Title: A method of analysing a blood sample of a subject for the presence of a disease marker

FIELD OF THE INVENTION

The invention is in the field of medical diagnostics, in particular in the field of disease diagnostics and monitoring. The invention is directed to markers for the detection of disease, to methods for detecting disease, and to a method for determining the efficacy of a disease treatment.

BACKGROUND OF THE INVENTION

In clinical practice there is a strong need to be able to detect disease in its earliest stages, to predict disease progression, and to implement patient-tailored therapy. Early detection of in particular neoplastic disease (cancer) is critical to ensure favourable treatment of the disease. In spite of numerous advances in medical research, cancer remains a major cause of death worldwide. When patients seek treatment, they are generally exhibiting symptoms of distant metastases, meaning that too often the cancer is detected too late.

Lung, prostate, breast, and colon cancer are the most common tumours, and in order to facilitate appropriate remedial action by surgical resection, radiotherapy, chemotherapy, or other known treatment methods there is a need for rapid and simple methods for the early diagnoses of cancer. The availability of good diagnostic methods for cancer is also important to assess patient responses to treatment, or to assess recurrence due to re-growth at the original site or metastasis.

Several types of cancer markers, such as, for example, oncogene products, growth factors and growth factor receptors, angiogenic factors, proteases, adhesion factors and tumour suppressor gene products, etc, are presently known and are not only considered essential for early diagnosis, but

also for differential diagnosis of patients with uncertain clinical abnormalities such as for distinguishing malignant from benign abnormalities; for predicting the likelihood of response in a particular patient with established malignancy to a selected therapeutic method of treatment; and for providing information concerning the risk, presence, status, or future behaviour of the malignancy in a human or animal subject. Currently, the ability to detect and diagnose cancer through the detection of tumour or cancer markers is an area of widespread interest and as a consequence the need exists for reproducible and reliable methods of identifying new and more useful cancer markers in patient specimens.

Glioblastoma is the most common and most aggressive type of primary brain tumor in humans. The disease is difficult to diagnose and even harder to treat due, in part, to the blood-brain barrier that hinders the delivery of therapeutic agents and detection of potentially important diagnostic markers. Diagnostic markers for glioblastoma are available, but are specific for the tumour tissue itself and require a tumour sample.

Improved screening and detection methods are needed in order to detect cancer in an early phase and to follow the progression of the disease. In the case of cancer we are at a state where we do not only need to detect the tumour, but also need to detect it before it has reached a point of no return, where the treatment becomes palliative instead of curative. People at risk, as well as patients with recurring cancer, should be monitored extensively. Furthermore, since tumours can respond differently to different therapeutics, patient stratification is becoming of importance.

Genetic analysis using tumour biopsies has allowed the identification of many mutations that are useful for diagnosis of the cancer as well as for emerging patient stratification strategies. However, a disadvantage of current genetic analysis of tumours is the need for tumour biopsies, which are often impossible to dissect from patients. Furthermore, the use of biopsies is static and does not allow genetic monitoring of tumour progression or

recurrence over time. Moreover, many tumours are heterogeneous, resulting in potential false-positive or false-negative genetic characterization of biopsies of such tumours.

Recently, the use of circulating tumour cells for diagnosis and monitoring of tumour progression or recurrence showed the use of blood as a source of tumour-derived material, notably tissue fragments in the form of cells. However, the use of circulating tumour cells is inefficient for most cancers.

In general, a disease marker is defined as a compound of which the concentration is altered, preferably elevated, in a biological fluid from a diseased patient when compared to a normal healthy subject, and which may subsequently be used as a marker compound indicative of a disease. Yet, the identification of specific compounds, for instance proteins, in various body fluids as markers of disease, such as cancer, has been hampered by the lack of suitable techniques therefore.

Also in case of diseases other than cancer, markers may be available that are difficult to detect. This hampers early diagnosis of the disease.

The present invention aims to overcome the problem of the prior art that not all diseased tissues or disease types (e.g. tumours) result in circulating disease cells (e.g. circulating tumour cells). The present invention also aims to overcome the problem that protein markers for detecting diseases such as cancer are difficult to detect. Further, the present invention aims to provide methods that do not require biopsies, and allow extensive monitoring of patients.

SUMMARY

The present disclosure provides a method of analysing a blood sample of a subject for the presence of a disease marker, said method comprising the steps of a) extracting nucleic acid from anucleated blood cells, preferably thrombocytes, in said blood sample to provide an

anucleated blood cell-extracted nucleic acid fraction, and b) analysing said anucleated blood cell-extracted nucleic acid fraction for the presence of a disease marker, wherein said disease marker is a disease-specific mutation in a gene of a nucleated cell of said subject, or wherein said disease marker is a disease-specific expression profile of genes of a nucleated cell of said subject.

In a first aspect, there is provided a method of analysing a blood sample of a subject for the presence of a cancer marker, said method comprising the steps of

a) extracting nucleic acid from anucleated blood cells, in said blood sample to provide an anucleated blood cell-extracted nucleic acid fraction, and

b) analysing said anucleated blood cell-extracted nucleic acid fraction for the presence of a cancer marker,

wherein said cancer marker is a cancer-specific mutation in a gene of a nucleated cell of said subject, or

wherein said cancer marker is a cancer-specific expression profile of genes of a nucleated cell of said subject, wherein said nucleic acid is cancer-derived nucleic acid.

The term "anucleated blood cell" as used herein refers to a cell that lacks a nucleus. The term includes reference to both erythrocyte and thrombocyte. Preferred embodiments of anucleated cells in aspects of this invention are thrombocytes. The term "anucleated blood cell" preferably does not include reference to cells that lack a nucleus as a result of faulty cell division.

The term "nucleated cell" as used herein refers to a cell having a nucleus. The term includes reference to somatic cells, germ cells and stem cells, and may include cells from colon, pancreas, brain, bladder, breast, prostate, lung, breast, ovary, uterus, liver, kidney, spleen, thymus, thyroid, nerve tissue, connective tissue, blood, epithelial tissue, lymph node, bone, muscle and skin tissues. The nucleated cell is preferably a cell from a diseased tissue.

Thus, the present disclosure is generally aimed at analysing nucleic acids that have been transferred from cells that have a nucleus into cells that have no nucleus, wherein the cells that have no nucleus can be easily isolated from the blood stream and contain nucleic acid from the nucleated cells.

The term "nucleus" refers to the membrane-enclosed organelle found in eukaryotic cells that contains most of the cell's genetic material organized in the form of chromosomes. The genes within these chromosomes are the cell's nuclear genome. The interior of the nucleus contains a number of subnuclear bodies including the RNA-comprising nucleolus, which is mainly involved in the assembly of RNA-comprising ribosomes. After being produced in the nucleolus, ribosomes are exported to the cytoplasm where they translate mRNA.

An anucleated blood cell-extracted nucleic acid fraction preferably refers to a fraction comprising chromosomal DNA, ribosomal RNA, nucleolus RNA, and/or messenger RNA.

The term "gene" as used herein, and in particular in the phrasing "mutation in a gene of a nucleated cell" is meant to refer to any nucleic acid sequence, both chromosomal and extra-chromosomal, of a nucleated (somatic) cell, preferably a nuclear nucleic acid sequence, and may include transcribed and non-transcribed sequences as well as ribosomal RNA sequences, most preferably chromosomal sequences that are transcribed into RNA.

In a preferred embodiment of a method of the disclosure said disease-specific mutation is in a chromosomal gene.

In another preferred embodiment, said gene is not a gene from an anucleated blood cell.

In a preferred embodiment of a method of the disclosure said disease-specific expression profile is the expression profile of chromosomal genes. In particular of chromosomal genes from a nucleated cell the mRNA of which is present in a thrombocyte.

In another preferred embodiment of a method of the disclosure said nucleic acid is ribonucleic acid (RNA), more preferably messenger ribonucleic acid (mRNA).

In a preferred embodiment of a method of the disclosure said nucleic acid is not mtDNA. Hence, mitochondrial nucleic acid is preferably not an aspect of the present disclosure.

In another preferred embodiment of a method of analysing a blood sample according to the disclosure said step b) of analysing said anucleated blood cell-extracted nucleic acid fraction for the presence of a disease marker comprises the selective amplification of:

i) said mutation by reverse transcriptase polymerase chain reaction amplification using at least one nucleic acid mutation-specific amplification primer or probe, or

ii) a plurality of mRNAs by reverse transcriptase polymerase chain reaction amplification to determine the expression level of the chromosomal genes encoding said mRNAs to thereby provide an expression profile for said genes and comparing said expression profile to a reference profile.

The blood sample is preferably outside the body.

In a preferred embodiment of a method of the disclosure the disease is selected from the group consisting of cancer, autoimmune disease, skin diseases, eye disease, endocrine diseases, neurological disorders, and cardiovascular diseases.

In another preferred embodiment of a method of the disclosure said disease is selected from the group consisting of autoimmune disease, skin diseases, eye disease, endocrine diseases, neurological disorders, and cardiovascular diseases.

In another preferred embodiment of a method of the disclosure said disease is cancer.

In yet another preferred embodiment of a method of the disclosure said cancer is a solid tumour cancer, preferably selected from colon, pancreas, brain, bladder, breast, prostate, lung, breast, ovary, uterus, liver, kidney, spleen, thymus, thyroid, nerve tissue, epithelial tissue, lymph node, bone, muscle and skin.

In preferred embodiments of aspects of the disclosure the auto-immune disease is selected from the group consisting of Achlorhydra Autoimmune Active Chronic Hepatitis; Acute Disseminated Encephalomyelitis; Acute hemorrhagic leukoencephalitis; Addison's Disease; Agammaglobulinemia; Alopecia areata; Amyotrophic Lateral Sclerosis; Ankylosing Spondylitis; Anti-GBM/TBM Nephritis; Antiphospholipid syndrome; Antisynthetase syndrome; polyarticular Arthritis; Atopic allergy; Atopic Dermatitis; Autoimmune Aplastic Anemia; Autoimmune cardiomyopathy; Autoimmune enteropathy; Autoimmune hemolytic anemia; Autoimmune hepatitis; Autoimmune inner ear disease; Autoimmune

lymphoproliferative syndrome; Autoimmune peripheral neuropathy; Autoimmune pancreatitis; Autoimmune polyendocrine syndrome; Autoimmune progesterone dermatitis; Autoimmune thrombocytopenic purpura; Autoimmune uveitis; Balo disease/Balo concentric sclerosis; Bechets Syndrome; Berger's disease; Bickerstaff's encephalitis; Blau syndrome; Bullous Pemphigoid; Castleman's disease; Celiac disease; Chagas disease; Chronic Fatigue Immune Dysfunction Syndrome; Chronic inflammatory demyelinating polyneuropathy; Chronic recurrent multifocal osteomyelitis; Chronic lyme disease; Chronic obstructive pulmonary disease; Churg-Strauss syndrome; Cicatricial Pemphigoid; Coeliac Disease; Cogan syndrome; Cold agglutinin disease; Complement component 2 deficiency; Cranial arteritis; CREST syndrome; Crohns Disease; Cushing's Syndrome; Cutaneous leukocytoclastic angiitis; Dego's disease; Dercum's disease; Dermatitis herpetiformis; Dermatomyositis; Diabetes mellitus type 1; Diffuse cutaneous systemic sclerosis; Dressler's syndrome; Discoid lupus erythematosus; Eczema; Endometriosis; Enthesitis-related arthritis; Eosinophilic fasciitis; Eosinophilic gastroenteritis; Epidermolysis bullosa acquisita; Erythema nodosum; Essential mixed cryoglobulinemia; Evan's syndrome; Fibrodysplasia ossificans progressiva; Fibromyalgia/Fibromyositis; Fibrosing aveolitis; Gastritis; Gastrointestinal pemphigoid; Giant cell arteritis; Glomerulonephritis; Goodpasture's syndrome; Graves' disease; Guillain-Barré syndrome; Hashimoto's encephalitis; Hashimoto's thyroiditis; Haemolytic anaemia; Henoch-Schonlein purpura; Herpes gestationis; Hidradenitis suppurativa; Hughes syndrome; Hypogammaglobulinemia; Idiopathic Inflammatory Demyelinating Diseases; Idiopathic pulmonary fibrosis; Idiopathic thrombocytopenic purpura; IgA nephropathy; Inclusion body myositis; Inflammatory demyelinating polyneuopathy; Interstitial cystitis; Irritable Bowel Syndrome (IBS); Juvenile idiopathic arthritis; Juvenile rheumatoid arthritis; Kawasaki's Disease; Lambert-Eaton myasthenic syndrome; Leukocytoclastic vasculitis; Lichen planus; Lichen sclerosus; Linear IgA

disease; Lou Gehrig's Disease; Lupoid hepatitis; Lupus erythematosus; Majeed syndrome; Ménière's disease; Microscopic polyangiitis; Miller-Fisher syndrome; Mixed Connective Tissue Disease; Morphea; Mucha-Habermann disease; Muckle–Wells syndrome; Multiple Myeloma; Multiple Sclerosis; Myasthenia gravis; Myositis; Narcolepsy; Neuromyelitis optica; Neuromyotonia; Ocular cicatricial pemphigoid; Opsoclonus myoclonus syndrome; Ord thyroiditis; Palindromic rheumatism; PANDAS; Paraneoplastic cerebellar degeneration; Paroxysmal nocturnal hemoglobinuria; Parry Romberg syndrome; Parsonnage-Turner syndrome; Pars planitis; Pemphigus; Pemphigus vulgaris; Pernicious anaemia; Perivenous encephalomyelitis; POEMS syndrome; Polyarteritis nodosa; Polymyalgia rheumatica; Polymyositis; Primary biliary cirrhosis; Primary sclerosing cholangitis; Progressive inflammatory neuropathy; Psoriasis; Psoriatic Arthritis; Pyoderma gangrenosum; Pure red cell aplasia; Rasmussen's encephalitis; Raynaud phenomenon; Relapsing polychondritis; Reiter's syndrome; Restless leg syndrome; Retroperitoneal fibrosis; Rheumatoid arthritis; Rheumatoid fever; Sarcoidosis; Schizophrenia; Schmidt syndrome; Schnitzler syndrome; Scleritis; Scleroderma; Sjögren's syndrome; Spondyloarthropathy; Sticky blood syndrome; Still's Disease; Stiff person syndrome; Subacute bacterial endocarditis (SBE); Susac's syndrome; Sweet syndrome; Sydenham Chorea; Sympathetic ophthalmia; Takayasu's arteritis; Temporal arteritis; Tolosa-Hunt syndrome; Transverse Myelitis; Ulcerative Colitis; Undifferentiated connective tissue disease; Undifferentiated spondyloarthropathy; Vasculitis; Vitiligo; Wegener's granulomatosis; Wilson's syndrome; and Wiskott-Aldrich syndrome.

In other preferred embodiments of aspects of the disclosure the skin disease is selected from the group consisting of Acneiform eruptions; Autoinflammatory syndromes; Chronic blistering; Conditions of the mucous membranes; Conditions of the skin appendages; Conditions of the subcutaneous fat; Congenital anomalies; Connective tissue diseases (such as

Abnormalities of dermal fibrous and elastic tissue); Dermal and subcutaneous growths; Dermatitis (including Atopic Dermatitis, Contact Dermatitis, Eczema, Pustular Dermatitis, and Seborrheic Dermatitis); Disturbances of pigmentation; Drug eruptions; Endocrine-related skin disease; Eosinophilic; Epidermal nevi, neoplasms, cysts; Erythemas; Genodermatoses; Infection-related skin disease; Lichenoid eruptions; Lymphoid-related skin disease; Melanocytic nevi and neoplasms (including Melanoma); Monocyte- and macrophage-related skin disease; Mucinoses; Neurocutaneous; Noninfectious immunodeficiency-related skin disease; Nutrition-related skin disease; Papulosquamous hyperkeratotic (including Palmoplantar keratodermas); Pregnancy-related skin disease; Pruritic; Psoriasis; Reactive neutrophilic; Recalcitrant palmoplantar eruptions; Resulting from errors in metabolism; Resulting from physical factors (including Ionizing radiation-induced); Urticaria and angioedema; Vascular-related skin disease.

In other preferred embodiments of aspects of the disclosure the endocrine disease is selected from the group consisting of Adrenal disorders; Glucose homeostasis disorders; Thyroid disorders; Calcium homeostasis disorders and Metabolic bone disease; Pituitary gland disorders ; and Sex hormone disorders.

In other preferred embodiments of aspects of the disclosure the eye disease is selected from the group consisting of H00-H06 Disorders of eyelid, lacrimal system and orbit; H10-H13 Disorders of conjunctiva; H15-H22 Disorders of sclera, cornea, iris and ciliary body; H25-H28 Disorders of lens; H30-H36 Disorders of choroid and retina (including H30 Chorioretinal inflammation, H31 Other disorders of choroid, H32 Chorioretinal disorders in diseases classified elsewhere, H33 Retinal detachments and breaks, H34 Retinal vascular occlusions, H35 Other retinal disorders, and H36 Retinal disorders in diseases classified elsewhere); H40-H42 Glaucoma; H43-H45 Disorders of vitreous body and globe; H46-H48 Disorders of optic nerve and visual pathways; H49-H52 Disorders of ocular muscles, binocular movement,

accommodation and refraction; H53-H54.9 Visual disturbances and blindness; and H55-H59 Other disorders of eye and adnexa.

In other preferred embodiments of aspects of the disclosure the neurological disorder is selected from the group consisting of Abarognosis; Acquired Epileptiform Aphasia; Acute disseminated encephalomyelitis; Adrenoleukodystrophy; Agenesis of the corpus callosum; Agnosia; Aicardi syndrome; Alexander disease; Alien hand syndrome; Allochiria; Alpers' disease; Alternating hemiplegia; Alzheimer's disease; Amyotrophic lateral sclerosis (see Motor Neurone Disease); Anencephaly; Angelman syndrome; Angiomatosis; Anoxia; Aphasia; Apraxia; Arachnoid cysts; Arachnoiditis; Arnold-Chiari malformation; Arteriovenous malformation; Ataxia Telangiectasia; Attention deficit hyperactivity disorder; Auditory processing disorder; Autonomic Dysfunction; Back Pain; Batten disease; Behcet's disease; Bell's palsy; Benign Essential Blepharospasm; Benign Intracranial Hypertension; Bilateral frontoparietal polymicrogyria; Binswanger's disease; Blepharospasm; Bloch-Sulzberger syndrome; Brachial plexus injury; Brain abscess; Brain damage; Brain injury; Brain tumor; Brown-Séquard syndrome; Canavan disease; Carpal tunnel syndrome; Causalgia; Central pain syndrome; Central pontine myelinolysis; Centronuclear myopathy; Cephalic disorder; Cerebral aneurysm; Cerebral arteriosclerosis; Cerebral atrophy; Cerebral gigantism; Cerebral palsy; Cerebral vasculitis; Cervical spinal stenosis; Charcot-Marie-Tooth disease; Chiari malformation; Chorea; Chronic fatigue syndrome; Chronic inflammatory demyelinating polyneuropathy (CIDP); Chronic pain; Coffin Lowry syndrome; Coma; Complex regional pain syndrome; Compression neuropathy; Congenital facial diplegia; Corticobasal degeneration; Cranial arteritis; Craniosynostosis; Creutzfeldt-Jakob disease; Cumulative trauma disorders; Cushing's syndrome; Cytomegalic inclusion body disease (CIBD); Cytomegalovirus Infection; Dandy-Walker syndrome; Dawson disease; De Morsier's syndrome; Dejerine-Klumpke palsy; Dejerine-Sottas disease; Delayed sleep phase syndrome; Dementia; Dermatomyositis;

Developmental dyspraxia; Diabetic neuropathy; Diffuse sclerosis; Dravet syndrome; Dysautonomia; Dyscalculia; Dysgraphia; Dyslexia; Dystonia; Empty sella syndrome; Encephalitis; Encephalocele; Encephalotrigeminal angiomatosis; Encopresis; Epilepsy; Erb's palsy; Erythromelalgia; Essential tremor; Fabry's disease; Fahr's syndrome; Fainting; Familial spastic paralysis; Febrile seizures; Fisher syndrome; Friedreich's ataxia; Fibromyalgia; Gaucher's disease; Gerstmann's syndrome; Giant cell arteritis; Giant cell inclusion disease; Globoid Cell Leukodystrophy; Gray matter heterotopia; Guillain-Barré syndrome; HTLV-1 associated myelopathy; Hallervorden-Spatz disease; Head injury; Headache; Hemifacial Spasm; Hereditary Spastic Paraplegia; Heredopathia atactica polyneuritiformis; Herpes zoster oticus; Herpes zoster; Hirayama syndrome; Holoprosencephaly; Huntington's disease; Hydranencephaly; Hydrocephalus; Hypercortisolism; Hypoxia; Immune-Mediated encephalomyelitis; Inclusion body myositis; Incontinentia pigmenti; Infantile phytanic acid storage disease; Infantile Refsum disease; Infantile spasms; Inflammatory myopathy; Intracranial cyst; Intracranial hypertension; Joubert syndrome; Karak syndrome; Kearns-Sayre syndrome; Kennedy disease; Kinsbourne syndrome; Klippel Feil syndrome; Krabbe disease; Kugelberg-Welander disease; Kuru; Lafora disease; Lambert-Eaton myasthenic syndrome; Landau-Kleffner syndrome; Lateral medullary (Wallenberg) syndrome; Learning disabilities; Leigh's disease; Lennox-Gastaut syndrome; Lesch-Nyhan syndrome; Leukodystrophy; Lewy body dementia; Lissencephaly; Locked-In syndrome; Lou Gehrig's disease (See Motor Neurone Disease); Lumbar disc disease; Lumbar spinal stenosis; Lyme disease - Neurological Sequelae; Machado-Joseph disease (Spinocerebellar ataxia type 3); Macrencephaly; Macropsia; Megalencephaly; Melkersson-Rosenthal syndrome; Menieres disease; Meningitis; Menkes disease; Metachromatic leukodystrophy; Microcephaly; Micropsia; Migraine; Miller Fisher syndrome; Mini-stroke (transient ischemic attack); Mitochondrial myopathy; Mobius syndrome; Monomelic amyotrophy; Motor Neurone Disease; Motor skills

disorder; Moyamoya disease; Mucopolysaccharidoses; Multi-infarct dementia; Multifocal motor neuropathy; Multiple sclerosis; Multiple system atrophy; Muscular dystrophy; Myalgic encephalomyelitis; Myasthenia gravis; Myelinoclastic diffuse sclerosis; Myoclonic Encephalopathy of infants; *Myoclonus*; *Myopathy*; *Myotubular myopathy*; *Myotonia congenita*; Narcolepsy; Neurofibromatosis; Neuroleptic malignant syndrome; Neurological manifestations of AIDS; Neurological sequelae of lupus; Neuromyotonia; Neuronal ceroid lipofuscinosis; Neuronal migration disorders; Niemann-Pick disease; Non 24-hour sleep-wake syndrome; Nonverbal learning disorder; O'Sullivan-McLeod syndrome; Occipital Neuralgia; Occult Spinal Dysraphism Sequence; Ohtahara syndrome; Olivopontocerebellar atrophy; Opsoclonus myoclonus syndrome; Optic neuritis; Orthostatic Hypotension; Overuse syndrome; Palinopsia; Paresthesia; Parkinson's disease; Paramyotonia Congenita; Paraneoplastic diseases; Paroxysmal attacks; Parry-Romberg syndrome; Pelizaeus-Merzbacher disease; Periodic Paralyses; Peripheral neuropathy; Persistent Vegetative State; Pervasive developmental disorders; Photic sneeze reflex; Phytanic acid storage disease; Pick's disease; Pinched nerve; Pituitary tumors; PMG; Polio; Polymicrogyria; Polymyositis; Porencephaly; Post-Polio syndrome; Postherpetic Neuralgia (PHN); Postinfectious Encephalomyelitis; Postural Hypotension; Prader-Willi syndrome; Primary Lateral Sclerosis; Prion diseases; Progressive hemifacial atrophy; Progressive multifocal leukoencephalopathy; Progressive Supranuclear Palsy; Pseudotumor cerebri; Rabies; Ramsay-Hunt syndrome (Type I and Type II); Rasmussen's encephalitis; Reflex neurovascular dystrophy; Refsum disease; Repetitive motion disorders; Repetitive stress injury; Restless legs syndrome; Retrovirus-associated myelopathy; Rett syndrome; Reye's syndrome; *Rhythmic Movement Disorder*; Romberg syndrome; Saint Vitus dance; Sandhoff disease; Schizophrenia; Schilder's disease; Schizencephaly; Sensory integration dysfunction; Septo-optic dysplasia; Shaken baby syndrome; Shingles; Shy-Drager syndrome; Sjögren's

syndrome; Sleep apnea; Sleeping sickness; Snatiation; Sotos syndrome; Spasticity; Spina bifida; Spinal cord injury; Spinal cord tumors; Spinal muscular atrophy; Spinocerebellar ataxia; Steele-Richardson-Olszewski syndrome; Stiff-person syndrome; Stroke; Sturge-Weber syndrome; Subacute sclerosing panencephalitis; Subcortical arteriosclerotic encephalopathy; Superficial siderosis; Sydenham's chorea; Syncope; Synesthesia; Syringomyelia; Tarsal tunnel syndrome; Tardive dyskinesia; Tarlov cyst; Tay-Sachs disease; Temporal arteritis; Tetanus; Tethered spinal cord syndrome; Thomsen disease; Thoracic outlet syndrome; Tic Douloureux; Todd's paralysis; Tourette syndrome; Toxic encephalopathy; Transient ischemic attack; Transmissible spongiform encephalopathies; Transverse myelitis; Traumatic brain injury; Tremor; Trigeminal neuralgia; Tropical spastic paraparesis; Trypanosomiasis; Tuberous sclerosis; Von Hippel-Lindau disease; Viliuisk Encephalomyelitis ; Wallenberg's syndrome; Werdnig-Hoffman disease; West syndrome; Whiplash; Williams syndrome; Wilson's disease; and Zellweger syndrome.

In other preferred embodiments of aspects of the disclosure the cardiovascular disease is selected from the group consisting of Aneurysm; Angina; Atherosclerosis; Cerebrovascular Accident (Stroke); Cerebrovascular disease; Congestive Heart Failure; Coronary Artery Disease; Myocardial infarction (Heart Attack); and Peripheral vascular disease.

In another aspect, the present disclosure provides a method of diagnosing disease in a subject using the method of analysing a blood sample according to the disclosure. Hence, in another preferred embodiment of a method of the disclosure, said method of analysing a blood sample according to the disclosure is part of a method of diagnosing disease in a subject, and wherein the presence of said disease marker in said anucleated blood cell-extracted nucleic acid fraction is indicative of said subject suffering from said disease.

The present disclosure provides a method for determining the efficacy of a disease treatment in a subject, comprising the steps of:

- analysing a blood sample of a subject for the presence of a disease marker using the method of analysing a blood sample according to the disclosure at a first time point to thereby provide a first value for the level of said disease marker in said subject;
- analysing a blood sample of said subject for the presence of a disease marker using the method of analysing a blood sample according to the disclosure at a second time point that is earlier or later, preferably later, than said first time point, to thereby provide a second value for the level of said disease marker in said subject, wherein said subject has been subjected to a disease treatment between said first and second time point, and
- comparing said first and second value to determine the efficacy of said disease treatment in said subject.

In a second aspect, there is provided a method for determining the stage of cancer or the efficacy of a cancer treatment in a subject, comprising the steps of:

- analysing a blood sample of a subject for the presence of a cancer marker using the method as defined above at a first time point to thereby provide a first value for the level of said cancer marker in said subject,
- analysing a blood sample of said subject for the presence of a cancer marker using the method as defined above at a second time point to thereby provide a second value for the level of said cancer marker in said subject, wherein said subject has been subjected to a cancer treatment between said first and second time point, and
- comparing said first and second value to determine the efficacy of said cancer treatment in said subject.

The skilled artisan will understand that treatment prior to the first time point and subsequent measurements at a second, later, time point without any disease treatment having occurred between said time points, is included in the present disclosure for determining the efficacy of a disease treatment.

The present disclosure provides a method for determining the stage of disease. In order to determine the stage of disease, it is beneficial to correlate disease marker values as determined by methods of this disclosure to disease stages. A single measurement of the disease marker may than be compared to one or more reference values to obtain an indication of the stage of the disease.

The present disclosure provides a method for determining the stage of disease in a subject, comprising the steps of:

- analysing a blood sample of a subject for the presence of a disease marker using the method of analysing a blood sample of a subject for the presence of a

disease marker according to the present disclosure to thereby provide a test value for the level of said disease marker in said subject,

- providing a reference value for the level of said disease marker wherein said reference value is correlated to a particular stage of disease, and
- comparing said test and reference value to determine the stage of disease in said subject.

In a third aspect, there is provided a method for determining the stage of a cancer in a subject, comprising the steps of:

- analysing a blood sample of a subject for the presence of a cancer marker using the method as defined above to thereby provide a test value for the level of said cancer marker in said subject,
- providing a reference value for the level of said cancer marker wherein said reference value is correlated to a particular stage of cancer, and
- comparing said test and reference value to determine the stage of cancer in said subject.

The present disclosure provides a kit of parts adapted for performing a method of the disclosure as described herein above, the kit comprising a packaging material which comprises at least one of:

- a container for holding anucleated blood cells, preferably thrombocytes, separated from a blood sample;
- an agent for extracting nucleic acids from said anucleated blood cells;
- an agent for selectively amplifying from said nucleic acids extracted from said anucleated blood cells a disease-specific marker as described herein above, such as a disease-specific mutation in a gene of a nucleated cell of a subject or a disease-specific expression profile of nucleic acid from a nucleated cell of said subject, for instance by reverse transcriptase polymerase chain reaction amplification, and
- a printed or electronic instruction for performing a method of the disclosure as described herein above,

the kit further comprising:

- a reference for said disease marker, wherein said reference is indicative for the presence or absence of said disease marker in said anucleated blood cells-extracted nucleic acid fraction.

In a fourth aspect, there is provided a kit of parts adapted for performing the method recited above, the kit comprising a packaging material which comprises at least one of:

- a container for holding anucleated blood cells separated from a blood sample of a subject;

- an agent for extracting nucleic acids from said anucleated blood cells;

- an agent for selectively amplifying from said nucleic acids extracted from said anucleated blood cells a cancer-specific mutation in a gene of a nucleated cell of said subject by reverse transcriptase polymerase chain reaction amplification, and

- a printed or electronic instruction for performing the method recited above, the kit further comprising:

- a reference for said cancer marker, wherein said reference is indicative for the presence or absence of said cancer marker in said anucleated blood cells-extracted nucleic acid fraction.

In a preferred embodiment of a kit according to the present disclosure said reference is a reference value for the level of nucleic acids comprising said disease-specific mutation in anucleated blood cells in a healthy control subject or in a control subject suffering from disease, or wherein said reference is a reference expression profile, for instance for a plurality of

mRNAs in anucleated blood cells from a healthy control subject or from a control subject suffering from disease.

In another preferred embodiment of a kit according to the present disclosure said agent or instruction is selected from a particle or fluorescent marker-labeled anti-anucleated blood cell antibody (preferably a fluorescent marker-labeled anti-thrombocyte antibody), an instruction for bead-based anucleated blood cells isolation (preferably thrombocyte isolation), an instruction for FACS sorting of anucleated blood cells (preferably of thrombocytes), an instruction for anucleated blood cell (preferably thrombocyte) recovery by centrifugation, or negative selection of non-anucleated blood cell components (preferably non-thrombocyte components).

The present disclosure provides a device for diagnosing disease, the device comprising a support and at least one agent for specifically determining a level and/or activity of at least one nucleic acid mutant in a anucleated blood cells sample of the subject, said agent being attached to said support, and a computer-readable medium having computer-executable instructions for performing a method of the disclosure as described herein above.

In a fifth aspect, there is provided a device for diagnosing cancer, the device comprising a support and at least one agent for specifically determining a level and/or activity of at least one nucleic acid mutant in an anucleated blood cell sample of the subject attached to said support, and a computer-readable medium having computer-executable instructions for performing the method recited above.

In a preferred embodiment of a device according to the present disclosure, said at least one agent is an oligonucleotide probe or sequencing primer.

In a preferred embodiment of a device according to the present disclosure, the device comprises a lateral flow device, a dipstick or a cartridge for performing a nucleic acid hybridization reaction between an anucleated blood cells-extracted nucleic acid and at least one nucleic acid mutation-specific amplification primer or oligonucleotide probe, or between an anucleated blood cells-extracted nucleic acid and a plurality of gene-specific amplification primers or oligonucleotide probes for providing an disease-specific gene expression profile.

DESCRIPTION OF THE DRAWINGS

Figure 1 displays RNA profiles as analyzed using an Agilent Bioanalyzer Picochip (Agilent Technologies, Inc.), with the length of the RNA (in number of nucleotides) on the X-axis, and the amount of RNA (in fluorescence units) on the Y-axis. Here depicted, RNA derived from microvesicles in the blood serum fraction (left), RNA derived from microvesicles in the blood plasma fraction (middle) or RNA derived from thrombocytes (right). It is shown that 1) RNA is present in microvesicles in serum and plasma and in thrombocytes, 2) microvesicles isolated from plasma samples contain less RNA than microvesicles isolated from serum samples, and 3) thrombocytes isolated from plasma samples contain RNAs of various sizes, including important fractions of relatively long RNA chains (>200 nucleotides (nt), and even >1000 nucleotides).

Figure 2 displays the findings of tumour derived genetic material found in thrombocytes from patients with brain tumours. Blood samples from patients (P1-14) were taken (whole blood tube (serum (S)) and anticoagulant-EDTA blood (plasma (P)). From the plasma tube, thrombocytes (T) were collected by centrifugation protocol. As controls, thrombocytes were collected from healthy individuals (C1-6). Some patients lack the serum sample, indicated by X in figure 2, and some have pooled serum and plasma samples indicated by SP in figure 2. Using nested PCR for RNA detection, the mutant EGFRvIII (V3) could be detected in thrombocytes of 4 glioblastoma patients out of 15 (27%) (P4, P5, P9, P10). This is in line with the published literature where mutant EGFRvIII is found in 20% of high grade gliomas (Liu et al. 2005). These experiments do provide the proof of principle that thrombocytes can be used as a biomarker source for the diagnosis of cancer by the identification of tumour-derived nucleic acids.

Figure 3. (A) U87 glioma-derived microvesicles were labelled with PKH67 green fluorescent dye and incubated with isolated platelets. After 15 and 60 min of incubation in the presence and absence of microvesicles the

platelets were washed and subjected to FACS analysis of PKH67 fluorescence. In addition, the platelets were stained and analyzed by confocal microscopy to determine microvesicle uptake. RNA was isolated from RNase-treated platelets after incubation with microvesicles under different conditions. RT-PCR was performed to detect EGFRvIII RNA. MV/MVEGFRvIII: microvesicles isolated from U87/U87-EGFRvIII cells. (B) RNA was isolated from platelets from healthy control subjects or glioma patients and subjected to RT-PCR analysis. Corresponding glioma tissue biopsies served as control. PC = U87-EGFRvIII RNA; NC = H20; nd = not determined; * indicates positive signal. (C) RNA as in (B) was subjected to gene expression arrays. Heat map of top-30 glioma biomarkers is shown on the left. Individual expression levels for the top-10 RNAs depicted on the right. Dashed line = BG (background).

Figure 4. RNA was isolated from platelets from healthy control subjects (n = 8) and prostate cancer patients (n = 12) and subjected to PCA3, PSA, and GAPDH RT-PCR analysis. * indicates weak positive signal.

Figure 5 shows the probe sequences used for the detection of the genes displayed in Figure 3C.

DETAILED DESCRIPTION OF THE INVENTION

As used herein, the term "cancer" refers to a disease or disorder resulting from the proliferation of oncogenically transformed cells. "Cancer" shall be taken to include any one or more of a wide range of benign or malignant tumours, including those that are capable of invasive growth and metastasis through a human or animal body or a part thereof, such as, for example, via the lymphatic system and/or the blood stream. As used herein, the term "tumour" includes both benign and malignant tumours or solid growths, notwithstanding that the present invention is particularly directed to the diagnosis or detection of malignant tumours and solid cancers. Cancers further include but are not limited to carcinomas, lymphomas, or sarcomas, such as, for example, ovarian cancer, colon cancer, breast cancer, pancreatic

cancer, lung cancer, prostate cancer, urinary tract cancer, uterine cancer, acute lymphatic leukaemia, Hodgkin's disease, small cell carcinoma of the lung, melanoma, neuroblastoma, glioma (e.g. glioblastoma), and soft tissue sarcoma, lymphoma, melanoma, sarcoma, and adenocarcinoma. In preferred embodiments of aspects of the present invention, thrombocyte cancer is disclaimed.

The term "cancer-derived" as used herein refers to origination from a cancer or cancer cell.

The term "cancer-derived nucleic acid " shall be taken to mean any nucleic acid that is indicative of cancer in the subject, specifically and in most preferred embodiments a mutant DNA or RNA indicating the presence in the cancer of a mutant gene that is expressed by or is present in a cancer cell of the subject, of which mutant gene the nucleic acid sequence is altered relative to the normal gene of a healthy control subject. The term "cancer-derived nucleic acid " shall also be taken to include (i) a nucleic acid that is produced by, expressed by, or present in a cancer cell but not in a normal healthy (non-cancerous) cell, or whose production or expression is altered (enhanced or reduced) by or in a cancer cell compared to a normal cell; or (ii) a nucleic acid that is produced by, expressed by, or present in a normal cell but not by or in a cancer cell. Hence, the nucleic acid need not be a mutant nucleic acid having a mutated sequence but may be a normal nucleic acid having a wild-type (non-cancer) sequence, but whose profile or expression level is altered in a cancer cell relative to a normal cell. In one preferred embodiment, the cancer-derived nucleic acid is a mutant nucleic acid (DNA, cDNA, or RNA) specific for the cancer, preferably an RNA transcript. In another very preferred embodiment, the cancer-derived nucleic acid is a nucleic acid expression profile indicative of being cancer-derived or cancer-specific, as explained in detail herein.

As used herein the term "cancer marker" refers to in particular to a cancer marker gene or a cancer marker gene expression profile. As used herein, the term "cancer marker gene" refers to a gene whose sequence or

expression level, alone or in combination with other genes, is correlated with cancer or prognosis of cancer. The correlation may relate to either an increased or decreased expression of the gene reflected in an increased or decreased presence of the RNA expression product of said gene in the nucleic acid fraction obtainable from thrombocytes. For example, the expression of the gene may be indicative of cancer, or lack of expression of the gene may be correlated with poor prognosis in a cancer patient. In the case of prostate cancer AMACR, PCA3 and PSA are suitable cancer markers. In the case of colorectal cancer KRAS mutations are suitable cancer markers. In the case of lung carcinoma EGFR mutations are suitable cancer markers. In the case of melanoma BRAF mutations are suitable cancer markers. In the case of glioma EGFRvIII mutations are suitable cancer markers. Other suitable cancer markers may be derived from Tables 1 and 2 as provided herein or from the Examples or Figures. The skilled person will understand that many other cancer markers may be employed in aspects and embodiments of this invention.

As used herein, the term "stage of cancer" refers to a qualitative or quantitative assessment of the level of advancement of a cancer. Criteria used to determine the stage of a cancer include, but are not limited to, the size of the tumor, whether the tumor has spread to other parts of the body and where the cancer has spread (e.g., within the same organ or region of the body or to another organ).

The term "cancer" in the terms "cancer derived", "cancer marker", "cancer marker gene", and/or "stage of cancer" may be generalized to the term "disease" as the definitions for cancer are generally applicable to all diseases as indicated herein.

The term "disease-derived" as used herein refers to origination from a disease or diseased cell.

The term "disease-derived nucleic acid " shall be taken to mean any nucleic acid that is indicative of a disease in the subject, specifically and in most preferred embodiments a mutant DNA or RNA indicating the presence in

the disease of a mutant gene that is expressed by or is present in a diseased cell of the subject, of which mutant gene the nucleic acid sequence is altered relative to the normal gene of a healthy control subject. The term "disease-derived nucleic acid " shall also be taken to include (i) a nucleic acid that is produced by, expressed by, or present in a diseased cell but not in a normal healthy (non-diseased) cell, or whose production or expression is altered (enhanced or reduced) by or in a diseased cell compared to a normal cell; or (ii) a nucleic acid that is produced by, expressed by, or present in a normal cell but not by or in a diseased cell. Hence, the nucleic acid need not be a mutant nucleic acid having a mutated sequence but may be a normal nucleic acid having a wild-type (non-cancer) sequence, but whose profile or expression level is altered in a diseased cell relative to a normal cell. In one preferred embodiment, the disease-derived nucleic acid is a mutant nucleic acid (DNA, cDNA, or RNA) specific for the disease, preferably an RNA transcript. In another very preferred embodiment, the disease-derived nucleic acid is a nucleic acid expression profile indicative of being disease-derived or disease-specific, as explained in detail herein.

As used herein the term “disease marker” refers to in particular to a disease marker gene or a disease marker gene expression profile. As used herein, the term "disease marker gene" refers to a gene whose sequence or expression level, alone or in combination with other genes, is correlated with disease or prognosis of the disease. The correlation may relate to either an increased or decreased expression of the gene reflected in an increased or decreased presence of the RNA expression product of said gene in the nucleic acid fraction obtainable from thrombocytes. For example, the expression of the gene may be indicative of a disease, or lack of expression of the gene may be correlated with poor prognosis in a patient.

As used herein, the term “stage of disease” refers to a qualitative or quantitative assessment of the level of advancement of a disease. Criteria used to determine the stage of a disease include, but are not limited to, whether the

disease has spread to other parts of the body and where the disease has spread to (e.g., within the same organ or region of the body or to another organ).

The term disease as used herein refers to cancer, autoimmune disease, skin diseases, eye disease, endocrine diseases, neurological disorders, and cardiovascular diseases.

Thus, diseases that in addition to cancer can be detected using the means and methods of the present invention include for instance the following auto-immune diseases: Achlorhydra Autoimmune Active Chronic Hepatitis; Acute Disseminated Encephalomyelitis; Acute hemorrhagic leukoencephalitis; Addison's Disease; Agammaglobulinemia; Alopecia areata; Amyotrophic Lateral Sclerosis; Ankylosing Spondylitis; Anti-GBM/TBM Nephritis; Antiphospholipid syndrome; Antisynthetase syndrome; polyarticular Arthritis; Atopic allergy; Atopic Dermatitis; Autoimmune Aplastic Anemia; Autoimmune cardiomyopathy; Autoimmune enteropathy; Autoimmune hemolytic anemia; Autoimmune hepatitis; Autoimmune inner ear disease; Autoimmune lymphoproliferative syndrome; Autoimmune peripheral neuropathy; Autoimmune pancreatitis; Autoimmune polyendocrine syndrome; Autoimmune progesterone dermatitis; Autoimmune thrombocytopenic purpura; Autoimmune uveitis; Balo disease/Balo concentric sclerosis; Bechets Syndrome; Berger's disease; Bickerstaff's encephalitis; Blau syndrome; Bullous Pemphigoid; Castleman's disease; Celiac disease; Chagas disease; Chronic Fatigue Immune Dysfunction Syndrome; Chronic inflammatory demyelinating polyneuropathy; Chronic recurrent multifocal osteomyelitis; Chronic lyme disease; Chronic obstructive pulmonary disease; Churg-Strauss syndrome; Cicatricial Pemphigoid; Coeliac Disease; Cogan syndrome; Cold agglutinin disease; Complement component 2 deficiency; Cranial arteritis; CREST syndrome; Crohns Disease; Cushing's Syndrome; Cutaneous leukocytoclastic angiitis; Dego's disease; Dercum's disease; Dermatitis herpetiformis; Dermatomyositis; Diabetes mellitus type 1; Diffuse cutaneous systemic sclerosis; Dressler's syndrome; Discoid lupus erythematosus; Eczema;

Endometriosis; Enthesitis-related arthritis; Eosinophilic fasciitis; Eosinophilic gastroenteritis; Epidermolysis bullosa acquisita; Erythema nodosum; Essential mixed cryoglobulinemia; Evan's syndrome; Fibrodysplasia ossificans progressiva; Fibromyalgia/Fibromyositis; Fibrosing aveolitis; Gastritis; Gastrointestinal pemphigoid; Giant cell arteritis; Glomerulonephritis; Goodpasture's syndrome; Graves' disease; Guillain-Barré syndrome; Hashimoto's encephalitis; Hashimoto's thyroiditis; Haemolytic anaemia; Henoch-Schonlein purpura; Herpes gestationis; Hidradenitis suppurativa; Hughes syndrome; Hypogammaglobulinemia; Idiopathic Inflammatory Demyelinating Diseases; Idiopathic pulmonary fibrosis; Idiopathic thrombocytopenic purpura; IgA nephropathy; Inclusion body myositis; Inflammatory demyelinating polyneuopathy; Interstitial cystitis; Irritable Bowel Syndrome (IBS); Juvenile idiopathic arthritis; Juvenile rheumatoid arthritis; Kawasaki's Disease; Lambert-Eaton myasthenic syndrome; Leukocytoclastic vasculitis; Lichen planus; Lichen sclerosus; Linear IgA disease; Lou Gehrig's Disease; Lupoid hepatitis; Lupus erythematosus; Majeed syndrome; Ménière's disease; Microscopic polyangiitis; Miller-Fisher syndrome; Mixed Connective Tissue Disease; Morphea; Mucha-Habermann disease; Muckle–Wells syndrome; Multiple Myeloma; Multiple Sclerosis; Myasthenia gravis; Myositis; Narcolepsy; Neuromyelitis optica; Neuromyotonia; Occular cicatricial pemphigoid; Opsoclonus myoclonus syndrome; Ord thyroiditis; Palindromic rheumatism; PANDAS; Paraneoplastic cerebellar degeneration; Paroxysmal nocturnal hemoglobinuria; Parry Romberg syndrome; Parsonnage-Turner syndrome; Pars planitis; Pemphigus; Pemphigus vulgaris; Pernicious anaemia; Perivenous encephalomyelitis; POEMS syndrome; Polyarteritis nodosa; Polymyalgia rheumatica; Polymyositis; Primary biliary cirrhosis; Primary sclerosing cholangitis; Progressive inflammatory neuropathy; Psoriasis; Psoriatic Arthritis; Pyoderma gangrenosum; Pure red cell aplasia; Rasmussen's encephalitis; Raynaud phenomenon; Relapsing polychondritis; Reiter's syndrome; Restless leg

syndrome; Retroperitoneal fibrosis; Rheumatoid arthritis; Rheumatoid fever; Sarcoidosis; Schizophrenia; Schmidt syndrome; Schnitzler syndrome; Scleritis; Scleroderma; Sjögren's syndrome; Spondyloarthropathy; Sticky blood syndrome; Still's Disease; Stiff person syndrome; Subacute bacterial endocarditis (SBE); Susac's syndrome; Sweet syndrome; Sydenham Chorea; Sympathetic ophthalmia; Takayasu's arteritis; Temporal arteritis; Tolosa-Hunt syndrome; Transverse Myelitis; Ulcerative Colitis; Undifferentiated connective tissue disease; Undifferentiated spondyloarthropathy; Vasculitis; Vitiligo; Wegener's granulomatosis; Wilson's syndrome; and Wiskott-Aldrich syndrome.

Apart from the above diseases, aspects of the present invention are also applicable to the prognosis and diagnosis of the following skin diseases: Acneiform eruptions; Autoinflammatory syndromes; Chronic blistering; Conditions of the mucous membranes; Conditions of the skin appendages; Conditions of the subcutaneous fat; Congenital anomalies; Connective tissue diseases (such as Abnormalities of dermal fibrous and elastic tissue); Dermal and subcutaneous growths; Dermatitis (including Atopic Dermatitis, Contact Dermatitis, Eczema , Pustular Dermatitis, and Seborrheic Dermatitis); Disturbances of pigmentation; Drug eruptions; Endocrine-related skin disease; Eosinophilic; Epidermal nevi, neoplasms, cysts; Erythemas; Genodermatoses; Infection-related skin disease; Lichenoid eruptions; Lymphoid-related skin disease; Melanocytic nevi and neoplasms (including Melanoma); Monocyte- and macrophage-related skin disease; Mucinoses; Neurocutaneous; Noninfectious immunodeficiency-related skin disease; Nutrition-related skin disease; Papulosquamous hyperkeratotic (including Palmoplantar keratodermas); Pregnancy-related skin disease; Pruritic; Psoriasis; Reactive neutrophilic; Recalcitrant palmoplantar eruptions; Resulting from errors in metabolism; Resulting from physical factors (including Ionizing radiation-induced); Urticaria and angioedema; Vascular-related skin disease.

Apart from the above diseases, aspects of the present invention are also applicable to the prognosis and diagnosis of the following endocrine diseases: Adrenal disorders; Glucose homeostasis disorders; Thyroid disorders; Calcium homeostasis disorders and Metabolic bone disease; Pituitary gland disorders ; and Sex hormone disorders.

Apart from the above diseases, aspects of the present invention are also applicable to the prognosis and diagnosis of the following eye diseases: H00-H06 Disorders of eyelid, lacrimal system and orbit; H10-H13 Disorders of conjunctiva; H15-H22 Disorders of sclera, cornea, iris and ciliary body; H25-H28 Disorders of lens; H30-H36 Disorders of choroid and retina (including H30 Chorioretinal inflammation, H31 Other disorders of choroid, H32 Chorioretinal disorders in diseaseas classified elsewhere, H33 Retinal detachments and breaks, H34 Retinal vascular occlusions, H35 Other retinal disorders, and H36 Retinal disorders in diseases classified elsewhere); H40-H42 Glaucoma; H43-H45 Disorders of vitreous body and globe; H46-H48 Disorders of optic nerve and visual pathways; H49-H52 Disorders of ocular muscles, binocular movement, accommodation and refraction; H53-H54.9 Visual disturbances and blindness; and H55-H59 Other disorders of eye and adnexa.

Apart from the above diseases, aspects of the present invention are also applicable to the prognosis and diagnosis of the following neurological disorders: Abarognosis; Acquired Epileptiform Aphasia; Acute disseminated encephalomyelitis; Adrenoleukodystrophy; Agenesis of the corpus callosum; Agnosia; Aicardi syndrome; Alexander disease; Alien hand syndrome; Allochiria; Alpers' disease; Alternating hemiplegia; Alzheimer's disease; Amyotrophic lateral sclerosis (see Motor Neurone Disease); Anencephaly; Angelman syndrome; Angiomatosis; Anoxia; Aphasia; Apraxia; Arachnoid cysts; Arachnoiditis; Arnold-Chiari malformation; Arteriovenous malformation; Ataxia Telangiectasia; Attention deficit hyperactivity disorder; Auditory processing disorder; Autonomic Dysfunction; Back Pain; Batten disease; Behcet's disease; Bell's palsy; Benign Essential Blepharospasm;

Benign Intracranial Hypertension; Bilateral frontoparietal polymicrogyria; Binswanger's disease; Blepharospasm; Bloch-Sulzberger syndrome; Brachial plexus injury; Brain abscess; Brain damage; Brain injury; Brain tumor; Brown-Séquard syndrome; Canavan disease; Carpal tunnel syndrome; Causalgia; Central pain syndrome; Central pontine myelinolysis; Centronuclear myopathy; Cephalic disorder; Cerebral aneurysm; Cerebral arteriosclerosis; Cerebral atrophy; Cerebral gigantism; Cerebral palsy; Cerebral vasculitis; Cervical spinal stenosis; Charcot-Marie-Tooth disease; Chiari malformation; Chorea; Chronic fatigue syndrome; Chronic inflammatory demyelinating polyneuropathy (CIDP); Chronic pain; Coffin Lowry syndrome; Coma; Complex regional pain syndrome; Compression neuropathy; Congenital facial diplegia; Corticobasal degeneration; Cranial arteritis; Craniosynostosis; Creutzfeldt-Jakob disease; Cumulative trauma disorders; Cushing's syndrome; Cytomegalic inclusion body disease (CIBD); Cytomegalovirus Infection; Dandy-Walker syndrome; Dawson disease; De Morsier's syndrome; Dejerine-Klumpke palsy; Dejerine-Sottas disease; Delayed sleep phase syndrome; Dementia; Dermatomyositis; Developmental dyspraxia; Diabetic neuropathy; Diffuse sclerosis; Dravet syndrome; Dysautonomia; Dyscalculia; Dysgraphia; Dyslexia; Dystonia; Empty sella syndrome; Encephalitis; Encephalocele; Encephalotrigeminal angiomatosis; Encopresis; Epilepsy; Erb's palsy; Erythromelalgia; Essential tremor; Fabry's disease; Fahr's syndrome; Fainting; Familial spastic paralysis; Febrile seizures; Fisher syndrome; Friedreich's ataxia; Fibromyalgia; Gaucher's disease; Gerstmann's syndrome; Giant cell arteritis; Giant cell inclusion disease; Globoid Cell Leukodystrophy; Gray matter heterotopia; Guillain-Barré syndrome; HTLV-1 associated myelopathy; Hallervorden-Spatz disease; Head injury; Headache; Hemifacial Spasm; Hereditary Spastic Paraplegia; Heredopathia atactica polyneuritiformis; Herpes zoster oticus; Herpes zoster; Hirayama syndrome; Holoprosencephaly; Huntington's disease; Hydranencephaly; Hydrocephalus; Hypercortisolism; Hypoxia; Immune-Mediated encephalomyelitis; Inclusion

body myositis; Incontinentia pigmenti; Infantile phytanic acid storage disease; Infantile Refsum disease; Infantile spasms; Inflammatory myopathy; Intracranial cyst; Intracranial hypertension; Joubert syndrome; Karak syndrome; Kearns-Sayre syndrome; Kennedy disease; Kinsbourne syndrome; Klippel Feil syndrome; Krabbe disease; Kugelberg-Welander disease; Kuru; Lafora disease; Lambert-Eaton myasthenic syndrome; Landau-Kleffner syndrome; Lateral medullary (Wallenberg) syndrome; Learning disabilities; Leigh's disease; Lennox-Gastaut syndrome; Lesch-Nyhan syndrome; Leukodystrophy; Lewy body dementia; Lissencephaly; Locked-In syndrome; Lou Gehrig's disease (See Motor Neurone Disease); Lumbar disc disease; Lumbar spinal stenosis; Lyme disease - Neurological Sequelae; Machado-Joseph disease (Spinocerebellar ataxia type 3); Macrencephaly; Macropsia; Megalencephaly; Melkersson-Rosenthal syndrome; Menieres disease; Meningitis; Menkes disease; Metachromatic leukodystrophy; Microcephaly; Micropsia; Migraine; Miller Fisher syndrome; Mini-stroke (transient ischemic attack); Mitochondrial myopathy; Mobius syndrome; Monomelic amyotrophy; Motor Neurone Disease; Motor skills disorder; Moyamoya disease; Mucopolysaccharidoses; Multi-infarct dementia; Multifocal motor neuropathy; Multiple sclerosis; Multiple system atrophy; Muscular dystrophy; Myalgic encephalomyelitis; Myasthenia gravis; Myelinoclastic diffuse sclerosis; Myoclonic Encephalopathy of infants; Myoclonus; Myopathy; Myotubular myopathy; Myotonia congenita; Narcolepsy; Neurofibromatosis; Neuroleptic malignant syndrome; Neurological manifestations of AIDS; Neurological sequelae of lupus; Neuromyotonia; Neuronal ceroid lipofuscinosis; Neuronal migration disorders; Niemann-Pick disease; Non 24-hour sleep-wake syndrome; Nonverbal learning disorder; O'Sullivan-McLeod syndrome; Occipital Neuralgia; Occult Spinal Dysraphism Sequence; Ohtahara syndrome; Olivopontocerebellar atrophy; Opsoclonus myoclonus syndrome; Optic neuritis; Orthostatic Hypotension; Overuse syndrome; Palinopsia; Paresthesia; Parkinson's disease; Paramyotonia Congenita; Paraneoplastic diseases;

Paroxysmal attacks; Parry-Romberg syndrome; Pelizaeus-Merzbacher disease; Periodic Paralyses; Peripheral neuropathy; Persistent Vegetative State; Pervasive developmental disorders; Photic sneeze reflex; Phytanic acid storage disease; Pick's disease; Pinched nerve; Pituitary tumors; PMG; Polio; Polymicrogyria; Polymyositis; Porencephaly; Post-Polio syndrome; Postherpetic Neuralgia (PHN); Postinfectious Encephalomyelitis; Postural Hypotension; Prader-Willi syndrome; Primary Lateral Sclerosis; Prion diseases; Progressive hemifacial atrophy; Progressive multifocal leukoencephalopathy; Progressive Supranuclear Palsy; Pseudotumor cerebri; Rabies; Ramsay-Hunt syndrome (Type I and Type II); Rasmussen's encephalitis; Reflex neurovascular dystrophy; Refsum disease; Repetitive motion disorders; Repetitive stress injury; Restless legs syndrome; Retrovirus-associated myelopathy; Rett syndrome; Reye's syndrome; Rhythmic Movement Disorder; Romberg syndrome; Saint Vitus dance; Sandhoff disease; Schizophrenia; Schilder's disease; Schizencephaly; Sensory integration dysfunction; Septo-optic dysplasia; Shaken baby syndrome; Shingles; Shy-Drager syndrome; Sjögren's syndrome; Sleep apnea; Sleeping sickness; Snatiation; Sotos syndrome; Spasticity; Spina bifida; Spinal cord injury; Spinal cord tumors; Spinal muscular atrophy; Spinocerebellar ataxia; Steele-Richardson-Olszewski syndrome; Stiff-person syndrome; Stroke; Sturge-Weber syndrome; Subacute sclerosing panencephalitis; Subcortical arteriosclerotic encephalopathy; Superficial siderosis; Sydenham's chorea; Syncope; Synesthesia; Syringomyelia; Tarsal tunnel syndrome; Tardive dyskinesia; Tarlov cyst; Tay-Sachs disease; Temporal arteritis; Tetanus; Tethered spinal cord syndrome; Thomsen disease; Thoracic outlet syndrome; Tic Douloureux; Todd's paralysis; Tourette syndrome; Toxic encephalopathy; Transient ischemic attack; Transmissible spongiform encephalopathies; Transverse myelitis; Traumatic brain injury; Tremor; Trigeminal neuralgia; Tropical spastic paraparesis; Trypanosomiasis; Tuberous sclerosis; Von Hippel-Lindau disease; Viliuisk Encephalomyelitis ; Wallenberg's syndrome; Werdnig-Hoffman disease; West

syndrome; Whiplash; Williams syndrome; Wilson's disease; and Zellweger syndrome.

Apart from the above diseases, aspects of the present invention are also applicable to the prognosis and diagnosis of the following cardiovascular diseases: Aneurysm; Angina; Atherosclerosis; Cerebrovascular Accident (Stroke); Cerebrovascular disease; Congestive Heart Failure; Coronary Artery Disease; Myocardial infarction (Heart Attack); and Peripheral vascular disease.

As used herein, "nucleic acid" includes reference to a deoxyribonucleotide or ribonucleotide polymer in either single-or double-stranded form, and unless otherwise limited, encompasses known analogues having the essential nature of natural nucleotides in that they hybridize to single-stranded nucleic acids in a manner similar to naturally occurring nucleotides (e. g., peptide nucleic acids).

The term "RNA" refers to ribonucleic acid, a molecule of RNA encoding for a protein product or non-coding for a protein product (such as miRNAs but not excluding other non-coding RNAs). RNA is transcribed from a DNA template.

As used herein the term "mutant" refers to a nucleic acid compound, protein, molecule, vector or cell resulting from mutation of the native wild type coding sequence or subunits thereof.

As used herein the term "mutation" refers to any change that alters a native coding sequence either by displacement, addition, deletion, insertion, cross-linking, or other destruction or substitution of one or more nucleotides of the native coding sequence, including naturally occurring splice variants. In particular, the mutation provides a gene that causes the cell to be a cancer cell. Such mutations include inherited and acquired mutations of tumor suppressor genes and/or oncogenes.

By "amplified" is meant the construction of multiple copies of a nucleic acid sequence or multiple copies complementary to the nucleic acid

sequence using at least one of the nucleic acid sequences as a template. Amplification systems include the polymerase chain reaction (PCR) system, ligase chain reaction (LCR) system, nucleic acid sequence based amplification (NASBA, Cangene, Mississauga, Ontario), Q-Beta Replicase systems, transcription-based amplification system (TAS), and strand displacement amplification (SDA). See, e. g., Diagnostic Molecular Microbiology. Principles and Applications, D. H. Persing et al., Ed., American Society for Microbiology, Washington, D. C. (1993). The product of amplification is termed an amplicon.

The term "hybrid" refers to a double-stranded nucleic acid molecule, or duplex, formed by hydrogen bonding between complementary nucleotides. The terms "hybridise" or "anneal" refer to the process by which single strands of nucleic acid sequences form double-helical segments through hydrogen bonding between complementary nucleotides.

The term "oligonucleotide" refers to a short sequence of nucleotide monomers (usually 6 to 100 nucleotides) joined by phosphorous linkages (e.g., phosphodiester, alkyl and aryl-phosphate, phosphorothioate), or non-phosphorous linkages (e.g., peptide, sulfamate and others). An oligonucleotide may contain modified nucleotides having modified bases (e.g., 5-methyl cytosine) and modified sugar groups (e.g., 2'-O-methyl ribosyl, 2'-O-methoxyethyl ribosyl, 2'-fluoro ribosyl, 2'-amino ribosyl, and the like). Oligonucleotides may be naturally-occurring or synthetic molecules of double- and single-stranded DNA and double- and single-stranded RNA with circular, branched or linear shapes and optionally including domains capable of forming stable secondary structures (e.g., stem-and-loop and loop-stem-loop structures).

The term "primer" as used herein refers to an oligonucleotide which is capable of annealing to the amplification target allowing a DNA polymerase to attach thereby serving as a point of initiation of DNA synthesis when placed under conditions in which synthesis of primer extension product which is complementary to a nucleic acid strand is induced, i.e., in the presence of

nucleotides and an agent for polymerization such as DNA polymerase and at a suitable temperature and pH. The (amplification) primer is preferably single stranded for maximum efficiency in amplification. Preferably, the primer is an oligodeoxy ribonucleotide. The primer must be sufficiently long to prime the synthesis of extension products in the presence of the agent for polymerization. The exact lengths of the primers will depend on many factors, including temperature and source of primer. A "pair of bi-directional primers" as used herein refers to one forward and one reverse primer as commonly used in the art of DNA amplification such as in PCR amplification.

The term "probe" refers to a single-stranded oligonucleotide sequence that will recognize and form a hydrogen-bonded duplex with a complementary sequence in a target nucleic acid sequence analyte or its cDNA derivative.

The terms "stringency" or "stringent hybridization conditions" refer to hybridization conditions that affect the stability of hybrids, e.g., temperature, salt concentration, pH, formamide concentration and the like. These conditions are empirically optimised to maximize specific binding and minimize non-specific binding of primer or probe to its target nucleic acid sequence. The terms as used include reference to conditions under which a probe or primer will hybridise to its target sequence, to a detectably greater degree than other sequences (e.g. at least 2-fold over background). Stringent conditions are sequence dependent and will be different in different circumstances. Longer sequences hybridise specifically at higher temperatures. Generally, stringent conditions are selected to be about 5°C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength and pH. The T_m is the temperature (under defined ionic strength and pH) at which 50% of a complementary target sequence hybridises to a perfectly matched probe or primer. Typically, stringent conditions will be those in which the salt concentration is less than about 1.0 M Na^+ ion, typically about 0.01 to 1.0 M Na^+ ion concentration (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30°C for short probes or primers (e.g. 10 to 50 nucleotides) and

at least about 60°C for long probes or primers (e.g. greater than 50 nucleotides). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide. Exemplary low stringent conditions or "conditions of reduced stringency" include hybridization with a buffer solution of 30% formamide, 1 M NaCl, 1% SDS at 37°C and a wash in 2x SSC at 40°C. Exemplary high stringency conditions include hybridization in 50% formamide, 1 M NaCl, 1% SDS at 37°C, and a wash in 0.1x SSC at 60°C. Hybridization procedures are well known in the art and are described in e.g. Ausubel et al, Current Protocols in Molecular Biology, John Wiley & Sons Inc., 1994.

"Subject" as used herein includes, but is not limited to, mammals, including, e.g., a human, a non-human primate, a mouse, a pig, a cow, a goat, a cat, a rabbit, a rat, a guinea pig, a hamster, a degu, a horse, a monkey, a sheep, or other non-human mammal; and non-mammal animals, including, e.g., a non-mammalian vertebrate, such as a bird (e.g., a chicken or duck) or a fish, and an invertebrate. The subject may be a healthy animal or human subject undergoing a routine well-being check up. Alternatively, the subject may be at risk of having a disease (e.g., a genetically predisposed subject, a subject with medical and/or family history of cancer, a subject who has been exposed to carcinogens, occupational hazard, environmental hazard] and/or a subject who exhibits suspicious clinical signs of a disease [e.g., blood in the stool or melena, unexplained pain, sweating, unexplained fever, unexplained loss of weight up to anorexia, changes in bowel habits (constipation and/or diarrhoea), tenesmus (sense of incomplete defecation, for rectal cancer specifically), anaemia and/or general weakness). According to another embodiment, the subject may be a patient diagnosed with the disease and is performing a routine check-up, in-between treatments.

The term "thrombocyte", as used herein, refers to blood platelets, i.e. the small, irregularly-shaped cell fragments do not have a nucleus containing DNA, and that circulate in the blood of mammals. Thrombocytes are 2-3 μm in

diameter, and are derived from fragmentation of precursor megakaryocytes. The average lifespan of a thrombocyte is 5 to 9 days. Thrombocytes are involved and play an essential role in haemostasis, leading to the formation of blood clots.

The term "blood" as used herein refers to whole blood (including plasma and cells) and includes arterial, capillary and venous blood.

The term "nucleated cell" as used herein preferably refers to a Bartholin's gland cell; Salivary gland mucous cell; Salivary gland serous cell; Von Ebner's gland cell; Mammary gland cell; Lacrimal gland cell; Ceruminous gland cell; Eccrine sweat gland cell; Apocrine sweat gland cell; Gland of Moll cell; Sebaceous gland cell; Bowman's gland cell; Brunner's gland cell; Seminal vesicle cell; Prostate gland cell; Bulbourethral gland cell; Gland of Littre cell; Uterus endometrium cell; Isolated goblet cell; Stomach lining mucous cell; Gastric gland zymogenic cell; Gastric gland oxyntic cell; Pancreatic acinar cell; Paneth cell; Type II pneumocyte; Clara cell; Anterior pituitary cell; Intermediate pituitary cell; Magnocellular neurosecretory cell; Thyroid gland cell; Parathyroid gland cells ; Adrenal gland cells ; Leydig cell; Theca interna cell; Corpus luteum cell; Juxtaglomerular cell; Macula densa cell; Peripolar cell; Mesangial cell; Blood vessel and lymphatic vascular endothelial fenestrated cell; Blood vessel and lymphatic vascular endothelial continuous cell; Blood vessel and lymphatic vascular endothelial splenic cell; Synovial cell; Serosal cell; Squamous cell; Columnar cell; Dark cell; Vestibular membrane cell; Stria vascularis basal cell; Stria vascularis marginal cell; Cell of Claudius; Cell of Boettcher; Choroid plexus cell; Pia-arachnoid squamous cell; Pigmented ciliary epithelium cell; Nonpigmented ciliary epithelium cell; Corneal endothelial cell; Peg cell; Respiratory tract ciliated cell; Oviduct ciliated cell; Uterine endometrial ciliated cell; Rete testis ciliated cell; Ductulus efferens ciliated cell; Ciliated ependymal cell; Epidermal keratinocyte; Epidermal basal cell; Keratinocyte; Nail bed basal cell; Medullary hair shaft cell; Cortical hair shaft cell; Cuticular hair shaft cell; Cuticular hair root sheath cell; External

hair root sheath cell; Hair matrix cell; Surface epithelial cell; basal cell; Urinary epithelium cell; Auditory inner hair cell; Auditory outer hair cell; Primary sensory neurons; Merkel cell; Olfactory receptor neuron; Photoreceptor cells; Carotid body cell (blood pH sensor); Hair cell; Taste bud cell; Cholinergic neural cell; Adrenergic neural cell; Peptidergic neural cell; Inner pillar cell; Outer pillar cell; Inner phalangeal cell; Outer phalangeal cell; Border cell; Hensen cell; Vestibular apparatus supporting cell; Taste bud supporting cell; Olfactory epithelium supporting cell; Schwann cell; Satellite cell; Enteric glial cell; Astrocyte; Neuron cells; Oligodendrocyte; Spindle neuron; Anterior lens epithelial cell; Crystallin-containing lens fiber cell; Hepatocyte ; Adipocytes: ; Liver lipocyte; Kidney glomerulus parietal cell; Kidney glomerulus podocyte; Kidney proximal tubule brush border cell; Loop of Henle thin segment cell; Kidney distal tubule cell; Kidney collecting duct cell; pneumocyte; Pancreatic duct cell; Nonstriated duct cell; Duct cell; Intestinal brush border cell; Exocrine gland striated duct cell; Gall bladder epithelial cell; Ductulus efferens nonciliated cell; Epididymal principal cell; Epididymal basal cell; Ameloblast epithelial cell; Planum semilunatum epithelial cell; Organ of Corti interdental epithelial cell; Loose connective tissue fibroblasts; Corneal fibroblasts; Tendon fibroblasts; Bone marrow reticular tissue fibroblasts; Other nonepithelial fibroblasts; Pericyte; Nucleus pulposus cell; Cementoblast/cementocyte; Odontoblast/odontocyte ; Hyaline cartilage chondrocyte; Fibrocartilage chondrocyte; Elastic cartilage chondrocyte; Osteoblast/osteocyte; Osteoprogenitor cell; Hyalocyte ; Stellate cell; Hepatic stellate cell; Pancreatic stelle cell; Skeletal muscle cell; Satellite cell; Heart muscle cell; Smooth muscle cell; Myoepithelial cell; Megakaryocyte ; Monocyte; Connective tissue macrophage ; Epidermal Langerhans cell; Osteoclast; Dendritic cell; Microglial cell; Neutrophil granulocyte; Eosinophil granulocyte; Basophil granulocyte; Mast cell; Helper T cell; Suppressor T cell; Cytotoxic T cell; Natural Killer T cell; B cell; Natural killer cell; Reticulocyte; Melanocyte; Retinal pigmented epithelial cell; Oogonium/Oocyte; Spermatid;

Spermatocyte; Spermatogonium cell; Spermatozoon; Ovarian follicle cell; Sertoli cell; Thymus epithelial cell; and Interstitial kidney cell.

Targeted therapy and personalized medicine are critically depending on disease profiling and the development of companion diagnostics. Mutations in disease-derived nucleic acids can be highly predictive for the response to targeted treatment. However, obtaining easily accessible high-quality nucleic acids remains a significant developmental hurdle. Blood generally contains 150,000-350,000 thrombocytes (platelets) per microliter, providing a highly available biomarker source for research and clinical use. Moreover, thrombocyte isolation is relatively simple and is a standard procedure in blood bank/haematology labs. Since platelets do not contain a nucleus, their RNA transcripts - needed for functional maintenance - are derived from bone marrow megakaryocytes during thrombocyte origination. It has now been found that thrombocytes may take up RNA during circulation via various transfer mechanisms. Tumor cells release an abundant collection of genetic material, some of which is secreted by microvesicles in the form of mutant RNA. During circulation in the blood stream thrombocytes absorb the genetic material secreted by cancer cells and other diseased cells, serving as an attractive platform for the companion diagnostics of cancer and other diseases as indicated above in the context of personalized medicine.

In the Examples below it is shown that platelets isolated from healthy human control subjects have the ability to take up RNA from RNA-containing microvesicles derived from human brain tumor cells (glioma), after which they contain tumor-associated RNA, including for instance mutant EGFRvIII mRNA in the case of glioma patients. Hence, it was determined that circulating platelets isolated from glioma patients contain RNA biomarkers. RT-PCR was used to confirm that mutant EGFRvIII mRNA found in the thrombocytes reflects the presence of glioma tissues

The presence of tumor-markers messages is not unique to platelets from glioma patients but is more generally applicable for a wide range of

diseases as identified herein. Messenger RNAs coding for the prostate cancer markers PCA3 and PSA could be demonstrated in platelets from prostate cancer patients, whereas these markers were absent in platelets from healthy control subjects.

Apart from detecting gene mutations associated with cancer or other diseases, the present inventors also found that gene expression arrays could be used to classify a thrombocytes nucleic acid sample as being that of a subject suffering from a specific type of (solid tumour) cancer or other disease. It was established that mRNA expression profiles obtained with nucleic acids extracted from platelets isolated from healthy control subjects or extracted from platelets isolated from glioma patients differed specifically. Distinct mRNA expression profiles were obtained and a minimal glioma biomarker signature could be detected, as shown for the Top-30 hits in figure 3C. The distinct profile as shown in Figure 3C comprises a significant increase in the expression of the following genes: WFDC1, Kremen1, DEF4A, ARG1, FKBP5, ACRC, ENST0328043, A_32_P167111, MAP2, ECTL8, UNC13B,TP53I3, FDXR, BX119718, SORT1, PFN4, C1QTNF5, A_24_P237896, PGLYRP1, SEC14L2, BC018626, MAOB, TCN1, AMOTL1, TSP50, CD109, A_24_P927015, THC2325987, C18orf1, and LIN28 (some of these gene names are referred to with reference to the Microarray Accession number, e.g. the oligonucleotide probe of the Agilent Chip). It will be understood that this profile is not limitative to the scope of the present invention, since the skilled person is well aware how to obtain other suitable gene expression profiles using the methods of the present invention for other cancers, and for other diseases in general.

The present inventors have now found that blood platelets contain cancer markers and disease markers in the form of tumor-derived or tumor-associated or disease-derived nucleic acids or nucleic acid expression profiles and that these platelets may serve as a diagnostic platform for the molecular

profiling of cancer and other diseases as identified herein. This is highly useful in the context of personalized medicine.

The present invention provides a novel and easy-to-use method to isolate circulating disease-derived material (e.g. disease markers as used herein) for genetic analysis. The present inventors isolated tumor-derived RNA from circulating thrombocytes, yielding pure RNA and thereby providing an easy way to extract high quality RNA from low amounts of blood. Thrombocyte nucleic acid (NA) isolation and subsequent analysis presents a marked increase in the diagnostic sensitivity of circulating NA in blood.

The present inventors found that in diseased patients circulating thrombocytes contain significant amounts of disease-derived RNA. This disease-derived RNAs presents unique genetic information about the disease, which may be used to determine disease type, extent of disease and possibly the susceptibility of the disease to therapeutic treatment. The thrombocyte RNA can be analyzed for the presence of specific disease-derived RNAs, as demonstrated herein for the EGFRvIII mutant RNA derived from glioma tumours.

The present invention describes a method of finding specific transcripts derived from nucleated cells of disease origin within anucleated blood cells such as thrombocytes extracted from blood. This approach is robust and easy. This is attributed to the rapid and straight forward extraction procedures and the quality of the extracted NA. Within the clinical setting, thrombocytes extraction (from blood samples) is already implemented in general biological sample collection and therefore it is foreseen that the implementation into the clinic is relatively easy.

The present invention provides a general method for analysing blood of a subject for the presence of a disease-derived nucleic acid and a method of diagnosing disease in a subject using said general method. When reference is herein made to a method of the invention, both embodiments are referred to.

A method of the invention can be performed on any suitable body sample comprising anucleated blood cells, such as for instance a tissue sample comprising blood, but preferably said sample is whole blood.

A blood sample of a subject can be obtained by any standard method, for instance by venous extraction.

The amount of blood needed is not particularly limited. Depending on the methods employed, the skilled person will be capable of establishing the amount of sample required to perform the various steps of the method of the present invention and obtain sufficient NA for genetic analysis. Generally, such amounts will comprise a volume ranging from 0.01 µl to 100 ml.

The body sample may be analyzed immediately following collection of the sample. Alternatively, analysis according to the method of the present invention can be performed on a stored body sample or on a stored fraction of anucleated blood cells thereof, preferably thrombocytes. The body sample for testing, or the fraction of anucleated blood cells thereof, can be preserved using methods and apparatuses known in the art. In a collected anucleated blood cell fraction, the thrombocytes are preferably maintenance in inactivated state (ie. in non-activated state). In that way, the cellular integrity and the disease-derived nucleic acids are best preserved.

In case the fraction of anucleated blood cells is a thrombocyte fraction, this platelet isolated fraction does preferably not include platelet poor plasma or platelet rich plasma (PRP). Further isolation of the platelets is preferred for optimal resolution.

The body sample may suitably be processed otherwise, for instance, it may be purified, or digested, or specific compounds may be extracted therefrom. Depending upon the method of characterizing the NA present in the anucleated blood cells in said sample, which method preferably involves RT-PCR, the anucleated blood cells may be extracted from the sample by methods known to the skilled person and be transferred to any suitable medium for extraction of the NA therefrom should the analysis method so

require. The recipient subject's body sample may be treated to remove abundant nucleic acid degrading enzymes (like RNases, DNases) therefrom, in order to prevent early destruction of the nucleic acids.

Thrombocyte extraction from the body sample of the subject may involve any available method. In transfusion medicine, thrombocytes are often collected by apheresis, a medical technology in which the blood of a donor or patient is passed through an apparatus that separates out one particular constituent and returns the remainder to the circulation. The separation of individual blood components is done with a specialized centrifuge. Plateletpheresis (also called thrombopheresis or thrombocytapheresis) is the apheresis process of collecting thrombocytes. Modern automatic plateletpheresis allows blood donors to give a portion of their thrombocytes, while keeping their red blood cells and at least a portion of blood plasma. Although it is possible to provide the body sample comprising thrombocytes as envisioned herein by apheresis, it is often easier to collect whole blood and isolate the thrombocyte fraction therefrom by centrifugation. Generally, in such a protocol, the thrombocytes are first separated from the other blood cells by a centrifugation step of about 120 x g for about 20 minutes at room temperature to obtain a platelet rich plasma (PRP) fraction. The thrombocytes are then washed (for instance in PBS-EDTA) to remove plasma proteins and enrich for thrombocytes. Wash steps are generally carried out at 850 - 1000 x g for about 10 min at room temperature. Further enrichments can be carried out to yield more pure thrombocyte fractions.

Platelet isolation generally involves blood sample collection in Vacutainer tubes containing anticoagulant citrate dextrose (e.g. 36 ml citric acid, 5 mmol/l KCl, 90 mmol/l NaCl, 5 mmol/l glucose, 10 mmol/l EDTA pH 6.8). A suitable protocol for platelet isolation is described in Ferretti et al. (J Clin Endocrinol Metab 2002; 87:2180–2184). This method involves a preliminary centrifugation step (1,300 rpm per 10 min) to obtain platelet-rich plasma (PRP). Platelets are then washed three times in an anti-aggregation

buffer (Tris–HCl 10 mmol/l; NaCl 150 mmol/l; EDTA 1 mmol/l; glucose 5 mmol/l; pH 7.4) and centrifuged as above, to avoid any contamination with plasma proteins and to remove any residual erythrocytes. A final centrifugation at 4,000 rpm for 20 min may then be performed to isolate platelets. The platelet pellet may be washed (e.g. in phosphate buffered saline For quantitative determination of disease marker levels, the protein concentration of platelet membranes may be used as internal reference. Such protein concentrations may be determined by the method of Bradford (Anal Biochem 1976; 72:248–254), using serum albumin as standard.

Following the provision of the body sample of the subject, and the extraction therefrom of the anucleated blood cells, the anucleated blood cells of the subject are screened for the presence of disease-specific nucleic acids. If disease-specific nucleic acids are encountered in the anucleated blood cells of the subject, or if disease-specific nucleic acids are encountered in the anucleated blood cells of the subject at a higher level than in the anucleated blood cells in an unaffected blood sample of a control subject, which disease-specific nucleic acids are considered to originate from a diseased cell or tissue residing in the subject, said subject is diagnosed with disease as defined herein.

Disease-specific nucleic acids (RNA and/or DNA disease markers) are defined as originating from disease cells that contain mutations or no mutations in the nucleic acid sequences that are associated with or specific to the disease, and also include disease-derived anucleated blood cells nucleic acids which are up- or down-regulated as compared to nucleic acids in anucleated blood cells from healthy donors. Hence, the terms "disease-specific nucleic acids" and "disease-derived nucleic acids" are used interchangeable herein. It will be appreciated that non-mutated genes can be identified and used for disease diagnostics. If certain genes are overexpressed in certain diseases, these nucleic acids may be transferred to anucleated blood cells. However, if these nucleic acids are already present in anucleated blood cells of

healthy subjects one can expect an increase in the number of nucleic acid copies in anucleated blood cells of such diseased patients. Hence, quantification of the copy number of certain genes (by quantitative PCR or microarrays e.g.) in anucleated blood cells may be beneficial in certain embodiments of aspects of this invention for detecting the presence of a diseases overexpressing such genes.

A further step in a method of the invention is the provision of an anucleated blood cells-extracted nucleic acid fraction. Such a nucleic acid fraction is subsequently used for the detection of a disease marker therein. An anucleated blood cells-extracted nucleic acid fraction may be obtained by any NA extraction method available. Usually RNA extraction is performed by using chaotropic reagents. The first step in isolating total RNA from cells or tissue is to break open the cells under denaturing conditions. In 1979, Chirgwin et al. (Biochemistry, 18[24]:5294-9, 1979) devised a method for the efficient isolation of total RNA by homogenization in a 4 M solution of the potent protein denaturant guanidinium thiocyanate with 0.1 M 2-mercaptoethanol to break protein disulfide bonds. RNA was then isolated by ethanol extraction or by ultracentrifugation through cesium chloride. In 1987 Chomczynski and Sacchi (Analytical Biochemistry, 162[1]:156-9, 1987) modified this method to devise a rapid single-step extraction procedure using a mixture of guanidinium thiocyanate and phenol-chloroform, a method especially useful for processing large numbers of samples or for isolation of RNA from small quantities of cells or tissue. Any commercial kit can also be used for the extraction of RNA, non-limiting examples thereof include Ambion's RNAqueous™ system, Bio101's RNaid Plus kit, Bioline Ltd.'s RNAce kits, CLONTECH's NucleoSpin® RNA II and NucleoTrap mRNA kits, Invitrogen Corp.'s S.N.A.P. Total RNA Isolation Kit and QIAGEN's RNeasy kits.

The detection of a disease-derived nucleic acid in the extracted nucleic acid sample may occur by any genetic analysis technique available that

is suitable for the detection of nucleic acid sequence mutations or expression profiles in nucleic acids that are specific for the disease. Usually, such sequence mutations can be easily detected by selective nucleic acid hybridization, involving the formation of a duplex nucleic acid structure formed by selective hybridization with each other of two single-stranded nucleic acid sequences. Selective hybridization includes reference to hybridization, under stringent hybridization conditions, of a nucleic acid sequence to a specified nucleic acid target sequence to a detectably greater degree (e. g., at least 2-fold over background) than its hybridization to non-target nucleic acid sequences and to the substantial exclusion of non-target nucleic acids. Selectively hybridizing sequences typically have about at least 80% sequence identity, preferably 90% sequence identity, and most preferably 100% sequence identity (i. e., complementary) with each other.

Alternatively, detection of a disease-derived nucleic acid may occur through sequencing technologies such as DNA and RNA sequencing.

When detecting sequence mutations in RNA, or expression profiles of RNA, it is preferred that the RNA is transcribed into cDNA prior to the detection of sequence mutations therein or quantitation of the amount expressed.

RNA can be reverse transcribed into cDNA using RNA-dependent DNA polymerases such as, for example, reverse transcriptases from viruses, retrotransposons, bacteria, etc. These can have RNase H activity, or reverse transcriptases can be used that are so mutated that the RNase H activity of the reverse transcriptase was restricted or is not present (e.g. MMLV-RT RNase H$^-$). RNA-dependent DNA synthesis (reverse transcription) can also be carried by enzymes that show altered nucleic acid dependency through mutation or modified reaction conditions and thus obtain the function of the RNA-dependent DNA polymerase. Commercial kits are available to reverse transcribe RNA into cDNA.

Once the RNA is reverse transcribed into cDNA, the DNA sequence can be analysed for the presence of cancer-specific mutations or expression profiles can be determined using for instance selective nucleic acid hybridization as described above. Such techniques are well known in the art and may comprise selective amplification using amplification primers that are specific for the mutation to be detected or selective hybridization to nucleic acid arrays using mRNA-specific probes. Alternatively, general primers can be used to amplify the DNA comprising the suspected mutation and the mutation can than be detected in the amplicon by selective nucleic acid hybridization using probes that are specific for the mutation. Expression profiles are generally obtained using methods of quantitative hybridization well described in the art, an illustration of which is described in the Examples.

Methods of the invention can in principle be performed by using any nucleic acid amplification method, such as the Polymerase Chain Reaction (PCR; Mullis 1987, U.S. Pat. No. 4,683,195, 4,683,202, en 4,800,159) or by using amplification reactions such as Ligase Chain Reaction (LCR; Barany 1991, Proc. Natl. Acad. Sci. USA 88:189-193; EP Appl. No., 320,308), Self-Sustained Sequence Replication (3SR; Guatelli et al., 1990, Proc. Natl. Acad. Sci. USA 87:1874-1878), Strand Displacement Amplification (SDA; U.S. Pat. Nos. 5,270,184, en 5,455,166), Transcriptional Amplification System (TAS; Kwoh et al., Proc. Natl. Acad. Sci. USA 86:1173-1177), Q-Beta Replicase (Lizardi et al., 1988, Bio/Technology 6:1197), Rolling Circle Amplification (RCA; U.S. Pat. No. 5,871,921), Nucleic Acid Sequence Based Amplification (NASBA), Cleavase Fragment Length Polymorphism (U.S. Pat. No. 5,719,028), Isothermal and Chimeric Primer-initiated Amplification of Nucleic Acid (ICAN), Ramification-extension Amplification Method (RAM; U.S. Pat. Nos. 5,719,028 and 5,942,391) or other suitable methods for amplification of DNA.

In order to amplify DNA with a small number of mismatches to one or more of the amplification primers, an amplification reaction may be performed under conditions of reduced stringency (e.g. a PCR amplification using an

annealing temperature of 38°C, or the presence of 3.5 mM $MgCl_2$). The person skilled in the art will be able to select conditions of suitable stringency.

The primers herein are selected to be "substantially" complementary (i.e. at least 65%, more preferably at least 80% perfectly complementary) to their target regions present on the different strands of each specific sequence to be amplified. It is possible to use primer sequences containing e.g. inositol residues or ambiguous bases or even primers that contain one or more mismatches when compared to the target sequence. In general, sequences that exhibit at least 65%, more preferably at least 80% homology with the target DNA oligonucleotide sequences, are considered suitable for use in a method of the present invention. Sequence mismatches are also not critical when using low stringency hybridization conditions.

The detection of the amplification products can in principle be accomplished by any suitable method known in the art. The detection fragments may be directly stained or labelled with radioactive labels, antibodies, luminescent dyes, fluorescent dyes, or enzyme reagents. Direct DNA stains include for example intercalating dyes such as acridine orange, ethidium bromide, ethidium monoazide or Hoechst dyes.

Alternatively, the DNA fragments may be detected by incorporation of labelled dNTP bases into the synthesized DNA fragments. Detection labels which may be associated with nucleotide bases include e.g. fluorescein, cyanine dye or BrdUrd.

When using a probe-based detection system, a suitable detection procedure for use in the present invention may for example comprise an enzyme immunoassay (EIA) format (Jacobs et al., 1997, J. Clin. Microbiol. 35, 791795). For performing a detection by manner of the EIA procedure, either the forward or the reverse primer used in the amplification reaction may comprise a capturing group, such as a biotin group for immobilization of target DNA PCR amplicons on e.g. a streptavidin coated microtiter plate wells for subsequent EIA detection of target DNA amplicons (see below). The skilled

person will understand that other groups for immobilization of target DNA PCR amplicons in an EIA format may be employed.

Probes useful for the detection of the target DNA as disclosed herein preferably bind only to at least a part of the DNA sequence region as amplified by the DNA amplification procedure. Those of skill in the art can prepare suitable probes for detection based on the nucleotide sequence of the target DNA without undue experimentation as set out herein. Also the complementary sequences of the target DNA may suitably be used as detection probes in a method of the invention, provided that such a complementary strand is amplified in the amplification reaction employed.

Suitable detection procedures for use herein may for example comprise immobilization of the amplicons and probing the DNA sequences thereof by e.g. southern blotting. Other formats may comprise an EIA format as described above. To facilitate the detection of binding, the specific amplicon detection probes may comprise a label moiety such as a fluorophore, a chromophore, an enzyme or a radio-label, so as to facilitate monitoring of binding of the probes to the reaction product of the amplification reaction. Such labels are well-known to those skilled in the art and include, for example, fluorescein isothiocyanate (FITC), ß-galactosidase, horseradish peroxidase, streptavidin, biotin, digoxigenin, ^{35}S or ^{125}I. Other examples will be apparent to those skilled in the art.

Detection may also be performed by a so called reverse line blot (RLB) assay, such as for instance described by Van den Brule et al. (2002, J. Clin. Microbiol. 40, 779787). For this purpose RLB probes are preferably synthesized with a 5'amino group for subsequent immobilization on e.g. carboxylcoated nylon membranes. The advantage of an RLB format is the ease of the system and its speed, thus allowing for high throughput sample processing.

The use of nucleic acid probes for the detection of DNA fragments is well known in the art. Mostly these procedures comprise the hybridization of

the target DNA with the probe followed by post-hybridization washings. Specificity is typically the function of post-hybridization washes, the critical factors being the ionic strength and temperature of the final wash solution. For DNA-DNA hybrids, the T_m (the thermal melting point, i.e. the temperature under defined ionic strength and pH at which 50% of a complementary target sequence hybridizes to a perfectly matched probe) can be approximated from the equation of Meinkoth and Wahl (Anal. Biochem., 138: 267-284 (1984)): T_m = 81.5 °C + 16.6 (log M) + 0.41 (% GC)-0.61 (% form)-500/L; where M is the molarity of monovalent cations, % GC is the percentage of guanosine and cytosine nucleotides in the DNA, % form is the percentage of formamide in the hybridization solution, and L is the length of the hybrid in base pairs. The T_m is reduced by about 1 °C for each 1 % of mismatching; thus, the hybridization and/or wash conditions can be adjusted to hybridize to sequences of the desired identity. For example, if sequences with > 90% identity are sought, the T_m can be decreased 10 °C. Generally, stringent conditions are selected to be about 5 °C lower than the T_m for the specific sequence and its complement at a defined ionic strength and pH. However, severely stringent conditions can utilize a hybridization and/or wash at 1, 2, 3, or 4 °C lower than T_m; moderately stringent conditions can utilize a hybridization and/or wash at 6,7,8,9, or 10 °C lower than the T_m; low stringency conditions can utilize a hybridization and/or wash at 11, 12, 13, 14, 15, or 20 °C lower than T_m. Using the equation, hybridization and wash compositions, and desired T_m, those of ordinary skill will understand that variations in the stringency of hybridization and/or wash solutions are inherently described. If the desired degree of mismatching results in a T_m of less than 45 °C (aqueous solution) or 32 °C (formamide solution) it is preferred to increase the SSC concentration so that a higher temperature can be used. An extensive guide to the hybridization of nucleic acids is found in Tijssen, Laboratory Techniques in Biochemistry and Molecular Biology—Hybridization with Nucleic Acid Probes, Part I, Chapter 2 "Overview of principles of hybridization and the strategy of nucleic acid probe

assays", Elsevier. New York (1993); and Current Protocols in Molecular Biology, Chapter 2, Ausubel, et al., Eds., Greene Publishing and Wiley-Interscience, New York (1995).

Detection probes are preferably selected to be "substantially" complementary to one of the strands of the double stranded DNA amplicons generated by an amplification reaction in a method of the invention. Preferably the probes are substantially complementary to the immobilizable (e.g. biotin labelled) antisense strands of the amplicons generated from the target DNA.

It is allowable for detection probes to contain one or more mismatches to their target sequence. In general, sequences that exhibit at least 65%, more preferably at least 80% homology with the target DNA oligonucleotide sequences are considered suitable for use in a method of the present invention.

The step of analysing the anucleated blood cell-extracted nucleic acid fraction for the presence of a disease marker can thus be performed by standard nucleic acid analysis techniques. The step of determining whether there is an alteration in the level of said nucleic acid marker in said nucleic acid fraction with respect to an unaffected blood sample will involve (semi-) quantitative measurements of the amount of disease marker in the anucleated blood cells. A much preferred protocol for the detection of disease-specific markers in the nucleic acids isolated from anucleated blood cells is therefore quantitative reverse-transcription PCR (qRT-PCR) (Freeman et al., BioTechniques 26:112-125 (1999)).

An "unaffected blood sample" as referred to above refers to the level of the disease marker in anucleated blood cells of a healthy control subject or from the same subject prior to the onset of the disease. Since anucleated blood cell characteristics and quantities of anucleated blood cell components depend on, amongst other things, species and age, it is preferable that the non-diseased control anucleated blood cells come from a subject of the same species, age and from the same sub-population (e.g. smoker/nonsmoker). Alternatively, control data may be taken from databases and literature. It will be appreciated

that the control sample may also be taken from the diseased subject at a particular time-point, in order to analyze the progression of the disease.

Disease markers include cancer/specific mutations and cancer-specific mutations may include a wide variety of mutations known to be associated with cancer. A non-limiting list of examples of mutations for various cancers is provided at http://www.sanger.ac.uk/genetics/CGP/Census/ and in the Tables herein.

The invention further provides a kit for diagnosing disease in a subject, the kit comprising a packaging material which comprises at least one agent for specifically determining a level and/or activity of at least one nucleic acid mutant and/or nucleic acid profile in an anucleated blood cell sample of the subject. As used herein, the term "diagnosing" refers to determining the presence of a disease, classifying a disease, determining a severity of disease (grade or stage), monitoring disease progression, forecasting an outcome of the disease and/or prospects of recovery.

It will be appreciated that the tools necessary for detecting the disease-derived nucleic acid may be provided as a kit, such as an FDA-approved kit, which may contain one or more unit dosage form containing the active ingredient for detection of the disease-derived nucleic acid in anucleated blood cells by a method of the present invention.

Alternatively, the kit may comprise means for collecting the sample and specific amplification and/or detection primers packaged separately.

The kit may be accompanied by instructions for performing a method of the present invention.

For example, the kit may be comprised in a device such as a dipstick or a cartridge, (optionally comprised in a housing) to which a blood sample or an isolated and/or amplified anucleated blood cell nucleic acid sample may be applied and which detects a disease-derived or disease-specific nucleic acid or nucleic acid profile in said sample. The device may comprise any agent capable of specifically detecting the disease-derived nucleic acid. For example, the

device may comprise one or a combination of immobilized mutation-specific hybridization probes that bind the disease-derived nucleic acid and an indicator for detecting binding. In an embodiment of this invention, supports are provided in the device to which the hybridization probes are removably or fixedly attached.

According to one embodiment, the device may be a lateral flow device comprising inlet means for flowing a blood sample or an isolated and/or amplified anucleated blood cell nucleic acid sample into contact with the agents capable of detecting the disease-derived nucleic acid. The test device can also include a flow control means for assuring that the test is properly operating. Such flow control means can include control nucleic acids bound to a support which capture detection probes added to the sample as a means of confirming proper flow of sample fluid through the test device. Alternatively, the flow control means can include capture probes in the control region which capture control nucleic acids naturally present in said sample or added thereto as control, again indicating that proper flow is taking place within the device.

In another aspect, the present invention provides the use of device of the present invention for diagnosing disease in a subject using any one of the methods described herein above. Very suitable devices for use in diagnosing disease in a subject using any one of the methods described herein above include Platelet RNA chips such as for instance described in Nagalla & Bray (2010) Blood 115 (1): 2-3 and Gnatenko et al. Blood 115 (1): 7-14.

The invention will now be exemplified by means of the following non-limiting examples.

EXAMPLES

Example 1

Thrombocytes were isolated from blood samples of 4 glioblastoma patients and 4 healthy donors by centrifugation steps. The thrombocytes were then subjected to RNA extraction using Trizol RNA isolation. The purified

thrombocytic RNA samples were then converted to cDNA and analyzed by Agilent 4x44K expression microarrays using standard microarray protocols. This allowed the profiling of the mRNAs in the different thrombocyte preparations.

About 8500 RNA transcripts could not be detected by expression microarrays in platelets from healthy donors. These transcripts were present at levels below the detection limit of the Agilent 4x44K chip in thrombocytes from healthy donors. Hence, such RNAs may all be potential biomarkers for cancer diagnostics. Of the RNAs not detected by expression microarrays in thrombocytes from healthy donors, a substantial set of RNAs was detected in thrombocytes from glioblastoma patients. Table 1 summarizes unique thrombocytic RNA transcripts detected in thrombocytes from glioblastoma patients but not in thrombocytes from healthy donors by expression microarrays. Unique RNA transcripts detected in 4/4 patient samples (Table 1A) or in 3/4 patient samples (Table 1B), but not in any of the four control samples are summarized in Table 1.

Table 1. Unique thrombocytic RNA transcripts detected in thrombocytes from glioblastoma patients but not in thrombocytes from healthy donors by expression microarrays.

1A. Transcripts detected in thrombocytes in four out of four patient samples, but not detected in thrombocytes from control samples.

A_23_P207233	A_23_P47546	A_24_P452024	A_24_P642240	A_24_P654255
A_24_P712193	A_24_P816073	A_32_P142521	A_32_P167111	A_32_P35839
A_32_P59532	AA594975	AF035790	AF119839	AF130062
AI138440	AK098562	ASPM	AW269819	BC002534
BC024745	BC047055	BHMT	BM683433	BX118161
C10orf10	C9orf138	CCL16	CENPQ	CLN5
CLTCL1	COCH	CPA6	CUTL2	DKFZp547H025
DLSTP	DNAJC5B	ENST00000303697	ENST00000315208	ENST00000382726
FILIP1	GAL	GPR149	GTSE1	HAS3

HFE	HOXB6	HOXD11	IGF1	IL21
LDLR	LOC221710	LOC388160	LOC641999	LRRC2
LRRC4	MGC16291	MPDZ	MYCL1	NPR3
OLAH	OR2H1	PLK4	PNMA2	ROBO4
SEPT10	SLC14A1	SP2	SPANXB2	TAF5L
TCEAL7	THC2279825	THC2334717	THC2340924	THC2412206
TIMP4	TMPRSS3	TNFAIP6	TNK1	ZNF596

1B. Transcripts detected in thrombocytes in three out of four patient samples, but not detected in thrombocytes from control samples.

A_23_P72252	A_24_P195400	A_24_P195621	KRT8P23	A_24_P246777
A_24_P315255	A_24_P647965	A_24_P669822	A_24_P752208	A_24_P790361
A_24_P834066	A_24_P915245	A_24_P928453	A_24_P929126	A_24_P931713
A_24_P933278	A_24_P934497	A_24_P935492	A_32_P119949	A_32_P136427
A_32_P15328	A_32_P182135	A_32_P69993	A_32_P743731	A_32_P75311
A_32_P92274	AA420988	AA669267	AA843546	AA890136
AA918648	ABCA10	ABCB9	ACADL	ACE
ADAM32	AF119848	AF136408	AF217973	AF263545
AF315716	AF401032	AI291464	AI335947	AI885257
AK021897	AK057725	AK057935	AK074369	AK091028
AK096102	AK096991	AK130038	AL133089	ALDH5A1
ANKRD40	APOA1	APOA1	APOD	AW385956
AY358234	BC017851	BC037882	BC038740	BC041899
BC37295_3	BCL2L11	BF376089	BF435769	BF509481
BF826743	BHMT2	BHMT2	BM476468	BM681332
BPI	BQ028381	BX091616	BX647685	C15orf37
C17orf53	C18orf56	C20orf117	C3orf23	C4orf6
C4orf7	C6orf10	C6orf52	C9orf39	CART1
CC2D1A	CCL7	CDC2	CES4	CF527929
CITED4	CLDN4	CNTN2	COL1A1	COL5A2
COL6A1	COX11	CPLX2	CPNE6	CRB1
DB380193	DENND1A	DPPA5	DST	EFEMP1
EGFR	ENST00000254271	ENST00000258873	ENST00000272235	ENST00000295989
ENST00000299308	ENST00000300996	ENST00000315293	ENST00000335534	ENST00000354261
ENST00000354417	ENST00000355077	ENST00000355247	ENST00000356104	ENST00000369615
ENST00000374334	ENST00000374458	ENST00000375587	ENST00000381050	ENTPD8
EPS8L3	ERVWE1	ESX1	F8	FAM104B
FAM62C	FAM71B	FAM9C	FBXW10	FCRL4
FGFR1	FLJ25715	FLJ32312	FLJ37543	FLJ39582
FLJ39779	FNDC5	FRG2	FSIP2	FTCD
GAPDHS	GAS2L2	GAS8	GCKR	GLRA1
GPR143	GPR98	GUCA1C	HILS1	HLA-DRB6
HOXD3	HR44	IGF1	IGF1	IGSF4
INHBA	ITGAV	JPH1	KIAA0492	KIAA1661
KIF20A	KLHL9	KREMEN1	LCE3B	LENEP
LIFR	LOC222171	LOC339524	LOC348021	LOC388503

LOC390211	LOC440295	LOC642730	LOC643100	LOC643125
LOC648556	LOC92270	M31157	MGC39584	MGC43122
MRAP	MSTO1	MYLC2PL	MYO7A	NDST3
NF2	NNMT	NR1H4	NTN1	NTRK3
NTS	NXPH3	ODAM	OPCML	ORSH1
PALM2-AKAP2	PAX9	PCNXL2	PHC2	PKNOX1
PLAC1	POTE2	PPCDC	PPFIBP1	PPP1R14C
RBMY2EP	RCBTB1	RHOD	RRAGB	RSHL1
RSPO1	S72478	SAA4	SASS6	SDK1
SLC22A9	SLC26A9	SLCO1A2	SNAP25	SP5
SPBC25	STEAP1	SUFU	SUNC1	SYCE1
SYT12	TAS2R38	TAS2R4	TBC1D3	TBC1D8B
TCEB3C	TEK	THC2269604	THC2269920	THC2276996
THC2279230	THC2281591	THC2281747	THC2286878	THC2286962
THC2289112	THC2296760	THC2316481	THC2316929	THC2339079
THC2339904	THC2347643	THC2369034	THC2374304	THC2374505
THC2380237	THC2385918	THC2407039	THC2444579	THC2454812
THRSP	TM4SF20	TNFRSF13B	TREH	TRPA1
TRPC7	TSC22D2	TSHZ2	TTTY6	UGT8
UNC13B	USP2	USP6	VLDLR	WWTR1
X87895	ZNF28	ZNF57		

Table 2. Cancer-specific mutations for various cancers.

Symbol	GeneID	Chr Band	Tumour Types (Somatic Mutations)	Cancer Syndrome
ABL1	25	9q34.1	CML, ALL, T-ALL	
ABL2	27	1q24-q25	AML	
ACSL3	2181	2q36	prostate	
AF15Q14	57082	15q14	AML	
AF1Q	10962	1q21	ALL	
AF3p21	51517	3p21	ALL	
AF5q31	27125	5q31	ALL	
AKAP9	10142	7q21-q22	papillary thyroid	
AKT1	207	14q32.32	breast, colorectal, ovarian, NSCLC	
AKT2	208	19q13.1-q13.2	ovarian, pancreatic	
ALK	238	2p23	ALCL, NSCLC, Neuroblastoma	Familial neuroblastoma
ALO17	57714	17q25.3	ALCL	
APC	324	5q21	colorectal, pancreatic, desmoid, hepatoblastoma, glioma, other CNS	Adenomatous polyposis coli; Turcot syndrome
ARHGEF12	23365	11q23.3	AML	
ARHH	399	4p13	NHL	
ARNT	405	1q21	AML	
ASPSCR1	79058	17q25	alveolar soft part sarcoma	
ASXL1	171023	20q11.1	MDS, CMML	
ATF1	466	12q13	malignant melanoma of soft parts , angiomatoid fibrous histiocytoma	

ATIC	471	2q35	ALCL	
ATM	472	11q22.3	T-PLL, leukemia, lymphoma, medulloblastoma, glioma	Ataxia-telangiectasia
BCL10	8915	1p22	MALT	
BCL11A	53335	2p13	B-CLL	
BCL11B	64919	14q32.1	T-ALL	
BCL2	596	18q21.3	NHL, CLL	
BCL3	602	19q13	CLL	
BCL5	603	17q22	CLL	
BCL6	604	3q27	NHL, CLL	
BCL7A	605	12q24.1	BNHL	
BCL9	607	1q21	B-ALL	
BCR	613	22q11.21	CML, ALL, AML	
BHD	201163	17p11.2	renal, fibrofolliculomas, trichodiscomas	Birt-Hogg-Dube syndrome
BIRC3	330	11q22-q23	MALT	
BLM	641	15q26.1	leukemia, lymphoma, skin squamous cell , other cancers	Bloom Syndrome
BMPR1A	657	10q22.3	gastrointestinal polyps	Juvenile polyposis
BRAF	673	7q34	melanoma, colorectal, papillary thyroid, borderline ov, Non small-cell lung cancer (NSCLC), cholangiocarcinoma, pilocytic astrocytoma	
BRCA1	672	17q21	ovarian, breast,	Hereditary breast/ovarian cancer
BRCA2	675	13q12	breast, ovarian, pancreatic, leukemia (FANCB, FANCD1)	Hereditary breast/ovarian cancer
BRD3	8019	9q34	lethal midline carcinoma of young people	
BRD4	23476	19p13.1	lethal midline carcinoma of young people	
BRIP1	83990	17q22	AML, leukemia, breast	Fanconi anaemia J, breast cancer susceptiblity
BTG1	694	12q22	BCLL	
BUB1B	701	15q15	rhabdomyosarcoma	Mosaic variegated aneuploidy
C12orf9	93669	12q14.3	lipoma	
C15orf21	283651	15q21.1	prostate	
CANT1	124583	17q25	prostate	
CARD11	84433	7p22	DLBL	
CARS	833	11p15.5	ALCL	
CBFA2T1	862	8q22	AML	
CBFA2T3	863	16q24	AML	
CBFB	865	16q22	AML	
CBL	867	11q23.3	AML, JMML, MDS	
CBLB	868	3q13.11	AML	
CBLC	23624	19q13.2	AML	
CCND1	595	11q13	CLL, B-ALL, breast	
CCND2	894	12p13	NHL,CLL	
CCND3	896	6p21	MM	
CD74	972	5q32	NSCLC	
CD79A	973	19q13.2	DLBCL	
CD79B	974	17q23	DLBCL	

CDH1	999	16q22.1	lobular breast, gastric	Familial gastric carcinoma
CDH11	1009	16q22.1	aneurysmal bone cysts	
CDK4	1019	12q14	melanoma	Familial malignant melanoma
CDK6	1021	7q21-q22	ALL	
CDKN2A - p16(INK4a)	1029	9p21	melanoma, multiple other tumour types, pancreatic	Familial malignant melanoma
CDKN2A-p14ARF	1029	9p21	melanoma, multiple other tumour types, pancreatic	Familial malignant melanoma
CDKN2C	1031	1p32	glioma, MM	
CDX2	1045	13q12.3	AML	
CEBPA	1050	19q13.1	AML, MDS	
CEP1	11064	9q33	MPD, NHL	
CHCHD7	79145	8q11.2	salivary adenoma	
CHEK2	11200	22q12.1	breast	familial breast cancer
CHIC2	26511	4q11-q12	AML	
CHN1	1123	2q31-q32.1	extraskeletal myxoid chondrosarcoma	
CIC	23152	19q13.2	soft tissue sarcoma	
CLTC	1213	17q11-qter	ALCL, renal	
CLTCL1	8218	22q11.21	ALCL	
CMKOR1	57007	2q37.3	lipoma	
COL1A1	1277	17q21.31-q22	dermatofibrosarcoma protuberans, aneurysmal bone cyst	
COPEB	1316	10p15	prostate, glioma	
COX6C	1345	8q22-q23	uterine leiomyoma	
CREB1	1385	2q34	clear cell sarcoma, angiomatoid fibrous histiocytoma	
CREB3L2	64764	7q34	fibromyxoid sarcoma	
CREBBP	1387	16p13.3	AL, AML	
CRLF2	64109	Xp22.3; Yp11.3	B-ALL, Downs associated ALL	
CRTC3	64784	15q26.1	salivary gland mucoepidermoid	
CTNNB1	1499	3p22-p21.3	colorectal, cvarian, hepatoblastoma, others, pleomorphic salivary adenoma	
CYLD	1540	16q12-q13	cylindroma	Familial cylindromatosis
D10S170	8030	10q21	papillary thyroid, CML	
DDB2	1643	11p12	skin basal cell, skin squamous cell, melanoma	Xeroderma pigmentosum (E)
DDIT3	1649	12q13.1-q13.2	liposarcoma	
DDX10	1662	11q22-q23	AML*	
DDX5	1655	17q21	prostate	
DDX6	1656	11q23.3	B-NHL	
DEK	7913	6p23	AML	
DICER1	23405	14q32.13	pleuropulmonary blastoma	Familial Pleuropulmonary Blastoma
DUX4	22947	4q35	soft tissue sarcoma	
EGFR	1956	7p12.3-p12.1	glioma, NSCLC	Familial lung cancer
EIF4A2	1974	3q27.3	NHL	
ELF4	2000	Xq26	AML	
ELK4	2005	1q32	prostate	
ELKS	23085	12p13.3	papillary thyroid	
ELL	8178	19p13.1	AL	

ELN	2006	7q11.23	B-ALL	
EML4	27436	2p21	NSCLC	
EP300	2033	22q13	colorectal, breast, pancreatic, AML	
EPS15	2060	1p32	ALL	
ERBB2	2064	17q21.1	breast, ovarian, other tumour types, NSCLC, gastric	
ERCC2	2068	19q13.2-q13.3	skin basal cell, skin squamous cell, melanoma	Xeroderma pigmentosum (D)
ERCC3	2071	2q21	skin basal cell, skin squamous cell, melanoma	Xeroderma pigmentosum (B)
ERCC4	2072	16p13.3-p13.13	skin basal cell, skin squamous cell, melanoma	Xeroderma pigmentosum (F)
ERCC5	2073	13q33	skin basal cell, skin squamous cell, melanoma	Xeroderma pigmentosum (G)
ERG	2078	21q22.3	Ewing sarcoma, prostate, AML	
ETV1	2115	7p22	Ewing sarcoma, prostate	
ETV4	2118	17q21	Ewing sarcoma, Prostate carcinoma	
ETV5	2119	3q28	Prostate	
ETV6	2120	12p13	congenital fibrosarcoma, multiple leukemia and lymphoma, secretory breast, MDS, ALL	
EVI1	2122	3q26	AML, CML	
EWSR1	2130	22q12	Ewing sarcoma, desmoplastic small round cell tumor , ALL, clear cell sarcoma, sarcoma, myoepithelioma	
EXT1	2131	8q24.11-q24.13	exostoses, osteosarcoma	Multiple Exostoses Type 1
EXT2	2132	11p12-p11	exostoses, osteosarcoma	Multiple Exostoses Type 2
EZH2	2146	7q35-q36	DLBCL	
FACL6	23305	5q31	AML, AEL	
FANCA	2175	16q24.3	AML, leukemia	Fanconi anaemia A
FANCC	2176	9q22.3	AML, leukemia	Fanconi anaemia C
FANCD2	2177	3p26	AML, leukemia	Fanconi anaemia D2
FANCE	2178	6p21-p22	AML, leukemia	Fanconi anaemia E
FANCF	2188	11p15	AML, leukemia	Fanconi anaemia F
FANCG	2189	9p13	AML, leukemia	Fanconi anaemia G
FBXW7	55294	4q31.3	colorectal, endometrial, T-ALL	
FCGR2B	2213	1q23	ALL	
FEV	54738	2q36	Ewing sarcoma	
FGFR1	2260	8p11.2-p11.1	MPD, NHL	
FGFR1OP	11116	6q27	MPD, NHL	
FGFR2	2263	10q26	gastric. NSCLC, endometrial	
FGFR3	2261	4p16.3	bladder, MM, T-cell lymphoma	
FH	2271	1q42.1	lieomyomatosis, renal	hereditary leiomyomatosis and renal cell cancer
FIP1L1	81608	4q12	idiopathic hypereosinophilic syndrome	
FLI1	2313	11q24	Ewing sarcoma	
FLT3	2322	13q12	AML, ALL	
FNBP1	23048	9q23	AML	
FOXL2	668	3q23	granulosa-cell tumour of the ovary	
FOXO1A	2308	13q14.1	alveolar rhabdomyosarcomas	
FOXO3A	2309	6q21	AL	
FOXP1	27086	3p14.1	ALL	

FSTL3	10272	19p13	B-CLL	
FUS	2521	16p11.2	liposarcoma, AML, Ewing sarcoma, angiomatoid fibrous histiocytoma, fibromyxoid sarcoma	
FVT1	2531	18q21.3	B-NHL	
GAS7	8522	17p	AML*	
GATA1	2623	Xp11.23	megakaryoblastic leukemia of Downs Syndrome	
GATA2	2624	3q21.3	AML(CML blast transformation)	
GATA3	2625	10p15	breast	
GMPS	8833	3q24	AML	
GNAQ	2776	9q21	uveal melanoma	
GNAS	2778	20q13.2	pituitary adenoma	
GOLGA5	9950	14q	papillary thyroid	
GOPC	57120	6q21	glioblastoma	
GPC3	2719	Xq26.1	Wilms tumour	Simpson-Golabi-Behmel syndrome
GPHN	10243	14q24	AL	
GRAF	23092	5q31	AML, MDS	
HCMOGT-1	92521	17p11.2	JMML	
HEAB	10978	11q12	AML	
HEI10	57820	14q11.1	uterine leiomyoma	
HERPUD1	9709	16q12.2-q13	prostate	
HIP1	3092	7q11.23	CMML	
HIST1H4I	8294	6p21.3	NHL	
HLF	3131	17q22	ALL	
HLXB9	3110	7q36	AML	
HMGA1	3159	6p21	microfollicular thyroid adenoma, various benign mesenchymal tumors	
HMGA2	8091	12q15	lipoma	
HNRNPA2B1	3181	7p15	prostate	
HOOK3	84376	8p11.21	papillary thyroid	
HOXA11	3207	7p15-p14.2	CML	
HOXA13	3209	7p15-p14.2	AML	
HOXA9	3205	7p15-p14.2	AML*	
HOXC11	3227	12q13.3	AML	
HOXC13	3229	12q13.3	AML	
HOXD11	3237	2q31-q32	AML	
HOXD13	3239	2q31-q32	AML*	
HRAS	3265	11p15.5	infrequent sarcomas, rare other types, rhadomyosarcoma, ganglioneuroblastoma, bladder	Costello syndrome
HRPT2	3279	1q21-q31	parathyroid adenoma, mulitiple ossifying jaw fibroma	Hyperparathyroidism-jaw tumor syndrome
HSPCA	3320	14q32.31	NHL	
HSPCB	3326	6p12	NHL	
IDH1	3417	2q33.3	gliobastoma	
IDH2	3418	15q26.1	GBM	
IGH@	3492	14q32.33	MM, Burkitt lymphoma, NHL, CLL, B-ALL, MALT, MLCLS	

IGK@	50802	2p12	Burkitt lymphoma, B-NHL	
IGL@	3535	22q11.1-q11.2	Burkitt lymphoma	
IKZF1	10320	7p12.2	ALL	
IL2	3558	4q26-q27	intestinal T-cell lymphoma	
IL21R	50615	16p11	NHL	
IL6ST	3572	5q11	hepatocellular ca	
IRF4	3662	6p25-p23	MM	
IRTA1	83417	1q21	B-NHL	
ITK	3702	5q31-q32	peripheral T-cell lymphoma	
JAK1	3716	1p32.3-p31.3	ALL	
JAK2	3717	9p24	ALL, AML, MPD, CML	
JAK3	3718	19p13.1	acute megakaryocytic leukemia,	
JAZF1	221895	7p15.2-p15.1	endometrial stromal tumours	
JUN	3725	1p32-p31	sarcoma	
KDM5A	5927	12p11	AML	
KDM5C	8242	Xp11.22-p11.21	clear cell renal carcinoma	
KDM6A	7403	Xp11.2	renal, oesophageal SCC, MM	
KDR	3791	4q11-q12	NSCLC, angiosarcoma	
KIAA1549	57670	7q34	pilocytic astrocytoma	
KIT	3815	4q12	GIST, AML, TGCT, mastocytosis, mucosal melanoma, epithelioma	Familial gastrointestinal stromal tumour
KLK2	3817	19q13.41	prostate	
KRAS	3845	12p12.1	pancreatic, colorectal, lung, thyroid, AML, others	
KTN1	3895	14q22.1	papillary thryoid	
LAF4	3899	2q11.2-q12	ALL, T-ALL	
LASP1	3927	17q11-q21.3	AML	
LCK	3932	1p35-p34.3	T-ALL	
LCP1	3936	13q14.1-q14.3	NHL	
LCX	80312	10q21	AML	
LHFP	10186	13q12	lipoma	
LIFR	3977	5p13-p12	salivary adenoma	
LMO1	4004	11p15	T-ALL	
LMO2	4005	11p13	T-ALL	
LPP	4026	3q28	lipoma, leukemia	
LYL1	4066	19p13.2-p13.1	T-ALL	
MADH4	4089	18q21.1	colorectal, pancreatic, small intestine, *gastrointestinal polyps*	Juvenile polyposis
MAF	4094	16q22-q23	MM	
MAFB	9935	20q11.2-q13.1	MM	
MALT1	10892	18q21	MALT	
MAML2	84441	11q22-q23	salivary gland mucoepidermoid	
MAP2K4	6416	17p11.2	pancreatic, breast, colorectal	
MDM2	4193	12q15	sarcoma, glioma, colorectal, other	
MDM4	4194	1q32	GBM, bladder, retinoblastoma	

MDS1	4197	3q26	MDS, AML	
MDS2	259283	1p36	MDS	
MECT1	94159	19p13	salivary gland mucoepidermoid	
MEN1	4221	11q13	parathyroid tumors, parathyroid adenoma, pituitary adenoma, pancreatic islet cell, carcinoid	Multiple Endocrine Neoplasia Type 1
MET	4233	7q31	papillary renal, head-neck squamous cell	Familial Papillary Renal Cancer
MHC2TA	4261	16p13	NHL	
MITF	4286	3p14.1	melanoma	
MKL1	57591	22q13	acute megakaryocytic leukemia	
MLF1	4291	3q25.1	AML	
MLH1	4292	3p21.3	colorectal, endometrial, ovarian, CNS	Hereditary non-polyposis colorectal cancer, Turcot syndrome
MLL	4297	11q23	AML, ALL	
MLLT1	4298	19p13.3	AL	
MLLT10	8028	10p12	AL	
MLLT2	4299	4q21	AL	
MLLT3	4300	9p22	ALL	
MLLT4	4301	6q27	AL	
MLLT6	4302	17q21	AL	
MLLT7	4303	Xq13.1	AL	
MN1	4330	22q13	AML, meningioma	
MPL	4352	p34	MPD	Familial essential thrombocythemia
MSF	10801	17q25	AML*	
MSH2	4436	2p22-p21	colorectal, endometrial, ovarian	Hereditary non-polyposis colorectal cancer
MSH6	2956	2p16	colorectal, endometrial, ovarian	Hereditary non-polyposis colorectal cancer
MSI2	124540	17q23.2	CML	
MSN	4478	Xq11.2-q12	ALCL	
MTCP1	4515	Xq28	T cell prolymphocytic leukemia	
MUC1	4582	1q21	B-NHL	
MUTYH	4595	1p34.3-1p32.1	colorectal	Adenomatous polyposis coli
MYB	4602	6q22-23	adenoid cystic carcinoma	
MYC	4609	8q24.12-q24.13	Burkitt lymphoma, amplified in other cancers, B-CLL	
MYCL1	4610	1p34.3	small cell lung	
MYCN	4613	2p24.1	neuroblastoma	
MYH11	4629	16p13.13-p13.12	AML	
MYH9	4627	22q13.1	ALCL	
MYST4	23522	10q22	AML	
NACA	4666	12q23-q24.1	NHL	
NBS1	4683	8q21	NHL, glioma, medulloblastoma, rhabdomyosarcoma	Nijmegen breakage syndrome
NCOA1	8648	2p23	alveolar rhadomyosarcoma	
NCOA2	10499	8q13.1	AML	
NCOA4	8031	10q11.2	papillary thyroid	

NF1	4763	17q12	neurofibroma, glioma	Neurofibromatosis type 1
NF2	4771	22q12.2	meningioma, acoustic neuroma, renal	Neurofibromatosis type 2
NFIB	4781	9p24.1	adenoid cystic carcinoma, lipoma	
NFKB2	4791	10q24	B-NHL	
NIN	51199	14q24	MPD	
NONO	4841	Xq13.1	papillary renal cancer	
NOTCH1	4851	9q34.3	T-ALL	
NOTCH2	4853	1p13-p11	marginal zone lymphoma, DLBCL	
NPM1	4869	5q35	NHL, APL, AML	
NR4A3	8013	9q22	extraskelctal myxoid chondrosarcoma	
NRAS	4893	1p13.2	melanoma, MM, AML, thyroid	
NSD1	64324	5q35	AML	
NTRK1	4914	1q21-q22	papillary thyroid	
NTRK3	4916	15q25	congenital fibrosarcoma, Secretory breast	
NUMA1	4926	11q13	APL	
NUP214	8021	9q34.1	AML, T-ALL	
NUP98	4928	11p15	AML	
NUT	256646	q13	lethal midline carcinoma of young people	
OLIG2	10215	21q22.11	T-ALL	
OMD	4958	9q22.31	aneurysmal bone cysts	
P2RY8	286530	Xp22.3; Yp11.3	B-ALL, Downs associated ALL	
PAFAH1B2	5049	11q23	MLCLS	
PALB2	79728	16p12.1	Wilms tumor, medulloblastoma, AML ,breast	Fanconi anaemia N, breast cancer susceptibility
PAX3	5077	2q35	alveolar rhabdomyosarcoma	
PAX5	5079	9p13	NHL, ALL, B-ALL	
PAX7	5081	1p36.2-p36.12	alveolar rhabdomyosarcoma	
PAX8	7849	2q12-q14	follicular thyroid	
PBX1	5087	1q23	pre B-ALL, myoepithelioma	
PCM1	5108	8p22-p21.3	papillary thyroid, CML, MPD	
PCSK7	9159	11q23.3	MLCLS	
PDE4DIP	9659	1q12	MPD	
PDGFB	5155	22q12.3-q13.1	DFSP	
PDGFRA	5156	4q11-q13	GIST, idiopathic hypereosinophilic syndrome	
PDGFRB	5159	5q31-q32	MPD, AML, CMML, CML	
PER1	5187	17p13.1-17p12	AML, CMML	
PHOX2B	8929	4p12	neuroblastoma	familial neuroblastoma
PICALM	8301	11q14	TALL, AML,	
PIK3CA	5290	3q26.3	colorectal, gastric, gliobastoma, breast	
PIK3R1	5295	5q13.1	gliobastoma, ovarian, colorectal	
PIM1	5292	6p21.2	NHL	
PLAG1	5324	8q12	salivary adenoma	
PML	5371	15q22	APL, ALL	
PMS1	5378	2q31-q33	colorectal, endometrial, ovarian	Hereditary non-polyposis colorectal cancer

PMS2	5395	7p22	colorectal, endometrial, ovarian, medulloblastoma, glioma	Hereditary non-polyposis colorectal cancer, Turcot syndrome
PMX1	5396	1q24	AML	
PNUTL1	5413	22q11.2	AML	
POU2AF1	5450	11q23.1	NHL	
POU5F1	5460	6p21.31	sarcoma	
PPARG	5468	3p25	follicular thyroid	
PRCC	5546	1q21.1	papillary renal	
PRDM16	63976	1p36.23-p33	MDS, AML	
PRF1	5551	10q22	various leukaemia, lymphoma	
PRKAR1A	5573	17q23-q24	myxoma, endocrine, papillary thyroid	Carney complex
PRO1073	29005	11q31.1	renal cell carcinoma (childhood epithelioid)	
PSIP2	11168	9p22.2	AML	
PTCH	5727	9q22.3	skin basal cell , medulloblastoma	Nevoid Basal Cell Carcinoma Syndrome
PTEN	5728	10q23.3	harmartoma, glioma, prostate, endometrial	Cowden Syndrome, Bannayan-Riley-Ruvalcaba syndrome
PTPN11	5781	12q24.1	JMML, AML, MDS	
RAB5EP	9135	17p13	CMML	
RAD51L1	5890	14q23-q24.2	lipoma, uterine leiomyoma	
RAF1	5894	3p25	pilocytic astrocytoma	
RANBP17	64901	5q34	ALL	
RAP1GDS1	5910	4q21-q25	T-ALL	
RARA	5914	17q12	APL	
RB1	5925	13q14	retinoblastoma, sarcoma, breast, small cell lung	Familial retinoblastoma
RBM15	64783	1p13	acute megakaryocytic leukemia	
RECQL4	9401	8q24.3	osteosarcoma, skin basal and sqamous cell	Rothmund-Thompson Syndrome
REL	5966	2p13-p12	Hodgkin Lymphoma	
RET	5979	10q11.2	medullary thyroid, papillary thyroid, pheochromocytoma	Multiple endocrine neoplasia 2A/2B
ROS1	6098	6q22	glioblastoma, NSCLC	
RPL22	6146	1p36.31	AML, CML	
RPN1	6184	3q21.3-q25.2	AML	
RUNX1	861	21q22.3	AML, preB- ALL, T-ALL	
RUNXBP2	7994	8p11	AML	
SBDS	51119	7q11	AML, MDS	Schwachman-Diamond syndrome
SDH5	54949	11q12.2	paraganglioma	Familial paraganglioma
SDHB	6390	1p36.1-p35	paraganglioma, pheochromocytoma	Familial paraganglioma
SDHC	6391	1q21	paraganglioma, pheochromocytoma	Familial paraganglioma
SDHD	6392	11q23	paraganglioma, pheochromocytoma	Familial paraganglioma
SEPT6	23157	Xq24	AML	
SET	6418	9q34	AML	
SETD2	29072	3p21.31	clear cell renal carcinoma	
SFPQ	6421	1p34.3	papillary renal cell	
SFRS3	6428	6p21	follicular lymphoma	
SH3GL1	6455	19p13.3	AL	
STL	6491	1p32	T-ALL	

SLC45A3	85414	1q32	prostate	
SMARCA4	6597	19p13.2	NSCLC	
SMARCB1	6598	22q11	malignant rhabdoid	Rhabdoid predisposition syndrome
SMO	6608	7q31-q32	skin basal cell	
SOCS1	8651	16p13.13	Hodgkin Lymphoma, PMBL	
SRGAP3	9901	3p25.3	pilocytic astrocytoma	
SS18	6760	18q11.2	synovial sarcoma	
SS18L1	26039	20q13.3	synovial sarcoma	
SSH3BP1	10006	10p11.2	AML	
SSX1	6756	Xp11.23-p11.22	synovial sarcoma	
SSX2	6757	Xp11.23-p11.22	synovial sarcoma	
SSX4	6759	Xp11.23	synovial sarcoma	
STK11	6794	19p13.3	NSCLC, pancreatic, jejunal harmartoma, ovarian, testicular	Peutz-Jeghers syndrome
STL	7955	6q23	B-ALL	
SUFU	51684	10q24.32	medulloblastoma	Medulloblastoma predisposition
SUZ12	23512	17q11.2	endometrial stromal tumours	
SYK	6850	9q22	MDS, peripheral T-cell lymphoma	
TAF15	8148	17q11.1-q11.2	extraskeletal myxoid chondrosarcomas, ALL	
TAL1	6886	1p32	lymphoblastic leukemia/biphasic	
TAL2	6887	9q31	T-ALL	
TCEA1	6917	8q11.2	salivary adenoma	
TCF1	6927	12q24.2	hepatic adenoma, hepatocellular ca	Familial Hepatic Adenoma
TCF12	6938	15q21	extraskeletal myxoid chondrosarcoma	
TCF3	6929	19p13.3	pre B-ALL	
TCL1A	8115	14q32.1	T-CLL	
TCL6	27004	14q32.1	T-ALL	
TET2	54790	4q24	MDS	
TFE3	7030	Xp11.22	papillary renal, alveolar soft part sarcoma, renal	
TFEB	7942	6p21	renal (childhood epithelioid)	
TFG	10342	3q11-q12	papillary thyroid, ALCL, NSCLC	
TFPT	29844	19q13	pre-B ALL	
TFRC	7037	3q29	NHL	
THRAP3	9967	1p34.3	aneurysmal bone cysts	
TIF1	8805	7q32-q34	APL	
TLX1	3195	10q24	T-ALL	
TLX3	30012	5q35.1	T-ALL	
TMPRSS2	7113	21q22.3	prostate	
TNFAIP3	7128	6q23	marginal zone B-cell lymphomas, Hodgkin's lymphoma, primary mediastinal B cell lymphoma	
TNFRSF17	608	16p13.1	intestinal T-cell lymphoma	
TNFRSF6	355	10q24.1	TGCT, nasal NK/T lymphoma, skin squamous cell ca -burn scar-related	
TOP1	7150	20q12-q13.1	AML*	

TP53	7157	17p13	breast, colorectal, lung, sarcoma, adrenocortical, glioma, multiple other tumour types	Li-Fraumeni syndrome
TPM3	7170	1q22-q23	papillary thyroid, ALCL	
TPM4	7171	19p13.1	ALCL	
TPR	7175	1q25	papillary thyroid	
TRA@	6955	14q11.2	T-ALL	
TRB@	6957	7q35	T-ALL	
TRD@	6964	14q11	T-cell leukemia	
TRIM27	5987	6p22	papillary thyroid	
TRIM33	51592	1p13	papillary thyroid	
TRIP11	9321	14q31-q32	AML	
TSC1	7248	9q34	hamartoma, renal cell	Tuberous sclerosis 1
TSC2	7249	16p13.3	hamartoma, renal cell	Tuberous sclerosis 2
TSHR	7253	14q31	toxic thyroid adenoma	
TTL	150465	2q13	ALL	
USP6	9098	17p13	aneurysmal bone cysts	
VHL	7428	3p25	renal, hemangioma, pheochromocytoma	von Hippel-Lindau syndrome
WAS	7454	Xp11.23-p11.22	lymphoma	Wiskott-Aldrich syndrome
WHSC1	7468	4p16.3	MM	
WHSC1L1	54904	8p12	AML	
WRN	7486	8p12-p11.2	osteosarcoma, meningioma, others	Werner Syndrome
WT1	7490	11p13	Wilms, desmoplastic small round cell tumor	Denys-Drash syndrome, Frasier syndrome, Familial Wilms tumor
WTX	139285	Xq11.1	Wilms tumour	
XPA	7507	9q22.3	skin basal cell, skin squamous cell, melanoma	Xeroderma pigmentosum (A)
XPC	7508	3p25	skin basal cell, skin squamous cell, melanoma	Xeroderma pigmentosum (C)
ZNF145	7704	11q23.1	APL	
ZNF198	7750	13q11-q12	MPD, NHL	
ZNF278	23598	22q12-q14	Ewing sarcoma	
ZNF331	55422	19q13.3-q13.4	follicular thyroid adenoma	
ZNF384	171017	12p13	ALL	
ZNF521	25925	18q11.2	ALL	
ZNF9	7555	3q21	aneurysmal bone cysts	
ZNFN1A1	10320	7p12	ALL, DLBL	

Example 2

Introduction

Diagnostic platforms which are highly predictive for diagnosing, monitoring, and stratifying cancer patients are key instruments in the development of personalized medicine. In this Example, it is demonstrated

that tumor cells transfer (mutant) RNA into blood platelets *in vitro*, and it is shown that blood platelets isolated from glioblastoma and prostate cancer patients contain the cancer-associated RNA biomarkers EGFRvIII, and PCA3 and PSA, respectively. Moreover, gene expression arrays revealed a distinct mRNA signature in platelets from glioma patients as compared to normal control subjects. Because platelets are easily accessible and isolated, they may form an attractive platform for the companion diagnostics of cancer.

Methods

Platelet isolation and tissue resection.

Platelets were isolated from whole blood collected in purple-cap BD Vacutainers containing EDTA anti-coagulant by standard centrifugation, and quality (activation and aggregation) as well as purity was assessed by microscopic analysis showing less than 0.1% contamination with red or white blood cells. Next, isolated platelet pellets were snap-frozen for further use. Glioma tissue resection and whole blood harvesting from glioma and prostate cancer patients was performed at the VU University medical center and Umeå University, as described elsewhere (J. Skog et al., Nat Cell Biol. 10(12), 1470-6 (2008)).

Microvesicle isolation, labeling, and transfer.

Microvesicles were isolated from U87-EGFRvIII glioma cells and labeled as described previously (J. Skog et al., Nat Cell Biol. 10(12), 1470-6 (2008)). After U87-dEGFR microvesicle incubation the platelets were washed and treated with RNAse enzymes to ensure the EGFRvIII RNA was delivered into the platelets and therefore protected from RNAse-mediated degradation. For confocal microscopy analysis the platelets were stained with texas red-conjugated wheat germ agglutinin to indicate platelet structure and analyzed for microvesicle uptake by the presence of green PKH67. RNA purification. RNA was isolated using miRvana (Ambion) or miRNeasy (Qiagen) protocols

according the manufacturer's instruction. RNA concentration and quality was determined using a Bioanalyzer 2100 with total RNA Pico chip (Agilent).

RT-PCR.

RT-PCR for EGFRvIII, PCA3, PSA, and GAPDH, was performed as described previously (J. Skog et al., Nat Cell Biol. 10(12), 1470-6 (2008)) using the following primer sets:

GAPDH primers:

forward 5′-GAAGGTGAAGGTCGGAGTC-3′,

reverse: 5′-TCAGAAGATGGTGATGGGATTTC-3′.

PSA primers:

forward 5'-ATGTGGGTCCCGGTTGTCTT-3',

reverse 5'-TCCCACAATCCGAGACAGGA-3'.

Nested PCA3 primers:

PCR1:

forward 5'-AGTCCGCTGTGAGTCT-3',

reverse 5'-CCATTTCAGCAGATGTGTGG-3';

PCR2:

forward 5'-ATCGACGGCACTTTCTGAGT-3',

reverse 5'-TGTGTGGCCTCAGATGGTAA-3'.

Nested EGFRvIII primers:

PCR1:

forward 5′-CCAGTATTGATCGGGAGAGC-3′,

reverse 5′-TGTGGATCCAGAGGAGGAGT-3′;

PCR2:

forward 5′-GAGCTCTTCGGGGAGCAG-3′,

reverse 5′-GCCCTTCGCACTTCTTACAC-3′.

Gene expression arrays.

The mRNA expression arrays were performed at the VU University Medical Center microarray core facility using Agilent 4x44K gene expression arrays. Platelet RNA integrity was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Inc.). RNA samples were labelled using the Agilent Low RNA Input Linear Amplification Kit Plus (5188-5340) according to the manufacturer's protocol.

Briefly, 25 ng of total RNA was amplified and reverse transcribed to cDNA using T7-polymerase and subsequently labelled with Cy3 or Cy5. Dye incorporation was measured using a Nanodrop ND-1000 spectrophotometer. Subsequently, cRNA was hybridized using the Agilent Gene Expression Hybridization Kit (5188-5242), according to the manufacturer's protocol. Briefly, 825 ng of Cy3 labelled cRNA was mixed with 825 ng of Cy5 labelled cRNA, fragmented for 30 min at 60°C in the dark and hybridized on an Agilent Hybridization Chamber Gasket Slide (G2534-60011) in a rotation oven at 65°C for 17 h. Slides scanned using an Agilent Microarray Scanner (G2565BA). Image analysis and array normalization was performed using feature extraction software version 9.5 (Agilent Technologies, Inc.). The Agilent GE2-v5_95 protocol was applied using default settings.

Statistical analysis.

The heat map (Fig 3C) of the gene expression data was generated using median centered arrays in Excel (Microsoft Office 2007 package) with the S.A.M. analysis plug-in, with a set false discovery rate <0.5%. The top-30 significantly differentially expressed genes are depicted using Heatmap Builder v1.1 software (King et al. Physiol Genomics. Sep 21 2005;23(1):103-118).).

Results

In this Example it is shown that platelets isolated from healthy human control subjects have the ability to take up RNA-containing microvesicles derived from human brain tumor cells (glioma), and contain tumor-associated RNA, including mutant EGFRvIII. Uptake of PKH67 labelled glioma-derived microvesicles is demonstrated in blood platelets by FACS analysis and confocal microscopy. In addition, it was shown that microvesicle-mediated transfer of mutant EGFRvIII RNA into platelets from healthy control subjects by RT-PCR occurs. Furthermore, it is determined that circulating platelets isolated from glioma patients, contain RNA biomarkers (see Fig. 3B). RT-PCR was used to determine whether mutant EGFRvIII mRNA was found in resected high-grade glioma tissues (n =18) and the result was compared to platelets from the same patient and to platelets from healthy control subjects (n = 30). The samples were coded and RT-PCR was performed in a blind assay. Four of the 18 (22.5%) glioma samples contained the EGFRvIII transcript, as observed before. Notably, EGFRvIII could be amplified from platelets in 3 out of these 4 EGFRvIII-positive patients (75%), and in none of the platelets of the healthy donors (n = 12), whereas GAPDH mRNA was detected in all platelet samples. A possible false negative signal was detected in the platelets of one patient only, which may be contributed to inadequate processing of the blood sample. Conversely, one patient with EGFRvIII-negative tissue sample was EGFRvIII-positive in the platelet sample, most likely due to heterogeneous distribution of EGFRvIII positive foci in high-grade gliomas.

To demonstrate that the presence of tumor-associated messages is not unique to platelets from glioma patients we report the presence of mRNAs coding for the prostate cancer markers PCA3 and PSA in platelets from prostate cancer patients (n = 12) and their absence in platelets from healthy control subjects (n = 10) (See Figure 4). Finally, using gene expression arrays it was determined the mRNA expression profiles of platelets isolated from healthy control subjects (n = 12), and glioma patients (n = 8). Distinct mRNA

expression profiles were obtained and a minimal glioma biomarker signature was detected (Fig. 1C, left panel). Interestingly, several of the potential biomarkers were barely detectable in control samples, whereas in the glioma samples they were highly expressed (Fig. 1C, right panel).

In conclusion, the findings of the present inventors demonstrate that blood platelets contain cancer markers in the form of tumor-derived or tumor-associated RNA and, therefore, may serve as a diagnostic platform for the molecular profiling of cancer in the context of personalized medicine.

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International Patent Application No.
PCT/NL2011/050518
Our letter of July 2, 2012

Amended claims

1. A method of analysing a blood sample of a subject for the presence of a cancer marker, said method comprising the steps of
a) extracting nucleic acid from anucleated blood cells, in said blood sample to provide an anucleated blood cell-extracted nucleic acid fraction, and
b) analysing said anucleated blood cell-extracted nucleic acid fraction for the presence of a cancer marker,
wherein said cancer marker is a cancer-specific mutation in a gene of a nucleated cell of said subject, or
wherein said cancer marker is a cancer-specific expression profile of genes of a nucleated cell of said subject, wherein said nucleic acid is cancer-derived nucleic acid.

2. The method of claim 1, wherein said anucleated blood cells are thrombocytes or erythrocytes.

3. The method of claim 1 wherein said cancer is a solid tumour cancer,.

4. The method of any one of the preceding claims, wherein said cancer-specific mutation is in a chromosomal gene, or wherein said cancer-specific expression profile is of chromosomal genes.

5. The method of any one of the preceding claims, wherein said nucleic acid is ribonucleic acid (RNA).

6. The method of any one of the preceding claims, wherein said step b) of analysing said anucleated blood cell-extracted nucleic acid fraction for the presence of a cancer marker comprises the selective amplification of
i) said mutation by reverse transcriptase polymerase chain reaction amplification using at least one nucleic acid mutation-specific amplification primer or probe, or
ii) a plurality of mRNAs by reverse transcriptase polymerase chain reaction amplification to determine the expression level of the chromosomal genes encoding said mRNAs to thereby provide an expression profile for said genes and comparing said expression profile to a reference profile.

7. The method of any one of the preceding claims, wherein said method is part of a method of diagnosing said cancer in a subject, and wherein the presence of said cancer marker in said anucleated blood cell-extracted nucleic acid fraction is indicative of said subject suffering from cancer.

8. A method for determining the stage of cancer or the efficacy of a cancer treatment in a subject, comprising the steps of:
- analysing a blood sample of a subject for the presence of a cancer marker using the method according to any one of claims 1-8 at a first time point to thereby provide a first value for the level of said cancer marker in said subject,
- analysing a blood sample of said subject for the presence of a cancer marker using the method according to any one of claims 1-7 at a second time point to thereby provide a second value for the level of said cancer marker in said subject, wherein said subject has been subjected to a cancer treatment between said first and second time point, and

- comparing said first and second value to determine the efficacy of said cancer treatment in said subject.

9. A method for determining the stage of a cancer in a subject, comprising the steps of:
- analysing a blood sample of a subject for the presence of a cancer marker using the method according to any one of claims 1-7 to thereby provide a test value for the level of said cancer marker in said subject,
- providing a reference value for the level of said cancer marker wherein said reference value is correlated to a particular stage of cancer, and
- comparing said test and reference value to determine the stage of cancer in said subject.

10. A kit of parts adapted for performing the method recited in any one of claims 1-9, the kit comprising a packaging material which comprises at least one of:
- a container for holding anucleated blood cells separated from a blood sample of a subject;
- an agent for extracting nucleic acids from said anucleated blood cells;
- an agent for selectively amplifying from said nucleic acids extracted from said anucleated blood cells a cancer-specific mutation in a gene of a nucleated cell of said subject by reverse transcriptase polymerase chain reaction amplification, and
- a printed or electronic instruction for performing the method recited in any one of claims 1-9,
the kit further comprising:
- a reference for said cancer marker, wherein said reference is indicative for the presence or absence of said cancer marker in said anucleated blood cells-extracted nucleic acid fraction.

11. The kit of claim 10, wherein said reference is a reference value for the level of nucleic acids comprising said cancer-specific mutation in thrombocytes in a healthy control subject or in a control subject suffering from said cancer, or
wherein said reference is a reference expression profile for said plurality of mRNAs in anucleated blood cells from a healthy control subject or from a control subject suffering from said cancer.

12. A kit of claim 10 or 11, wherein said agent is selected from a particle or fluorescent marker-labeled anti-anucleated blood cell antibody, or wherein said instruction is selected from an instruction for bead-based anucleated blood cells isolation, an instruction for FACS sorting of anucleated blood cells, an instruction for anucleated blood cell recovery by centrifugation, or negative selection of non-anucleated blood cell components.

13. A device for diagnosing cancer, the device comprising a support and at least one agent for specifically determining a level and/or activity of at least one nucleic acid mutant in an anucleated blood cell sample of the subject attached to said support, and
a computer-readable medium having computer-executable instructions for performing the method recited in any one of claims 1-9.

14. The device of claim 13, wherein said at least one agent is an oligonucleotide probe or sequencing primer.

15. The device of claim 13 or 14, comprising a lateral flow device, a dipstick or a cartridge for performing a nucleic acid hybridization reaction between:
- an anucleated blood cells-extracted nucleic acid and at least one nucleic acid mutation-specific amplification primer or oligonucleotide probe, wherein said

nucleic acid mutation-specific amplification primer or oligonucleotide probe is specific for a cancer-specific mutation, or

- an anucleated blood cells-extracted nucleic acid and a plurality of gene-specific amplification primers or oligonucleotide probes for providing an cancer-specific gene expression profile.