialic acid in children with malignancy or infections. Anticancer Res 1998; 18: 2793-2797.

III Romppanen J, Haapalainen T, Punnonen K, Penttilä I. Serum sialic acid and prostate-specific antigen in differential diagnosis of benign prostate hyperplasia and prostate cancer. Anticancer Res 2002; 22: 415-420.

IV Romppanen J, Punnonen K, Anttila P, Jakobsson T, Blake J, Niemelä O. Serum sialic acid as a marker of alcohol consumption: Effect of liver disease and heavy drinking. Alcohol Clin Exp Res 2002; 26: 1234-1238.

V Romppanen J, Romppanen EL, Halonen T, Eskelinen M, Punnonen K. Reference values of serum total sialic acid for healthy adult subjects and comparison of two different calibrators for quantitation of total sialic acid in HPAE-PAD. Clin Chem Lab Med 2003; In press.

CONTENTS

1 INTRODUCTION

Biological markers can be used to monitor cancer, predict the therapeutic response and prognosis of cancer, and in some certain situations even diagnose cancer. These markers, referred to as tumour markers, are naturally occurring or modified molecules that can be measured in serum, plasma, or other body fluids and their concentration becomes changed in the presence of cancer (Chan and Schwartz 2002). Before a tumour marker can be used in routine clinical diagnostics a massive amount of work is needed. After discovery of a promising marker, an assay system has to be developed and a thorough clinical evaluation has to be carried out (Hammond 2002).

Another increasing health problem is abuse of alcohol. This is a health risk that can lead to a broad range of medical and social problems, which impose a high burden on society. Biological markers could be useful in revealing objective information about alcohol intake. Although several biological markers are currently used routinely, all these markers have some limitations in their clinical efficiency.

N-acetylneuraminic acid (referred to as sialic acid, SA) is a negatively charged nine-carbon monosaccharide commonly attached by an α-glycosidic linkage to the non-reducing residues of the carbohydrate chains of glycoproteins and glycolipids (Schauer 1982). An elevation in the serum total sialic acid (TSA) concentration has been observed in a number of cancer types (Shamberger 1984). Recently SA has been suggested also to be a valuable diagnostic indicator for detecting and monitoring alcohol abuse (Pönniö et al. 1999a).

In the present investigation, the usefulness of sialic acid in clinical diagnostics of malignancy and benign tumours, and as an alcohol biomarker was evaluated. TSA reference values were determined with a method based on high performance anion exchange chromatography coupled with pulsed amperometric detection (HPAE-PAD).

2 REVIEW OF THE LITERATURE

2.1 Structure and biology of sialic acid

From over 30 acetylated derivatives of neuraminic acid, N-acetylneuraminic acid (referred to as sialic acid) is the most common in humans. It is a negatively charged nine-carbon monosaccharide with a molecular weight of 309 and a pK value of 2.6. The structure of sialic acid is presented in Figure 1. SA is commonly attached by an α-glycosidic linkage to the non-reducing residues of the carbohydrate chains of glycoproteins and glycolipids (Schauer 1982).

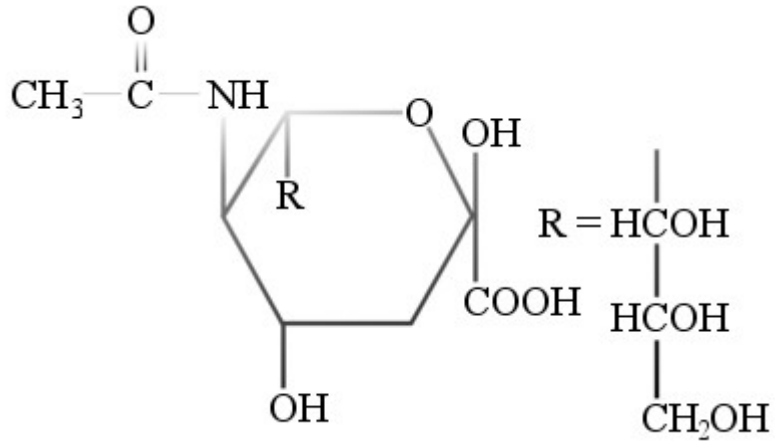

Figure 1. Structure of sialic acid.

Glucose is the precursor in the synthesis of sialic acid (Figure 2.). After several modifications an important intermediate, uridine-diphosphate-N-acetyl-D-glucosamine (UDP-GlcNAc), is formed and this is then converted to N-acetylmannosamine (ManNAc). Cytidine-monophosphate (CMP) SA can inhibit this step by feedback inhibition (Kornfeld et al. 1964; Sommar and Ellis 1972). Sialic acid is then produced from ManNAc via N-acetylmannosamine-6-phosphate (ManNAc-6-P) and N-acetylneuraminic acid-9-phosphate (ManNAc-9-P) as the intermediate compounds. The incorporation of SA into glycoconjugates takes place in the Golgi apparatus using CMP as the carrier (Carey and Hirschberg 1979). The degradation of sialoglycoproteins and sialoglycolipids occurs within lysosomes.

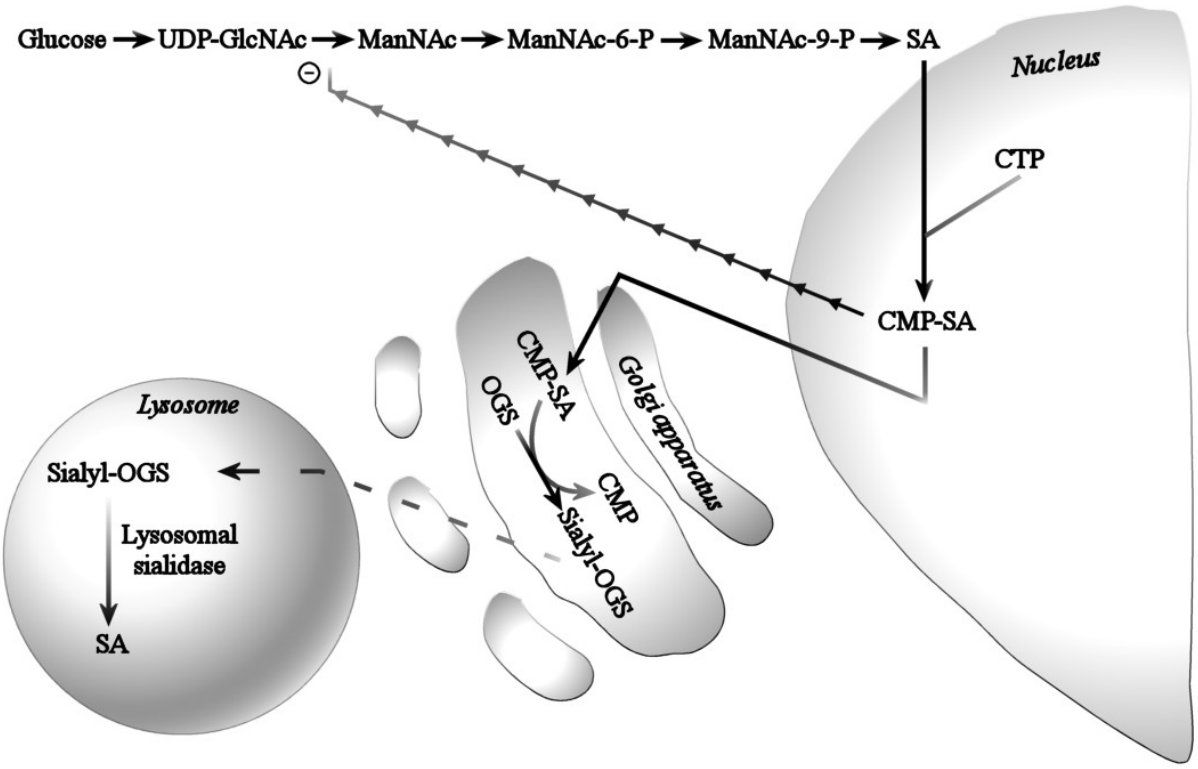

Figure 2. Metabolism of sialic acid.

There is only a minute amount of free sialic acid in tissues and body fluids, and no direct biologic role has been identified for this unbound sialic acid. On the contrary, bound sialic acid is of major importance in cell biology because of the external position of SA on glycoproteins and glycolipids, and on the outer cell membranes (Schauer 2000). SA participates in the stabilization of the conformation of glycoproteins and cellular membranes. It can hinder the action of some endoglycosidases and proteinases. Furthermore, SA can either mask the recognition sites or be a biological target allowing the recognition of a receptor protein. The negative charge present in sialic acid means that the compound takes part in binding and transport of positively charged molecules, and in the attraction and repulsion of cells and molecules (Crook et al. 1996; Schauer 2000). SA also contributes to the regulation of the permeability of the basement membranes in glomeruli (Crook et al. 1996).

Aberrant glycosylation processes in tumour cells may contribute to the biosynthesis of the carbohydrate structures so that malignant or transformed cells contain increased levels of sialic acid on their surfaces. Cell shape, anchorage and growth rate have been shown to influence the sialic acid content of the cell (Yogeeswaran 1983). In some studies, the total sialic acid content has been observed to increase in highly metastatic cells compared with non-metastatic cells (Skipski et al. 1981; Yogeeswaran and Salk 1981). However, in other studies

no consistent increase in total cellular sialic acid has been found, but highly metastatic cells have been observed to have significantly elevated amounts of neuraminidase-releasable SA and also an increased degree of sialylation of galactose and N-acetylgalactosamine groups compared with nonmetastatic cells (Yogeeswaran et al. 1979; Kloppel and Morre 1980).

Increased sialylation helps malignant cells to disguise their immunogenic sites, to increase the negative charge of the outer cell membrane so that the binding and killing by lymphocytes and macrophages can be impaired, and to hide the receptor sites for IgM antibodies, which kill cells by a complement-mediated reaction (Yogeeswaran 1983; Schauer 2000). Cell activation, transformation, and malignant growth increase the spontaneous shedding of cell surface components (Black 1980). In growing cells, the rate of carbohydrate synthesis is significantly higher compared with non-growing cells (Kaplan and Moskowitz 1975). Consequently, this shedding process is a normal event linked to the activation and growth periods of the normal cell, but in cancer cells shedding is a continuous and rapidly on-going phenomenon (Yogeeswaran 1983). In view of the above facts, the elevation of total sialic acid in serum in malignancy is not surprising. However, the mechanism is very complex and can lead to variable total SA results in different types of cancers and patient populations. This has also been found in studies using the normalization of total SA for total protein in serum and by measuring lipid-associated sialic acid (LASA), the sialic acid bound to sialic acid containing glycolipids, i.e. gangliosides (Dnistrian and Schwartz 1981; Plucinsky et al. 1986; Patel et al. 1988; Patel et al. 1991; Tautu et al. 1991; Schutter et al. 1992; Patel et al. 1994).

Elevated serum sialic acid concentrations have also been reported in patients with bacterial infections (Plucinsky et al. 1986; O'Kennedy et al. 1991; Seider et al. 1992) and rheumatoid arthritis (Stefenelli et al. 1985; Voigtmann et al. 1989). This increase in serum SA concentration may occur via changes in the biosynthesis and post-translational glycosylation processing of the acute-phase glycoproteins in the liver (van Dijk et al. 1991).

Recent studies have shown that the sialic acid concentration in serum may be elevated in alcoholics (Pönniö et al. 1999a; Sillanaukee et al. 1999). The proposed mechanism for the elevation is that ethanol can depress the activities of sialyltransferases in the Golgi apparatus (and synaptosomes) and can increase the activities of sialidase in the cytosol and plasma membranes (Xin et al. 1995; Hale et al. 1998).

2.2 Analysis of serum sialic acid

Serum sialic acid has been found to be stable for at least 6 months when stored frozen at -20°C (Plucinsky et al. 1986). It can be measured with several different analytical methods. The first methods for SA measurement were colorimetric. One of the most commonly used methods was the Warren thiobarbiturate method, where periodic acid oxidises free sialic acid to formyl pyruvic acid under acidic conditions. Formyl pyruvic acid then reacts with thiobarbituric acid to form a red chromophore with absorption maximum at 549 nm (Warren 1959). Svennerholm described the resorcinol method in which free sialic acid reacts with resorcinol and copper(II) ions to form a chromogen (Svennerholm 1957). Several other colorimetric assays, for example an acidic ninhydrin assay, direct Ehrlich and orsinol reactions, have been described (Schauer 1987). A number of compounds have been reported to cause interferences in the colorimetric assays, e.g. 2-deoxyribose, unsaturated fatty acids, lactose and maltose (Crook 1993).

Enzymatic assays using specific enzymes reacting with sialic acid can theoretically offer improved specificity. There are many enzymatic routes which can be used to lead to the formation of a colored or fluorescent end product after sialidase treatment of a sample. For example, acylneuraminate pyruvate lyase or N-acetylneuraminic acid aldolase together with pyruvate oxidase can be used to generate hydrogen peroxide, which can be converted to a red dye by peroxidase (Crook 1993). Enzymatic assays are easy to automate, and require only small amounts of sample and reagents. However, interference by endogenous pyruvate in the sample can occur.

Chromatographic techniques consist of thin-layer chromatography, gas chromatography with mass spectrometry and high-performance liquid chromatography (HPLC). Thin-layer chromatography techniques based on cellulose or silica gel plates and visualisation with colorimetric assay methods are very rarely used anymore (Crook 1993). Gas chromatography with mass spectrometry can be used to detect minute amounts of sialic acid with high specificity (Renlund et al. 1983), but these techniques require a time-consuming derivatization of the analyte. HPLC has been used to detect sialic acid from many kinds of samples, for example, from urine (Mononen 1986; Romppanen and Mononen 1995), cells (Renlund et al. 1986; Budd et al. 1992), and serum (Silver et al. 1981; Shukla and Schauer 1982).

Today, the method of choice for measuring sialic acid is the HPLC system with an anion-exchange column coupled with pulsed amperometric detection from Dionex (Manzi et al. 1990; Rohrer et al. 1998; Rohrer 2000). HPAE-PAD is a simple and effective way to

determine sialic acid without the need for derivatization. Pulsed amperometry detects only those compounds that contain functional groups that are oxidizable at the detection voltage employed. This leads to high sensitivity and specificity. Neutral or cationic sample components in the matrix, even if oxidizable, elute in the void volume of the anion-exchange column and do not interfere with the analyses.

LASA has been suggested to be the sialic acid bound to sialic acid containing glycolipids, i.e. gangliosides. The analysis of LASA can be done using any of the sialic acid measurements described above after isolation of glycolipids with the method by Katopodis et al. (Katopodis and Stock 1980; Katopodis et al. 1982). However, the lipid-extraction procedure results in co-extraction of glycoproteins containing sialic acid, acid-α-1-glycoprotein being the most significant (Voigtmann et al. 1989; Végh et al. 1991). Therefore the term LASA is somewhat misleading.

2.3 Reference values of serum total sialic acid

Serum TSA concentrations in healthy adult subjects have been determined with several methods in a number of studies. There are many methodological and other factors that can influence the measured serum TSA concentrations in reference individuals. Age, sex, smoking and use of contraceptive pills may affect serum TSA concentrations (Pönniö et al. 1999b) although there is no agreed confirmation of these findings (Lorentz et al. 1986; Lindberg et al. 1991). From the methodological point of view, the methods used differ drastically from each other. Furthermore there is no reference method for the measurement of TSA against which other methods can be calibrated. Major differences in detected serum values can be seen between different studies apparently using the same methodology (Table 1, e.g. see the spread of values obtained with the thiobarbiturate method).

Table 1. Detected values of TSA in serum of reference individuals.

Method	n	Mean value	Reference
Ehrlich	134	1.74 ± 0.24 mmol/l	(Shamberger 1984)
Resorcinol	42	2.18 ± 0.23 mmol/l	(Plucinsky et al. 1986)
Resorcinol	75 69	1.90 ± 0.18 mmol/l (male) 2.04 ± 0.19 mmol/l (female)	(Lindberg et al. 1997)
Resorcinol	45	1.94 ± 0.24 mmol/l	(Horgan 1982)
Thiobarbiturate	158	1.89 ± 0.23 mmol/l	(Stefenelli et al. 1985)
Thiobarbiturate	127 122	2.10 ± 0.53 mmol/l (male) 2.17 ± 0.48 mmol/l (female)	(Lorentz et al. 1986)
Thiobarbiturate	64	1.82 ± 0.44 mmol/l	(O'Kennedy et al. 1991)
Thiobarbiturate	60	1.93 ± 0.26 mmol/l	(Özben 1991)
Thiobarbiturate	88	1.45 ± 0.24 mmol/l	(Patel et al. 1991)
Thiobarbiturate	96 97	2.04 ± 0.34 mmol/l (male) 2.05 ± 0.35 mmol/l (female)	(Pönniö et al. 1999b)
Enzymatic	24	1.94 ± 0.29 mmol/l	(Sugahara et al. 1980)
Enzymatic	126	2.10 ± 0.45 mmol/l	(Gressner and Henn 1985)
Enzymatic	28	2.20 ± 0.26 mmol/l (children 6 m – 18 y)	(Seider et al. 1992)
Enzymatic	20	1.98 ± 0.67 mmol/l	(Crook et al. 1993)
HPLC with UV detection	30	0.64 ± 0.28 mmol/l	(Diamantopoulou et al. 1999)
HPLC with fluorometric detection	12	1.63 ± 0.12 mmol/l	(Li 1992)

Additionally, cleavage of SA from glycoconjugates using neuraminidase may underestimate the TSA concentration compared with acid hydrolysis (Crook 1993). As shown in Table 1, there are very few studies examining the TSA concentration in serum measured by HPLC. This method seems to obtain lower reference values than seen with other methods. However, also discrepant results have been published (Silver et al. 1981). In conclusion, uniform reference values for serum TSA cannot be determined. Instead, for each method, specific reference values have to be used.

2.4 Tumour markers

A tumour marker is a substance which can be measured in blood or other biological fluids and can be used to differentiate a tumour from normal tissue or to determine the presence of a tumour in a patient. A tumour marker can be produced by the tumour itself or by the host in response to a tumour (Chan and Schwartz 2002). Measurement of tumour markers can be used to monitor cancer, predict the therapeutic response and prognosis of cancer, and in some certain situations even screen and diagnose for cancer. The first identified cancer marker was monoclonal immunoglobulin light chain discovered by Bence-Jones in 1846. This Bence-Jones protein is still in clinical use in the diagnosis and prediction of the therapeutic response in cases of multiple myeloma. Subsequently the concentrations of many hormones, enzymes, and other proteins in biological fluids have been shown to reflect the presence of cancer in patients (Diamandis 2002). An ideal tumour marker should be specific for a certain type of cancer and be sensitive enough to detect small tumours and thus permit early diagnosis. Unfortunately most tumour markers do not fulfil these criteria.

2.4.1 Tumour markers in solid tumours

Recently, guidelines for the recommendations on clinical utility of tumour markers in breast cancer, gynaecological cancers, prostate cancer, colorectal cancer, neuroendocrine tumours, myeloma, and lung cancer have been published (Fleisher et al. 2002). A large number of tumour markers have been used or proposed as markers for breast cancer. CA 15-3 is a carbohydrate antigen expressed in several glandular structures including the mammary gland (Hilkens et al. 1984). Serum CA 15-3 is not useful for breast cancer diagnosis, but it has been shown to associate with the stage of breast cancer (Eskelinen et al. 1997). Carcinoembryonic antigen (CEA) levels in serum have been shown to be related to tumour size and nodal involvement of breast cancer. Simultaneous use of CEA and CA 15-3 allows early diagnosis of metastases in 60-80% of breast cancer patients (Molina et al. 1995). Proteolytic fragments of cytokeratins 8, 18 and 19 are measured in the tissue polypeptide antigen (TPA) assay (Sundström and Stigbrand 1994). Instead of the many epitopes recognized in the TPA assay, the tissue polypeptide-specific antigen (TPS) assay measures only the cell proliferation related epitope M_3 (Björklund and Björklund 1990). Serum TPA and TPS have been shown to have only limited value in the diagnosis of breast cancer (Eskelinen et al. 1994; Eskelinen et al. 1997). However, serum CA 15-3, TPS and TPA may be useful in estimating the response of the treatment in patients with advanced breast cancer (Sjöström et al. 2001).

Prostate-specific antigen (PSA) is the most important marker for the evaluation and even screening of prostate cancer (Fleisher et al. 2002). It is very sensitive (Stamey et al. 1987), but most of the patients with increased PSA values have benign prostatic hyperplasia (BPH) (Labrie et al. 1992). The specificity can be clearly improved by measuring the proportion of the free PSA to PSA-α1-antichymotrypsin complex in addition to total PSA (Stenman et al. 1991). Furthermore, the clinical findings from the digital rectal examination (DRE) and the transrectal ultrasonography (TRUS) are still used for prostate cancer diagnosis (Finne et al. 2002).

A few examples of the tumour markers used in clinical practice related to other solid tumours are CA 19-9, CEA, and CA 125. Serum CA 19-9 is elevated in adenocarcinoma of the pancreas, and other gastrointestinal cancers (Koprowski et al. 1979; Herlyn et al. 1982; Haglund et al. 1986) and may be useful in monitoring patients who are receiving treatment. Serum CEA in colorectal cancer can be used to monitor the response to therapy and to document the progressive course of the disease (Fleisher et al. 2002). One of the most widely used tumour markers for gynaecological cancers is CA 125 which is used for estimating the prognosis and monitoring of ovarian cancer and monitoring patients with endometrical cancer. However, serum CA 125 levels may be elevated also in benign gynaecologic conditions and during the first trimester in normal pregnancy (Fleisher et al. 2002).

2.4.2 Sialic acid as a tumour marker in solid tumours

An elevation in the serum TSA concentration has been observed in a number of cancer types (Shamberger 1984), including breast carcinoma (Hogan-Ryan et al. 1980; Topuz et al. 1986) and the majority of solid tumours in children (Seider et al. 1992). The TSA concentration in serum, when normalized for total protein (TP) and expressed as the TSA/TP ratio, has been found in one study to be a more specific marker compared with TSA alone (Plucinsky et al. 1986). However, in the later study published by the same researchers this better specificity could not be confirmed (Tautu et al. 1991). An elevation in the levels of serum LASA has been found in various malignancies including breast cancer (Schutter et al. 1992). However, the use of LASA as a tumour marker has been found to be restricted by the fact that the elevation of LASA seems to be mainly attributable to the unspecific co-extraction of inflammation associated glycoproteins containing sialic acid as mentioned above (Voigtmann et al. 1989; Végh et al. 1991).

In several studies, TSA, TSA/TP and LASA concentrations in serum have been found to be significantly elevated in breast cancer patients (Silver et al. 1981; Plucinsky et al. 1986; Patel et al. 1990a; Patel et al. 1990b), but the differentiation of breast cancer patients from those with benign breast disease has proved to be difficult (Patel et al. 1990a; Patel et al. 1990b).

Elevation in TSA concentration has been reported in the patients with prostate cancer compared with healthy individuals (Höbarth et al. 1993). TSA (Höbarth et al. 1993) and LASA levels (Dunzendorfer et al. 1981; Meyer et al. 1993) have been reported to increase especially in prostate cancer patients with metastases compared with patients without metastatic involvement. Higher levels of SA in association with the increasing burden imposed by the tumour have been found also in ovarian neoplasia (Berbec et al. 1999), cancer of the oral cavity (Rao et al. 1998), endometrial cancer (Paszkowska et al. 1998), cancer of stomach, breast, colorectal region and gall bladder (Tewarson et al. 1993), and thyroid cancer (Kökoglu et al. 1989).

2.4.3 Sialic acid as a tumour marker in haematological malignancies

In leukemias and lymphomas, the neoplastic cells can be identified and quantitated in peripheral blood and bone marrow cells. Thus, serum tumour markers are not commonly used in diagnosis or follow-up of haematological malignancies. The only exceptions are serum lactate dehydrogenase, which is widely used for risk stratification of patients with non-Hodgkin's lymphoma, and β2-microglobulin, which is used for estimating prognosis of myeloma and to some extent the prognosis of non-Hodgkin's lymphoma and B-cell chronic lymphocytic leukemia patients. (DiGiuseppe and Borowitz 2002)

An elevation in serum TSA has been reported in the majority of children with leukemias (Seider et al. 1992), in adults with acute myeloid leukemia (AML), chronic myeloid leukemia (CML) (Patel et al. 1988), acute lymphoblastic leukemia (ALL) (Patel et al. 1991), chronic lymphocytic leukemia (O'Kennedy et al. 1991) and lymphomas (Shamberger 1984; Voigtmann et al. 1989). Elevated TSA/TP values have been reported in the majority (63% - 90%) of AML, CML and ALL patients (Patel et al. 1994). An elevation of serum LASA has been found in various malignancies including leukemias and lymphomas (Dnistrian and Schwartz 1981; Patel et al. 1988; Voigtmann et al. 1989; Patel et al. 1991; Schutter et al. 1992; Patel et al. 1994).

2.5 Sialic acid in the diagnosis of alcohol abuse

2.5.1 Conventional markers of alcohol abuse

Serum γ-glutamyltransferase (GT) is the most widely used marker for the detection of alcohol abuse, but aspartate aminotransferase (AST), alanine aminotransferase (ALT), and erythrocyte mean corpuscular volume (MCV) have also been used (Stibler 1991; Bell et al. 1994; Yersin et al. 1995). Carbohydrate deficient transferrin (CDT) has been reported to be more specific compared with the markers mentioned above (Table 2) (Stibler 1991; Bell et al. 1994; Yersin et al. 1995). However, inborn errors of glycoprotein metabolism, rare transferrin isoform types, and liver diseases have been shown to produce false-positive serum CDT results (Bean and Peter 1994; Helander et al. 2001).

Table 2. Sensitivity and specificity of conventional biochemical alcohol markers.

Marker	Sensitivity	Specificity	Reference
GT	0.69	0.65	(Yersin et al. 1995)
	0.73	0.75	(Bell et al. 1994)
	0.69	0.59	(Kwoh-Gain et al. 1990)
	0.59	0.50	(Behrens et al. 1988)
AST	0.50	0.82	(Bell et al. 1994)
	0.69	0.68	(Kwoh-Gain et al. 1990)
ALT	0.35	0.86	(Bell et al. 1994)
	0.58	0.57	(Kwoh-Gain et al. 1990)
MCV	0.27	0.91	(Yersin et al. 1995)
	0.52	0.85	(Bell et al. 1994)
	0.73	0.76	(Kwoh-Gain et al. 1990)
	0.25	0.95	(Behrens et al. 1988)
CDT	0.58	0.82	(Yersin et al. 1995)
	0.69	0.92	(Bell et al. 1994)
	0.81	0.90	(Kwoh-Gain et al. 1990)
	0.81	0.91	(Behrens et al. 1988)

2.5.2 Sialic acid levels in alcohol abusers

Excessive ethanol intake has been proposed to affect post-translational glycosylation processes in the liver and result into the desialylation of transferrin and other glycoproteins (Malagolini et al. 1989; Stibler and Borg 1991; Guasch et al. 1992). Alcohol has been shown also to increase the activities of sialidase in the cytosol and plasma membranes (Xin et al. 1995; Hale et al. 1998). Recent studies have shown that the sialic acid concentration in serum

may be elevated in alcoholics (Pönniö et al. 1999a; Sillanaukee et al. 1999). In these studies serum SA was comparable with traditional alcohol markers especially in female subjects. However, the effects of alcohol consumption and the secondary effects of liver disease as the underlying mechanism for the increased marker values were not evaluated in those studies.

2.6 Statistical methods in the diagnosis of cancer and in the evaluation of the diagnostic accuracy of laboratory tests

2.6.1 ROC analysis

The receiver operating characteristic (ROC) curve is a graphical presentation of the relationship between sensitivity and specificity of a laboratory test (Beck and Shultz 1986). The sensitivity (Se) of a test is calculated using an equation (patients with positive test result / number of patients). The specificity (Sp) of a test is calculated using an equation (healthy subjects with negative test results / number of healthy subjects). The ROC curve is constructed by graphing Se as a function of 1-Sp over all possible diagnostic cut-off values of the test. When results from multiple tests have been obtained, the ROC plots can be graphed and compared together; the plot situated above and to the left of another plot indicates greater accuracy (Zweig and Campbell 1993). The area under the curve (AUC) is shown to represent the probability that a randomly chosen diseased subject is ranked correctly with greater suspicion than a randomly chosen non-diseased subject (Hanley and McNeil 1982). A nonparametric statistical comparison of the areas under their ROC curves obtained with two different diagnostic tests from the same sample of patients is an elegant and unbiased way to describe and compare the performance (accuracy) of these tests (Hanley and McNeil 1983; Beck and Shultz 1986; Zweig and Campbell 1993). The ROC analysis has become an essential part of reporting the diagnostic performance of any diagnostic test.

2.6.2 Determination of optimal cut-off value for a test

The optimal cut-off values for tumour markers can be determined by the Youden's index. The Youden's index is computed using the equation (Se + Sp – 1) for each threshold value (Youden 1950). The value that has the greatest Youden´s index is the optimal cut-off value. Another way of determining the optimal cut-off value is to calculate the positive-likelihood ratio value (Se / (1 – Sp)) for each threshold value. The one with the greatest positive-likelihood ratio is the optimal cut-off value (Albert 1982).

2.6.3 Logistic regression analysis

Logistic regression can be used to achieve a direct estimate of the probability of an event occurring based on values of a set of predictor variables (Press and Wilson 1978). For the case of more than one independent variable, the logistic regression model can be written as Prob(event) = $1/(1 + e^{-Z})$ where Z is the linear combination $Z = B_0 + B_1X_1 + B_2X_2 + \ldots + B_PX_P$ (B_{0-P} are the coefficients estimated from the data; X_{1-P} are the independent variables). Logistic regression coefficients are estimated using the maximum-likelihood method, i.e. those coefficients that make the observed results most likely are selected. In this way logistic regression analysis can be used to estimate the diagnostic value of individual variables as well as to combine different variables into a diagnostic algorithm. Since the logistic regression model is nonlinear, an iterative algorithm is necessary for parameter estimation. For example, Virtanen and co-workers showed that the use of a logistic regression model comprising the proportion of free to total PSA, DRE, and heredity of the prostate cancer as explanatory variables had significantly better diagnostic accuracy for prostate cancer than total PSA and the proportion of free to total PSA (Virtanen et al. 1999).

3 AIMS OF THE STUDY

The main purpose of the present study was to evaluate the usefulness of serum sialic acid in clinical diagnostics. The specific aims of this study were:

- To evaluate the diagnostic accuracy of sialic acid in differentiating between breast cancer and benign breast disease (I).

- To evaluate the diagnostic accuracy of sialic acid in differentiating between children with benign tumours and children with malignancy and to study the effect of infections on the levels of serum sialic acid (II).

- To evaluate the diagnostic accuracy of total sialic acid in differentiating between prostate cancer and BPH (III).

- To evaluate the clinical significance of serum total sialic acid as a biomarker for alcohol abuse (IV).

- To determine the reference values for serum total sialic acid using HPAE-PAD (V).

4 MATERIALS AND METHODS

Materials and chemicals used in this study are described in detail in the original papers (I-V).

4.1 Subjects and serum samples (I-V)

4.1.1 Study I, benign and malignant breast disease

This study consisted of patients treated for breast cancer, patients with benign breast disease, and healthy controls. The control group (n=56, median age 32 years, range 21-54) had no evidence of any type of cancer or inflammatory disease. The breast cancer patients (n=22, median age 58 years, range 32-86) underwent mastectomy and the diagnosis was validated histologically from the mastectomy specimen. The patients with benign breast disease (n=31, median age 52 years, range 9-78) underwent an operative biopsy under local or general anesthesia and the diagnosis was validated histologically. Serum samples were collected by venepuncture before surgery or biopsy and kept frozen (-20 °C) until analysed. All the procedures were in accordance with the Helsinki Declaration of 1975, as revised in 1983.

4.1.2 Study II, children with malignancy or infections

The study consisted of 98 children described in Table 3. Serum samples were available from each patient during the active stage of the disease, i.e., at the time of diagnosis, relapse or residual disease, and in some cases during remission. All of the serum samples were collected by venepuncture and kept frozen (-20 °C) until analysed. All the procedures were in accordance with the Helsinki Declaration of 1975, as revised in 1983.

Table 3. Children in study II.

Group	n	Median age (y)	Range (y)
Healthy controls	39	5.3	0.3 – 18.6
Children with acute infectious or inflammatory diseases	13	6.3	2.8 – 17.7
Children with benign tumours	7	4.2	0.4 – 15.9
Children with solid tumours	24	4.9	0.7 – 17.3
Children with leukemia	9	6.8	1.5 – 15.4
Children with malignancy and simultaneous infections	6	5.6	1.0 – 12.5

4.1.3 Study III, the diagnosis of prostate cancer

The study consisted of a total of 121 men in three different groups. Serum samples from 50 prostate cancer patients were collected at the time of diagnosis, during the active stage of disease, during good clinical response to the treatment, or during the last four months of terminal care. The BPH group consisted of 42 patients with increased PSA levels and the histological study from the sextant biopsies revealing benign prostatic adenoma tissue. The control group included 29 patients with PSA levels < 4 μg/l and no evidence of prostate cancer or hyperplasia in DRE or TRUS. All the serum samples were collected by venepuncture before DRE and TRUS and kept frozen (-20 °C) until analysed. All the procedures were in accordance with the Helsinki Declaration of 1975, as revised in 1983, and all of the study subjects had signed an informed consent form and the study was approved by the Ethical Committee of Kuopio University Hospital.

4.1.4 Study IV, serum sialic acid as a marker of alcohol consumption

Serum samples were collected from a total of 71 individuals. The group of alcoholics consisted of 32 alcoholics with biopsy proven liver disease (7 females and 25 males, mean age 50 years) and 19 heavy drinkers (2 females and 17 males, mean age 46 years) devoid of any clinical or biochemical evidence of liver dysfunction despite their drinking habits. The alcoholic liver disease (ALD) patients had a history of continuous ethanol consumption for at least five years in amounts exceeding 80 g/day. Liver biopsies had been obtained to confirm the diagnosis of alcoholic liver disease. Heavy drinkers had consumed a mean of 81±55 grams of ethanol per day during the previous four weeks preceeding blood sampling. The alcoholic patients were negative for serum markers for hepatitis B and for hepatitis C serology. The reference population with no history of alcohol abuse consisted of 20 healthy individuals (10 females and 10 males, mean age 51 years). All serum and biopsy samples were also used for routine diagnostic purposes and the research was conducted according to the provisions of the Declaration of Helsinki. Serum samples were stored at -70 °C until analysed.

4.1.5 Study V, reference values of serum total sialic acid

A reference population consisting of 105 healthy women and 45 healthy men was established. Serum samples from these individuals were collected by venepuncture and stored -20°C until analysed. All the procedures were in accordance with the Helsinki Declaration of 1975, as revised in 1983, and all the study subjects had signed an informed consent form.

4.2 Measurement of TSA and LASA in serum using an HPLC system with absorbance detection (I-II)

The TSA concentration in serum was analyzed by HPLC after liberation of sialic acid by hydrolysis (Shukla and Schauer 1982). Sialic acid-containing glycolipids were isolated (Katopodis and Stock 1980) and the LASA concentration was analysed by HPLC. Chromatography was performed in a column containing Aminex A-29-anion exchange resin (Bio-Rad Labs., Richmond, CA, USA) with a mobile phase of 0.75 mM sodium sulphate. Sialic acid was used as an assay calibrator (Sigma Chemical Co, St. Louis, MO, USA). A UV-detector set at a wavelength of 202 nm was used. Total protein was determined with a protein assay kit (Bio-Rad Labs., Richmond, CA, USA).

4.3 Measurement of TSA in serum using HPAE-PAD (III-V)

TSA in serum was liberated by acid hydrolysis. The assay calibrator (sialyllactose, Sigma Chemical Co, St. Louis, MO, USA) was subjected to a similar hydrolysis process. An internal standard, glucuronic acid, was added to serum samples and calibrators before hydrolysis. After hydrolysis, the samples were passed through Sep-Pak C18 column (Waters Corp., Milford, MA, USA). Chromatography was performed using an HPLC system with CarboPac PA-1 anion exchange column of pellicular resin with a PA1 Guard precolumn and pulsed amperometric detection (Dionex Corp., Sunnyvale, CA, USA) with a mobile phase of 100 mM sodium hydroxide and 150 mM sodium acetate. TSA was quantitated using a linear calibration curve (TSA concentration versus SA peak height per internal standard peak height).

In studies I-II, synthetic free SA (Sigma Chemical Co, St. Louis, MO, USA) and in studies III-V sialyllactose (Sigma Chemical Co, St. Louis, MO, USA) were used as the calibrators. To evaluate the effect of the different sialyllactose commercial preparates on the levels obtained for the sialic acid results, compounds from Sigma and Calbiochem (Calbiochem-Novabiochem Corp., La Jolla, CA, USA) were compared (Study V).

4.4 Measurement of total and free PSA in serum (III)

The total and free PSA concentrations in sera were measured simultaneously with the ProStatus PSA Free/Total Assay using time-resolved DELFIA immunofluorometry (EC&G Wallac, Turku, Finland) (Blijenberg et al. 1997). Some samples from the control and the prostate cancer groups were analyzed with the ProStatus PSA EQM Assay using time-resolved DELFIA immunofluorometry (EC&G Wallac, Turku, Finland).

4.5 Statistical evaluation

Statistical analyses were carried out by using the SPSS for Windows (SPSS Inc., Chicago, IL, USA). The distribution of the assay results was tested for normality in each group by the Lilliefors modification of the Kolmogorov-Smirnov goodness-of-fit test or ShapiroWilks test. Mann-Whitney U-test was used for analyzing the difference between two independent groups. Kruskall-Wallis oneway variance analysis was used for analyzing the difference between several independent groups and Wilcoxon signed ranks test for analyzing the difference between two dependent groups. The Spearman correlation analysis was used to test the correlation between different variables.

Logistic regression analysis was performed to describe the relationship between different sets of explanatory variables and prostate cancer in study III.

The diagnostic performance of the serum markers and logistic regression models were evaluated by ROC analysis. The areas under the ROC curves, standard errors, and the average correlations were calculated according to Beck and Schultz (Beck and Shultz 1986) and Hanley and McNeil (Hanley and McNeil 1982; Hanley and McNeil 1983). The Youden's index was used to determine the optimal cut-off limits.

5 RESULTS

5.1 Serum sialic acid in differentiating between breast cancer, benign breast disease and healthy subjects (I)

The serum TSA, TSA/TP and LASA concentrations in the breast cancer patients were significantly higher ($p<0.0001$) than those found in the healthy controls. The breast cancer patients had significantly higher LASA and TSA levels ($P<0.05$) than the patients with a benign breast disease. ROC analysis revealed that all the markers had low accuracy (AUCs<0.773) in differentiating between the healthy controls and the patients with benign breast disease or between the patients with benign breast disease and the breast cancer patients. According to the ROC analysis, the best differentiation between the healthy controls and the patients with breast cancer was found for serum TSA concentration. The area under the curve was 0.882, indicating a rather high accuracy.

The cut-off values for TSA, TSA/TP and LASA were determined using Youden's index. For differentiation between benign breast disease and cancer patients, the cut-off value for TSA was 2.47 mmol/l (Se = 0.86; Sp = 0.58), for TSA/TP 0.040 mmol/g (Se = 0.59; Sp = 0.84), and for LASA 358.0 μmol/l (Se = 0.64; Sp = 0.81), respectively. The best differentiation between healthy controls and cancer patients was obtained with serum TSA concentration (cut-off value 2.47 mmol/l; Se = 0.86; Sp = 0.84). The best differentiation between healthy controls and benign breast disease patients was also obtained with serum TSA concentration (cut-off value 2.21 mmol/l; Se = 0.87; Sp = 0.61).

5.2 Serum sialic acid in children with malignancy or infections (II)

5.2.1 Children with solid tumours and children with leukemia compared with healthy children

The serum TSA, TSA/TP and LASA values were significantly higher in children with solid tumours ($p<0.0001$ for all parameters) and leukemia ($p<0.0001$ for TSA and TSA/TP and $p<0.001$ for LASA) compared with healthy children. Using Youden's index, the TSA cut-off limit was set to 2.52 mmol/l to discriminate healthy children from pediatric patients with leukemia (Se = 1.00; Sp = 0.95), to 34.8 μmol/g for TSA/TP (Se = 1.00; Sp = 0.92), and to 710 μmol/l for LASA (Se = 0.78; Sp = 0.97), respectively. In the discrimination of healthy children from pediatric patients with solid tumours, the TSA cut-off limit was set to 2.26 mmol/l (Se = 0.96; Sp = 0.87), TSA/TP to 33.4 μmol/g (Se = 0.92; Sp = 0.92), and LASA to 566 μmol/l (Se = 0.58; Sp = 0.95). Serum TSA and TSA/TP accurately discriminated patients

with malignancies from healthy children (AUCs > 0.95). According to the ROC analysis, TSA and TSA/TP were superior to LASA in solid tumours ($p < 0.005$) and leukemias ($p < 0.05$).

5.2.2 Children with benign tumours

The serum TSA, TSA/TP or LASA values in patients with benign tumours did not differ significantly from healthy controls. However, these values were significantly higher ($p < 0.005$) in patients with leukemia compared with patients with benign tumours. Similarly, patients with solid tumours had significantly higher serum TSA and LASA values ($p < 0.005$), and TSA/TP ($p < 0.001$) values than patients with benign tumours. ROC analysis showed that all these markers reliably discriminated patients with malignancies from patients with benign tumours (AUCs > 0.83).

5.2.3 Differentiating children with simultaneous malignancy and infection from children with infectious diseases

Infectious diseases led to a significant increase in TSA, TSA/TP and LASA compared with healthy children ($p < 0.0001$ with each marker) and compared with patients with benign tumours ($p < 0.01$, $p < 0.05$, and $p < 0.005$, respectively). There were no statistically significant differences between infectious diseases and patients with leukemia or solid tumours in the absence of infection (AUCs below 0.65). However, patients with malignancy and simultaneous infection did have significantly higher serum TSA ($p < 0.05$) and TSA/TP ($p < 0.01$) levels than patients with only infectious diseases (AUCs 0.83 and 0.88, respectively).

5.3 Serum sialic acid and PSA in the diagnosis of prostate cancer and BPH (III)

5.3.1 Serum TSA and PSA in the healthy controls, the patients with BPH and the prostate cancer patients

The PSA values were significantly lower in healthy controls compared with patients with BPH or prostate cancer at diagnosis ($p < 0.001$). There were no statistically significant differences in the TSA values between the control and the BPH groups or between the control and the prostate cancer groups. According to ROC analysis, serum PSA could reliably discriminate patients with prostate cancer from healthy controls (AUC 0.991). Using the serum PSA cut-off limit with the maximum Youden's index value (PSA = 3.8 μg/l), Se was 1.00 and Sp was

0.93. Correspondingly, when the cut-off limit with the maximum positive likelihood ratio value (PSA = 4.2 μg/l) was used, Se was 0.94 and Sp was 0.97.

5.3.2 Differentiating BPH from prostate cancer

The diagnostic accuracy of the tumour markers and different logistic regression models was studied with two sets of patients. The first set (Set A) consisted of 46 prostate cancer patients and 42 BPH patients with serum PSA and TSA values and DRE results. The other set (Set B) consisted of 9 prostate cancer patients and 35 BPH patients with PSA values in the grey zone (from 4 μg/l to 10 μg/l), and with free to total PSA ratio, serum TSA values and DRE results.

Using the Set A patients, the diagnostic accuracy of PSA in discriminating patients with prostate cancer from BPH patients according to ROC analysis was 0.871. The diagnostic accuracy of TSA was poor (AUC 0.609). The logistic regression model taking into account the serum PSA and DRE accurately discriminated patients with prostate cancer from BPH patients (AUC 0.937). When the serum TSA values were added to the model, the diagnostic accuracy did not change (AUC 0.935).

Using the Set B patients, the diagnostic accuracy of PSA in discriminating the patients with prostate cancer from the BPH patients according to ROC analysis was 0.563. The corresponding diagnostic accuracy of TSA was 0.603. A logistic regression model combining the serum PSA and free to total PSA ratio values was created to discriminate the patients with prostate cancer from the BPH patients (AUC 0.803). By adding the DRE results to this logistic regression model, the AUC was elevated to 0.863, and by adding the serum TSA values, the AUC further improved to 0.895 and the diagnostic accuracy increased significantly ($p < 0.01$) when compared to that achieved with serum PSA alone.

5.3.3 Correlation of clinical data with the serum PSA and TSA values

There were no statistically significant differences in PSA values between the patients with prostate cancer at diagnosis, the patients with an active stage of the disease, and the patients during the last four months of terminal care. Interestingly, the TSA levels were statistically significantly higher ($p < 0.005$) in the patients during the last four months of terminal care compared to the patients with prostate cancer at diagnosis or during an active stage of the disease. A good response to treatment was reflected in a statistically significant ($p < 0.05$) reduction in the PSA values, but not in the TSA values ($p > 0.05$), compared with the combination of the three groups mentioned above.

5.3.4 Serum tumour markers in the follow-up of prostate cancer

Samples from seven prostate cancer patients in the progressive stage of the disease were collected during the follow-up period. The TSA levels increased in all of these patients, and all but two of them had also increasing PSA levels. The logistic regression model, taking into account the serum PSA and DRE results, classified these patients as prostate cancer patients throughout the follow-up periods (probability values 0.60 - 1.00), all but one having increasing or constantly maximal probability values.

5.4 Serum sialic acid as a marker of alcohol consumption (IV)

The mean sialic acid concentration in serum was found to be significantly higher in the alcoholics than in the healthy controls. Using the serum sialic acid cut-off limit with the maximum Youden's index value (TSA = 1.425 mmol/l), Se was 0.51 and Sp was 1.00. The diagnostic accuracy of the serum TSA according to ROC analysis was good (AUC 0.805).

When the alcoholics were divided into two groups according to the presence or absence of liver disease, the concentration of serum TSA was not found to differ significantly between the groups. However, all of the conventional markers of ethanol consumption, including GT, CDT, and aspartate amino transferase were significantly higher in alcoholics with liver disease than in those without liver disease ($p < 0.05$, $p < 0.05$, and $p < 0.01$, respectively). In the heavy drinkers without liver disease, serum TSA and GT were found to be the most sensitive markers of ethanol abuse. However, in patients with alcoholic liver disease, the conventional markers reflected the severity of liver disease and were elevated more often than serum TSA.

5.5 Reference values of serum TSA and comparison of different calibrators in quantitation of TSA in HPAE-PAD (V)

The serum TSA concentrations of reference groups in studies I - V are presented in Figure 3. The reference subjects were healthy adults except for study II, in where the reference group consisted of children, median age 5.3 y, and range 0.3-18.6 y.

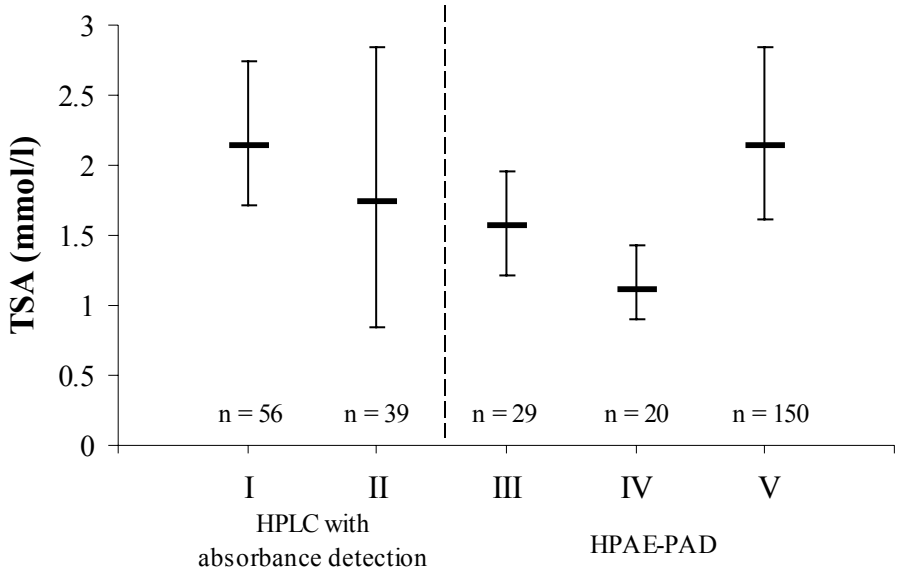

Figure 3. Serum TSA values of healthy subjects in Studies I – V. TSA values are presented as 2.5^{th} percentile, median, 97.5^{th} percentile. The number of the subjects in reference groups is presented for each study.

The sialic acid levels of the reference groups were different between studies I - IV. Different analytical methods and different types of calibrators were used in studies I-II (HPLC with absorbance detection; sialic acid) compared with studies III-V (HPAE-PAD; sialyllactose). The number of reference subjects in studies I – IV was limited, and the median age of these reference populations were different. In study V, a large reference population was collected (n = 150) for the assessment of reference values. Furthermore, to evaluate the effect of different sialyllactose calibrators to the levels of TSA results, preparations from Sigma and Calbiochem were compared. When CalBiochem's sialyllactose calibrator was used, TSA results were on average 28 % higher than with Sigma's sialyllactose compound.

Since there is no reference method against which the HPAE-PAD method could be calibrated, method dependent reference values for sialic acid concentrations were determined using Sigma's sialyllactose calibrator, i.e. the compound that gave serum TSA values closer to the median concentration (1.94 mmol/l) of the previous studies presented in Table 1, and the median concentrations in studies III and IV. There was no statistically significant difference in the TSA concentrations between men and women (Mann-Whitney U test, $p < 0.05$), and the concentrations did not correlate statistically significantly with age. The reference range for

TSA for both men and women was found to be 1.62 mmol/l – 2.85 mmol/l (2.5^{th} and 97.5^{th} centiles), with the median being 2.14 mmol/l.

6 DISCUSSION

6.1 Serum sialic acid in patients with malignancy, benign tumours or infectious diseases

TSA, TSA/TP and LASA values were significantly higher in the breast cancer group compared with the control group ($p < 0.0001$), which is in accordance with earlier studies (Silver et al. 1981; Dnistrian et al. 1982; Plucinsky et al. 1986; Patel et al. 1990a; Patel et al. 1990b). Furthermore, there was also a statistically significant difference in TSA and LASA between the benign and malignant breast tumour groups. This same observation for TSA was also reported by Patel and his co-workers (Patel et al. 1990a). However, discordant results have been reported for LASA (Dnistrian et al. 1982; Patel et al. 1990b).

In the present work we found significantly increased serum TSA, TSA/TP and LASA values in children with leukemia, solid tumours and infections compared with healthy children, which is in accordance with earlier studies in children (Seider et al. 1992) and adults (Dnistrian and Schwartz 1981; Shamberger 1984; Stefenelli et al. 1985; Plucinsky et al. 1986; Patel et al. 1988; Voigtmann et al. 1989; O'Kennedy et al. 1991; Patel et al. 1991; Patel et al. 1994).

The serum concentrations of TSA, TSA/TP and LASA may be increased through changes in the biosynthesis and post-translational glycosylation processing of the acute-phase glycoproteins in the liver (van Dijk et al. 1991) or the phenomenon may be related to the intensified cell metabolism and increased serum sialyltransferase activity expressed by the tumour cells (Warren et al. 1972; Rossowski and Srivastava 1983; Baker et al. 1987; Thampoe et al. 1989; Kanani et al. 1990; Brockhausen et al. 1995). However, malignancy with simultaneous infection increased the serum TSA and TSA/TP concentrations significantly more than infection alone. The present data, combined with earlier results, suggest that serum TSA and TSA/TP measurements could be useful adjuncts in exclusion, diagnosis and follow-up of breast cancer patients or children with malignancies. The possibility of infection superimposed on the underlying malignancy must, however, be taken into account when interpreting sialic acid values during the patient evaluation and follow-up.

In the present work serum PSA increased even in the case of early stages of prostate cancer, which is in accordance with the previous studies (Stamey et al. 1987; Lange et al. 1989; Oesterling 1991; Stenman 1997). On the contrary, the TSA response to the progression of the prostate cancer did not appear until the later stages of the cancer. These findings may be partly due to the very slow progression of prostate cancer and small volume of the tumour tissue at the time of diagnosis. At the terminal stage of the disease, the tumour tissue has

usually widely metastased and there may be also tissue breakdown with extended sialic acid release, as well as the acute phase response. This is in accordance with previous studies in which higher levels of serum sialic acid have been associated with the increasing burden imposed by the tumour (Kökoglu et al. 1989; Tewarson et al. 1993; Paszkowska et al. 1998; Rao et al. 1998; Berbec et al. 1999).

6.2 Diagnostic accuracy of sialic acid as a tumour marker

The ability of TSA measurement to discriminate breast cancer patients from reference people (Se = 0.86; Sp = 0.84) was found to be better than careful clinical breast examination (Se = 0.63, Sp = 0.83) and comparable with mammography combined with clinical breast examination (Se = 0.88; Sp = 0.83) (Fletcher et al. 1993). However, TSA, TSA/TP and LASA could not differentiate the patients with a benign breast disease from those with breast cancer. The low accuracy of the markers is due to the fact that there is also a moderate increase in the serum concentration of sialic acid-containing glycoproteins and glycolipids in patients with benign breast disease.

A logistic regression model combining the serum PSA, free to total PSA ratio values, DRE results, and serum TSA values was found to discriminate accurately BPH and prostate cancer in its early stages. This finding was very interesting, as the diagnostic accuracies of serum TSA and PSA were poor when they were used separately. This may be due to fact that the serum TSA levels were low in prostate cancer in its early stages, and quite high in BPH, and did not correlate to serum PSA values. This is in accordance with our earlier studies in which we found increased serum TSA levels in benign breast disease and in children with infections.

The present data suggest that the logistic regression model, where one combines laboratory measurements and the results of the clinical examination, may be useful especially in the differential diagnosis of benign and malignant prostate disease.

6.3 Clinical significance of serum sialic acid as a biomarker for alcohol abuse

Serum TSA concentrations were found to be significantly higher in alcohol abusers than in healthy controls, which is accordance with earlier studies (Sillanaukee et al. 1999). The sialic acid levels in our study were lower than those reported previously, which is most probably due to the different methods used and the differences in the calibration of these assays.

Most studies on the markers of alcohol consumption have failed to distinguish between the effects of alcohol and its secondary effects on liver disease as the underlying mechanism for

the elevated marker values. We found that the elevation in the serum TSA level was independent of liver status, offering good sensitivity and specificity.

The mechanisms that generate the elevated TSA levels in the serum of alcohol abusers may be caused by the ethanol-induced decrease in the activities of sialyltransferases in Golgi (and synaptosomes) and the increase in the activities of sialidase in the cytosol and plasma membranes (Xin et al. 1995; Hale et al. 1998). It is possible that the non-specificity of TSA could limit its clinical usefulness as a marker of alcohol abuse. However, cancer patients are not frequent in the patient group from whom markers of alcohol abuse are normally measured and any serious infection can be ruled out by clinical examinations and other laboratory tests.

6.4 Reference values for TSA in serum

The level of TSA results is affected by the specificity of the method for measurement of sialic acid and pre-treatment of calibrators and samples e.g. the method used to liberate sialic acid from glycoconjugate structures (Karamanos et al. 1990; Crook 1993). Furthermore, in this study it was found that also the different commercially available sialyllactose reagents contributed considerable differences to the measured TSA concentrations. Until a uniform standardisation of the TSA measurement can be established, method-dependent reference values will be needed.

In previously published studies, serum TSA levels have varied extensively. Our reference values for serum TSA concentrations using HPAE-PAD were in agreement with the median concentration of the previous studies. In contrast to previous reports (Lorentz et al. 1986; Lindberg et al. 1997; Pönniö et al. 1999b), no influence of gender or age to TSA values could be observed.

7 CONCLUSIONS

The aims of the present study were to evaluate the diagnostic accuracy of serum sialic acid in the clinical diagnosis of benign tumours and malignancy and as a biomarker for alcohol abuse. One of the aims was also to determine the reference values for serum total sialic acid. The main conclusions are:

- Both breast cancer and benign breast disease can cause an elevation in the concentration of sialic acid in serum and determination of sialic acid does not provide a reliable classification of undefined breast tumours.

- Serum TSA and TSA/TP could be useful adjuncts in the exclusion, diagnosis and follow-up of children with malignancies. However, an increase of the sialic acid values due to infection can hide the underlying malignancy, and thus this factor must be taken into account in the interpretation of increased sialic acid values.

- The logistic regression model combining TSA, PSA and free to total PSA ratio with DRE results has a good diagnostic accuracy in discriminating patients with prostate cancer from patients with BPH.

- Serum sialic acid measurements may be useful in the assessment of alcohol abuse especially in conditions where the secondary effects of liver disease hamper the use of the traditional markers.

- The observed serum concentrations of sialic acid measurements with different methods should be standardised. Until that occurs, every method should have its own reference values for healthy individuals. In the present work, TSA reference values were determined for the HPAE-PAD method.

In this thesis it was shown that the non-specificity of serum TSA may limit its clinical usefulness as a cancer marker. Elevated serum TSA levels were observed also in patients with benign tumours. However, in this thesis it was demonstrated that the elevation in the serum TSA concentration in alcoholics is independent of liver status and thus it may be of value as a marker of alcohol consumption in conditions where the traditional markers reflect the severity

of liver disease rather than alcohol consumption. Nonetheless, before one considers the use of serum TSA in the clinic for the assessment of ethanol-related problems, greater emphasis must be placed on the calibration of TSA assays.

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