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PHOTO QUIZ: Turning green with shock, see page 291

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EDITORIAL

Endocrine laboratory testing: why so complex?

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For a non-endocrinologist, laboratory testing involving hormones is often non-intuitive and sometimes flat-out complex. If one suspects hyperthyroidism, why not simply measure thyroid hormone? Or if a clinical suspicion of adrenal insufficiency exists, why don't we just measure plasma cortisol? The answer is simple: this approach does not work for endocrinology and a more intricate strategy is often required.

Apparently, there are several reasons that diagnostics in the world of endocrinology is less straightforward than in other areas of internal medicine. First, plasma concentrations of hormones are often extremely low. For example, plasma concentrations of insulin, free T4, or pituitary hormones are all in the picomolar range, i.e. a million times lower than that of major plasma proteins, such as albumin. It is only in the last decades that we are able to measure these small concentrations, initially by radioimmunoassays but more recently (by ever improving techniques) with more conventional enzyme-linked immunoassays. However, in the mean time we have learned to work with indirect measures of hormone activity and this may result in diagnostic strategies that have proven to work in the past, but are often not understandable at first sight for a new generation of physicians that has not grown up in times in which were not able to directly measure various hormones. However, it is not unlikely that newer diagnostic strategies will lean more strongly on direct measurements of hormones. Another difficult issue in measuring hormone concentrations is that these values are not stable over time and may rapidly fluctuate within a short time span (even within hours) due to a combination of external conditions and diurnal variation. These factors, on top of issues regarding assay variability and pre-analytical factors, render the establishment of normal values of utmost importance. In previous issues of the *Netherlands Journal of Medicine*, highly useful articles on reference values for various endocrine disorders have been published, for example on hypercortisolism and hypocortisolism, hyperprolactinaemia, hyperaldostenonism and excessive growth hormone.[1-5] These articles have proven to be useful in daily clinical practice but also in the establishment of guidelines or when reporting on individual cases in the literature.[6-9]

But apart from difficulties and solutions when measuring plasma hormone concentrations, direct measurement of hormones is often not sufficiently precise to establish a proper function or dysfunction of an endocrine axis. It is for this reason that endocrinologists often refer to function tests, which are able to provide a more dynamic answer, for example on how a target endocrine organ responds to a stimulus. In many cases, these function tests provide a more precise assessment of endocrine function and more accurately reflect the *in vivo* situation than a single measurement at a single point in time. Apparently, endocrine function is so subtle that regulatory pathways need to be challenged to provide adequate insight into their behaviour and to demonstrate endocrine derangement. But also these function tests require standardisation and proper cut-off values. In another series of articles in the *Netherlands Journal of Medicine* a number of these tests (TRH testing in hyperprolactinaemia, screening tests for hypercortisolism, and glucagon and clonidine testing in phaeochromocytoma) were extensively evaluated.[10-12] In this issue of the Journal a fourth paper on the application of the prolonged fasting test in the diagnosis of insulinoma is added.[13] Van Bon *et al*. clearly report on the utility of the prolonged fasting test for the detection of hypoglycaemia, due to insulinoma and also in patients who do not have this disease but display a surprisingly low glucose level without a proper explanation.

Standardisation and proper evaluation of diagnostic tests often receives less attention than evaluation of therapeutic interventions, which is an unwanted situation. In recent years a framework for proper assessment of diagnostic tests has been developed.[14] Briefly, tests should be standardised and validated and factors influencing the variability of the results should be understood. In addition, reference values for relevant populations should be assessed and the reliability of the test in terms of diagnostic power should be established. Furthermore, it should be evaluated whether

the test adds anything of value beyond information that is already available, e.g. from history, physical examination and other laboratory tests or imaging techniques. Ultimately, an assessment should be made whether the test result will have therapeutic or other clinically important consequences. The series of articles on endocrine testing in the *Netherlands Journal of Medicine* in recent years will certainly prove helpful in fulfilling all these prerequisites and may be of great relevance for endocrinologists and other internists.

REFERENCES

1. De Bos Kuil MJ, Endert E, Fliers E, Prummel MF, Romijn JA, Wiersinga WM. Establishment of reference values for endocrine tests. I: Cushing's syndrome. Neth J Med. 1998;53:153-63.
2. Le Moli R, Endert E, Fliers E, et al. Establishment of reference values for endocrine tests. II: Hyperprolactinemia. Neth J Med. 1999;55:71-5.
3. Endert E, Ouwehand A, Fliers E, Prummel MF, Wiersinga WM. Establishment of reference values for endocrine tests. Part IV: Adrenal insufficiency. Neth J Med. 2005;63:435-43.
4. Endert E, van Rooden M, Fliers E, Prummel MF, Wiersinga WM. Establishment of reference values for endocrine tests--part V: acromegaly. Neth J Med. 2006;64:230-5.
5. Baas SJ, Endert E, Fliers E, Prummel MF, Wiersinga WM. Establishment of reference values for endocrine tests. III: Primary aldosteronism. Neth J Med. 2003;61:37-43.
6. Muller AF, Berghout A, Wiersinga WM, Kooy A, Smits JW, Hermus AR. Thyroid function disorders--Guidelines of the Netherlands Association of Internal Medicine. Neth J Med. 2008;66:134-42.
7. Gilles R, den Heijer M, Ross AH, Sweep FC, Hermus AR, Wetzels JF. Thyroid function in patients with proteinuria. Neth J Med. 2008;66:483-5.
8. Abma EM, Kluin PM, Dullaart RP. Malignant aldosterone-producing adrenal tumour: reoccurrence with glucocorticoid excess without hyperaldosteronism. Neth J Med. 2008;66:252-5.
9. Van der Ven AC, Erdtsieck RJ. Changes of bone mineral density, quantitative ultrasound parameters and markers of bone turnover during treatment of hyperthyroidism. Neth J Med. 2008;66:428-32.
10. Le Moli R, Endert E, Fliers E, Prummel MF, Wiersinga WM. Evaluation of Endocrine Tests. A: the TRH test in patients with hyperprolactinaemia. Neth J Med. 2003;61:44-8.
11. Holleman F, Endert E, Prummel MF, van Vessem-Timmermans M, Wiersinga WM, Fliers E. Evaluation of Endocrine Tests. B: screening for hypercortisolism. Neth J Med. 2005;63:348-53.
12. Bisschop PH, Corssmit EP, Baas SJ, et al. Evaluation of Endocrine Tests. C: glucagon and clonidine test in phaeochromocytoma. Neth J Med. 2009;67:91-5.
13. Van Bon AC, Benhadi N, Endert E, Fliers E, Wiersinga WM. Evaluation of Endocrine Tests. D: the prolonged fasting test for insulinoma. Neth J Med. 2009;67:274-78.
14. Guyatt GH, Tugwell PX, Feeny DH, Haynes RB, Drummond M. A framework for clinical evaluation of diagnostic technologies. Can Med Assoc J. 1986;134:587-94.

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REVIEW

CD20-targeted therapy: a breakthrough in the treatment of non-Hodgkin's lymphoma

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ABSTRACT

Targeting the CD20 antigen on B lymphocytes with the monoclonal antibody rituximab has greatly improved the outcome of patients with B-cell malignancies. Despite the success of rituximab, resistance occurs in about half of the patients, resulting in non-response to treatment or early relapse of the original disease. A better understanding of the mechanism of rituximab resistance has lead to the development of novel, improved anti-CD20 antibodies. This review describes the development of CD20-targeted therapy from its historical background towards the next generation of anti-CD20 monoclonal antibodies and explains new strategies to overcome resistance.

KEYWORDS

Anti-CD20-therapy, CD20, non-Hodgkin's lymphoma, rituximab

INTRODUCTION

The goal of CD20-targeted therapy is to kill B lymphocytes by the use of monoclonal antibodies (MoAb) against the B-cell specific human CD20 molecule. Clinical success started by targeting non-Hodgkin's lymphoma (NHL) with rituximab, a chimeric anti-CD20 MoAb. The use of rituximab as a single agent or as an addition to chemotherapy in NHL patients can be considered as one of the most successful and worldwide accepted forms of immunotherapy so far.

However, despite its success, resistance occurs in about half of the NHL patients, resulting in non-response to treatment or early relapse of the original disease.

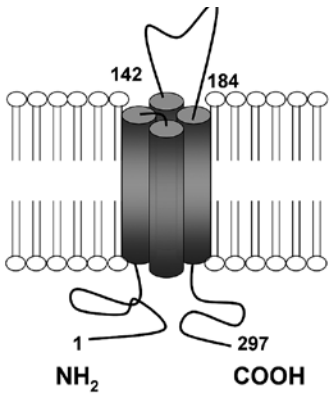
Rituximab eliminates CD20-positive cells mainly through three different mechanisms: complement-dependent cytotoxicity (CDC), antibody-dependent cellular cytotoxicity (ADCC) and the induction of apoptosis. Resistance to rituximab can be lymphoma-related or host-related. The preference for one of these mechanisms depends on the patient-specific microenvironment of the lymphoma. Based on the physiology of these factors, novel anti-CD20 antibodies are being developed.

This article reviews the development of CD20 targeting from its historical background towards the next generation of anti-CD20 monoclonal antibodies and explains the new strategies to overcome resistance.

HUMAN CD20

Expression of the human CD20 molecule is restricted to B-cell precursors and mature B cells (*figure 1*). CD20

Figure 1. *The human CD20 molecule*



expression is lost upon differentiation of the B cells towards plasma cells.[1-3] As shown in *figure 2*, CD20 is expressed within key B-cell development stages that give rise to B-cell NHL and chronic lymphocytic leukaemia (CLL).

CD20 is an ideal target for antibody-mediated therapy because CD20 is not expressed in haematopoetic stem cell B cells, so that the B-cell haematopoiesis and other cell lineages are not in danger. Moreover, CD20 is not expressed on plasma cells, which means that antibody therapy will not significantly decrease the immunoglobulin production against pathogens. Other advantages of targeting CD20 are that CD20 does not circulate in the plasma,[4] is not shed from the cell surface[5] and is not internalised[6] after antibody binding. Although CD20 is the most frequently antibody-targeted antigen in general, its exact function is still unknown. Actually, the CD20 antigen was discovered through generation of the first anti-CD20 monoclonal antibody. Balb/c mice were immunised with Burkitt's lymphoma cells and a new antibody was formed, called anti-B1, which recognised CD20.[1] Still no natural ligand is known for CD20 and our current understanding of the function of the CD20 molecule comes from ligation with different antibodies to CD20.[7-10] These experiments suggest that CD20 functions as a B-cell activating or proliferation molecule. Different antibodies have shown effects on B-cell proliferation, and some were able to block B-cell growth (reviewed in Deans *et al.*).[7] In general, ligation of CD20 with most antibodies (type I anti-CD20 MoAb) leads to the formation of signalling platforms (lipid rafts) and eventually to calcium flux and activation of caspase-3.[11] The formation of these signalling platforms and the downstream signalling cascade is probably in conjunction with the signalling potential of the B-cell receptor (BCR).[12]

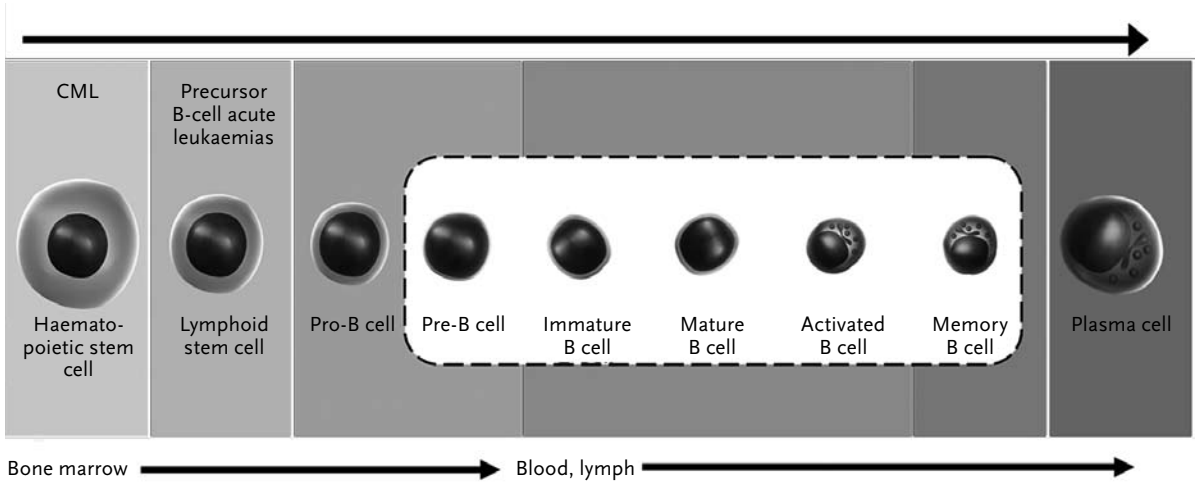
DEVELOPMENT OF THE ANTI-CD20 ANTIBODY RITUXIMAB

The first monoclonal antibody that recognised CD20, the murine anti-CD20 B1, was generated in 1980.[1] Because of their potential in the treatment of B-cell disorders, in the years thereafter anti-CD20 antibodies were genetically engineered for clinical application. In 1997, rituximab (MabThera®, Rituxan®) was the first MoAb approved specifically for the treatment of patients with relapsed or refractory CD20-positive low-grade (follicular) non-Hodgkin's lymphoma. Rituximab is a chimeric anti-CD20 antibody that is engineered as follows: the light and heavy chain variable regions from the murine 2B8 anti-CD20 antibody (IDEC-2B8), generated by immunising mice with a CD20-positive human lymphoma, are amplified by polymerase chain reaction and inserted into a cDNA mammalian chimeric antibody expression vector, which also contains the neomycin phosphotransferase gene (NEO). This vector is electroporated into Chinese hamster ovary (CHO) cells and under antibiotic pressure the cells stably secrete Ig levels.[13] The resulting chimeric antibody is purified and consists of a human kappa constant region, a human IgG Fc portion (IgG1), and a murine variable region, recognising the human CD20 protein.[13]

ACTION MECHANISMS OF RITUXIMAB

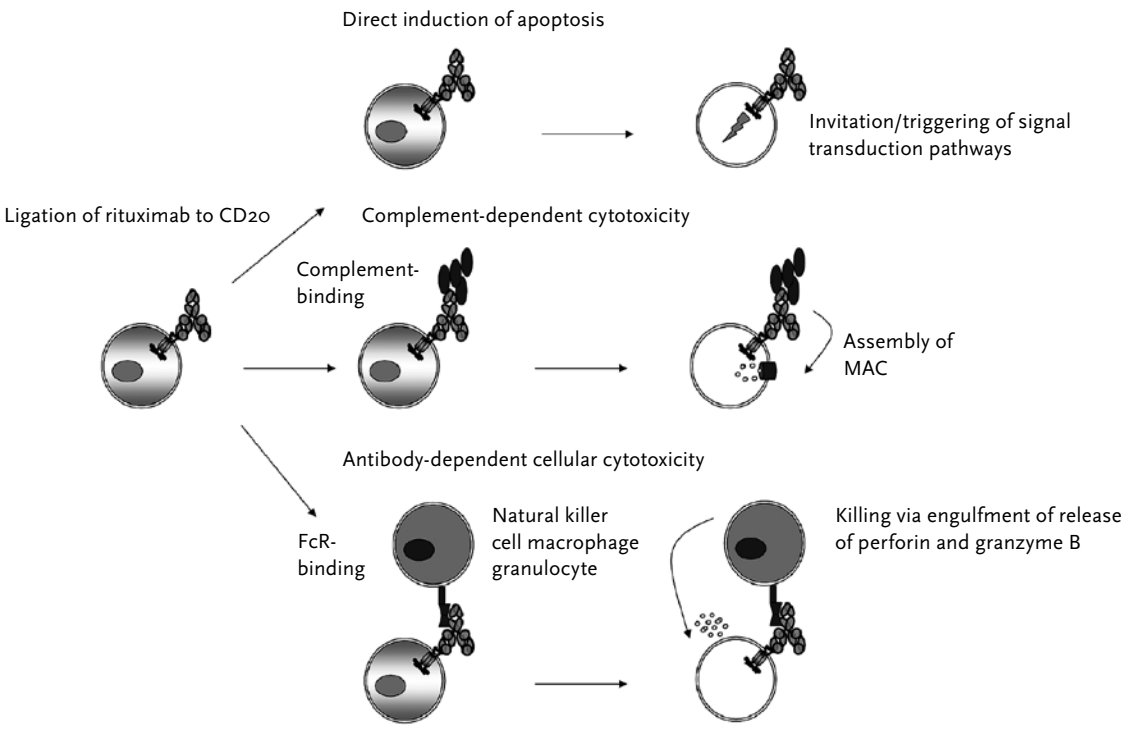
Upon ligation of CD20, rituximab triggers different effector mechanisms. Many *in vitro* and *in vivo* studies have been conducted to explore the most important one. *In vitro*, it is well established that there are three main modes of action of rituximab: 1) induction of apoptosis 2) CDC and 3) ADCC, as described below (*figure 3*).

Figure 2. *CD20 expression in B-cell development*



The CD20 antigen is first expressed during early pre-B-cell development and is lost during terminal plasma cell differentiation.

Figure 3. *The three main mechanisms of action of rituximab*



After rituximab ligation to CD20, three different effector mechanisms can be activated. 1) Direct induction of apoptosis may be caused by the activation of the death receptor pathway or the mitochondrial pathway by modulation of the bcl-2 gene family. But apoptosis-induced death can also bypass the mitochondrial pathway or caspases. 2) The Fc portion of the IgG1 tail of rituximab is able to bind C1q, the first component of the classical complement pathway. Binding of C1q triggers a proteolytic cascade, resulting in accumulation of C3b. C3b molecules act as opsonins for phagocyting cells but also bind to the C3 convertase to form a C5 convertase, leading to the generation of the membrane attack complex (MAC), which kills the cell. 3) The Fc portion is also recognised by cells expressing the Fc receptors (NK cells, granulocytes and macrophages). Cell killing is mediated by phagocytosis or the release of cytotoxic granules.

Rituximab and apoptosis

Data concerning the mechanism of the apoptotic effect of rituximab are conflicting. Different groups obtained different results, even if they used similar target cell lines.[7,14,15] It has been suggested that one of the late apoptotic pathways, caspase-3, is activated.[16] However, others documented that the apoptotic pathways are caspase or Fas ligand/ Fas death pathway and mitochondria independent, and do not require lipid raft formation.[14,17] Hyper-crosslinking of rituximab, either by a secondary antibody or by Fc bearing effector cells, generally increased the apoptosis.[7] An important observation is that within a treated cell population not all cells uniformly undergo apoptosis. This is the current focus of many groups that study rituximab resistance.

Rituximab and CDC

The Fc portion of rituximab is able to trigger the classic complement system, resulting in CDC. *In vitro*, C1q is bound efficiently by rituximab.[13,18] and simple CDC assays demonstrate that complement activation induces cell kill.[15,19,20] Rituximab-induced CDC has a variable degree of efficiency, which has been associated with expression of complement regulatory proteins (CRP) CD55 and CD59.[15,20,21] Whereas CD20 expression level has been suggested to be an important predictor of clinical CDC efficiency, several studies show contradictory results and no clear evidence for this relationship.[15,21-23]

Rituximab and ADCC

ADCC is mediated by effector cells expressing FcγRI (CD64), FcγRII (CD32) or FcγRIII (CD16). Effector cells, such as NK cells, granulocytes or macrophages, are able to recognise the Fc portion of rituximab, and kill the ligated cells by phagocytosis or the release of cytotoxic granules.[13,15,24,25] For ADCC, it has been demonstrated that the efficacy depends on polymorphisms of the effector cells.[25,26]

In the *in vitro* studies it was possible to investigate the mechanisms of rituximab separately, but this is more complex for *in vivo* studies. In several murine studies it was attempted to clarify the importance of each effector mechanism. Elegant mouse models using FcγR-deficient mice pointed out that clearing of CD20-expressing cells

was FcγR dependent for a panel of murine anti-CD20 MoAb[27] and rituximab.[28] Other groups demonstrated that complement was responsible for CD20-positive tumour clearance by rituximab.[9,29,30] However, there is no agreement in the literature about the dominance of one particular *in vivo* effector mechanism.

Also, some evidence concerning the mechanism of rituximab has been obtained in patients. One of the infusion-related side effects of rituximab is the complement consumption after administration,[31,32] indirectly confirming CDC. On the other hand, clinical responses have been correlated to polymorphisms in the FcγRIIIA gene,[26,33,34] indirectly confirming ADCC. In addition, a significant direct effect of rituximab cell kill by activating caspase-3 was demonstrated *in vivo* in patients with chronic lymphocytic leukaemia (CLL).[16]

CLINICAL APPLICATION OF RITUXIMAB

The first phase I trial in humans with rituximab as a single agent was conducted in 1993 for patients with relapsed low-grade B-cell lymphoma.[35] Within five single-agent trials, no severe toxicities were found and only infusion-related adverse events occurred within the first hours, in particular after the first infusion. The most common side effects were chills, fever, nausea, fatigue, headache and angio-oedema.[36] Several phase II and III trials studied the optimal schedules and dosing with or without chemotherapy, biologicals, and radiotherapy.[36] After approval in the USA in 1997 and in Europe in 1998, rituximab was included in the standard treatment of NHL. Rituximab works very efficiently in combination with chemotherapy. For diffuse large B-cell lymphoma (DLBCL), follicular lymphoma and mantle cell lymphoma, inclusion of rituximab in standard chemotherapy regimens significantly improved patients outcome with or without pretreatment[37-46] and is accepted as a standard first-line therapy for CD20-positive lymphomas. Moreover, if patients with low-grade lymphoma respond to single-agent rituximab therapy, progression-free survival and overall survival are substantially prolonged with scheduled maintenance treatment.[47,48] In patients who achieved complete or partial remission after the combination of chemotherapy and rituximab, maintenance with rituximab increased the overall and progression-free survival.[46-49] In addition, rituximab maintenance in patients treated after standard chemotherapy significantly increased the three-year progression-free survival from 33 to 68%.[49,50]

The therapeutic effect of rituximab, through the depletion of B cells, has also proven to be successful for patients with B-cell related autoimmune diseases. Examples are rheumatoid arthritis, autoimmune thrombocytopenic purpura, inflammatory skin diseases and pemphigus, systemic lupus erythematosus and other forms of vasculitis, diabetes, neurological diseases such as chronic inflammatory demyelinating polyneuropathy (CIDP) and multiple sclerosis[51-53] and chronic graft versus host disease after allogeneic stem cell transplantation.[54-57]

RITUXIMAB RESISTANCE

However, despite the success story, resistance of lymphoma B cells towards rituximab is observed in about half of the patients in the course of prolonged treatment. The precise mechanism of resistance to rituximab is unknown.

Resistance may be tumour-related or host-related. Tumour-related resistance could be the lower number of CD20 molecules per cell, the increased expression of complement regulatory proteins or expression of antiapoptotic genes. Host-related resistance is determined by polymorphisms in the FcγRIIIA gene effector cells.[26,33,34] The cellular microenvironment probably contributes to the dominant effector and resistance mechanism of rituximab.[58] There is a difference in the extent of B cell depletion in peripheral blood, lymph nodes and spleen. Also, within the lymph node there is a differential susceptibility of different B-cell subsets to MoAb treatment.[58,59] In a human-CD20 transgenic mouse model, Gong and colleagues demonstrated that circulating B cells are depleted mainly through the macrophages of the reticulo-endothelial system, while B cells within the marginal zone compartment in lymph nodes depend on CDC rather than FcγR-mediated depletion. In fact, marginal zone B cells that are trafficking from the marginal zone to the vasculature make them susceptible for depletion with MoAbs. B cells residing in the lymphoid tissues depend on the vasculature for accessibility of effector cells.[58] In addition, in some lymph node compartments (germinal centres) B cells receive additional survival signals. Exposure to these signals makes these cells less sensitive to anti-CD20.[58,59] The significance of the microenvironment in rituximab-induced cell death is also indirectly observed by differential responses to rituximab therapy in different subtypes of CD20-positive lymphomas (which have unique microenvironments), and is furthermore supported by the observation that molecular remissions in the blood and bone marrow induced by rituximab can occur in the setting of progressive nodal disease. More knowledge on and/or manipulation of the microenvironment may lead to developing a means to decrease or overcome rituximab resistance.

Several attempts have been made to improve rituximab efficacy and thereby to overcome resistance. For example, down-regulation of the antiapoptotic bcl-2 gene by antisense oligonucleotides may enhance the apoptotic effect of rituximab.[60] Other attempts were made to improve ADCC by immunostimulatory molecules such as IL-2, IL12, IL15 or CpG sequences.[61-63] or improving CDC by down-regulation of complement regulatory proteins, but with limited success.[20,22,23]

More promising is the next generation of monoclonal anti-CD20 antibodies (*figure 4*). In recent years, different murine, humanised and completely human anti-CD20 MoAbs have been developed (for nomenclature see *table 1*). These antibodies may bind to a different epitope or induce a specific mechanism of action. Another way to classify these antibodies is the ability to translocate CD20 into the lipid rafts. Anti-CD20 antibodies are either type I or type II (see also *table 2*).
Type I antibodies relocate CD20 molecules into lipid microdomains, which can act as signalling platforms. These antibodies are potent CDC inducers. Rituximab belongs to the type I antibodies. Type II antibodies do not redistribute CD20 into signalling platforms and do not induce CDC. However, type II antibodies promote strong homotypic adhesion and have a strong induction of direct cell death.

Table 1. *Nomenclature of therapeutic monoclonal antibodies*

Suffix to generic name	Origin
-omab	Murine
-amab	Rat
-emab	Hamster
-imab	Primate
-ximab	Chimeric
-zumab	Humanised
-umab	Human

Table 3 gives an overview of new anti-CD20 MoAbs in comparison with rituximab. They are summarised below.

HUMAN ANTIBODY (TYPE I)

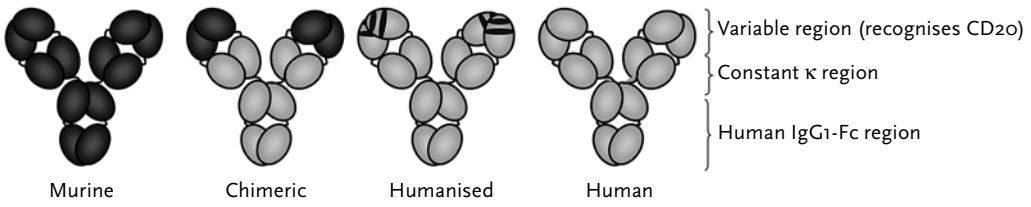
Ofatumumab

Ofatumumab is a completely human anti-CD20 antibody. Ofatumumab, a type I MoAb, is generated in human immunoglobulin transgenic mice. Compared with rituximab, it binds a different epitope on the CD20 molecule and has a slower off rate. Ofatumumab binds the small 7-mer loop of the human CD20 molecule, which is in a closer proximity to the cell membrane than the binding site of rituximab, which binds the larger 44-mer loop. This is probably the most important reason why ofatumumab is more potent than rituximab in inducing complement.[10,64] First clinical data with ofatumumab showed safe application and responses to therapy in

Table 2. *Differences between type I and II anti-CD20 monoclonal antibodies*

Type I MoAbs	Type II MoAbs
Localise CD20 to lipid rafts	Do not localise CD20 to lipid rafts
High CDC	Low CDC
ADCC activity	ADCC activity
Full number of binding sites/B-cell	Half number of binding sites/B-cell
Weak homotypic aggregation	Strong homotypic aggregation
Weak direct cell death induction	Strong direct cell death induction
Examples:	*Examples:*
Rituximab	GA101
Ocrelizumab	B1 (Tositumomab)
Ofatumumab	
Veltuzumab	
AME-133	
PRO131921	

Figure 4. *Development of monoclonal antibodies recognising CD20*



Murine anti-CD20 mAbs are generated by immunisation of mice with CD20-positive cells. In chimeric antibodies, the 30% that is of murine origin is the variable region that recognises the CD20 antigen. The variable regions are cloned into a chimeric antibody expression vector, resulting in an antibody which contains the constant κ region and the IgG1-Fc region of human origin. For humanised antibodies, also with cloning techniques, the variable region is modified to be more human. Humanised antibodies contain complementary-determining regions of murine origin, which recognise the CD20 antigen. Only 10% of the antibody is of murine origin. Human anti-CD20 mAbs are derived from human immunoglobulin transgenic mice. The latter antibodies are likely to be non-immunogenic in men.

Table 3. *Second- and third-generation anti-CD20 antibodies*

Antibody	Antibody specificity			Activity (compared with rituximab)			Additional features (compared with rituximab)	Clinical trials (www.clinicaltrials.gov)	References
	Type	Isotype	CDR	CDC	ADCC	Apoptosis			
Ofatumumab	I	IgG1	Human	+++	=	=	• Binds the small extracellular part of CD20 • Completely human • Slower off-rate	Phase I/II: RA, FL, CLL, WM, RRMS. Phase III: CLL, FL, DLBCL	10, 64-66
Ocrelizumab	I	IgG1	Humanised	=	=/+	=	Binds a different but overlapping epitope compared with rituximab	Phase I, II, III: RA Phase III: SLE Phase II: RRMS	59, 67, 68
PRO131921	I	IgG1	Humanised	=	++	=	Enhanced affinity for FcγRII	Phase I/II: CLL, NHL	69
Veltuzumab	I	IgG1	Humanised	=/+	=	=	Slower off-rate	Phase I/II: CLL, NHL, ITP	70-74
AME-133	I	IgG1	Humanised	=	+	=	Enhanced affinity for CD20	Phase I/II: NHL	75, 76
Tositumomab	II	IgG2A	Murine	-	=	++	Bound to radio-isotopes	Bound to radio-isotopes: NHL	9, 77, 79
GA-101	II	IgG1	Humanised	-	+++	+++	• High affinity for FcγRII • Strong induction of apoptosis	Phase I/II: NHL	79-81

CDR = complementary determining regions; IgG = immunoglobulin; CDC = complement-dependent cytotoxicity; ADCC = antibody-dependent cellular cytotoxicity; RA = rheumatoid arthritis; FL = follicular lymphoma; CLL = chronic lymphocytic leukaemia; RRMS = relapsing remitting multiple sclerosis; WM = Waldenstrom's macroglobulinaemia; DLBCL = diffuse large B cell lymphoma; sc = subcutaneous; SLE = systemic lupus erythematosus; ITP = idiopathic autoimmune thrombocytopenic purpura; NHL = non-Hodgkin's lymphoma.

rituximab-resistant patients.[65,66] Clinical responses to ofatumumab in a phase I/II trial are promising. In this trial in patients with follicular lymphoma, previously treated with rituximab, clinical responses with ofatumumab were up to 63% with a median time to progression of 32.8 months.[66] Ofatumumab is currently being used in different phase III trials.

HUMANISED ANTIBODIES (TYPE I)

Ocrelizumab (PRO70769 or rhuH27)

Ocrelizumab is derived from the murine 2H7 anti-CD20 antibody and humanised with recombination techniques. Ocrelizumab is a type I MoAb and has an IgG1 isotype. Compared with rituximab, ocrelizumab binds a different, but overlapping epitope on the large extracellular part of CD20 and shows a two to fivefold increased ADCC and three to fivefold decreased CDC, which might lessen infusion-related reactions.[67] In a phase I/II study, ocrelizumab was administered to rituximab-pretreated patients with relapsed/refractory follicular NHL. Ocrelizumab was well tolerated and showed a response rate of 36%.[67]

In cynomolgus monkeys ocrelizumab was shown to have the same B-cell depleting capability as rituximab.[59] In the ACTION study group, ocrelizumab in combination with methotrexate was studied in a phase I/II trial in the treatment of RA. Over a 72-week follow-up ocrelizumab appeared to be safe with minimal immunogenicity and longer duration of the B-cell depletion.[68] Currently, ocrelizumab is undergoing phase III clinical trials for RA and lupus nephritis, and phase II trials for multiple sclerosis.

Modification of ocrelizumab resulted in an antibody with improved binding to FCγRIIIa and possibly a better ADCC. This version of ocrelizumab, called PRO131921, is studied in a phase I/II trial in the treatment of relapsed or refractory CLL and indolent NHL.[69]

Veltuzumab (hA20, IMMU-106)

Veltuzumab is a type I, humanised IgG1 MoAb generated by using the same human framework as epratuzumab (humanised anti-CD22). The complementary determining regions (CDR) were taken from the parental murine A20. Compared with rituximab there is a single amino acid difference in CDR3-V_H. For this reason, veltuzumab has a slower off rate and improved *in vivo* activity.[70] *In vitro*, the three main mechanisms of action are similar to rituximab.[71] The first clinical studies have shown favourable safety and efficacy results in NHL patients with lower doses and less administrations of antibody.[72-74] Overall response rate in rituximab-pretreated patient with refractory or relapsed NHL was 44%.[74] In a phase I/II study, subcutaneous administration of veltuzumab in NHL and CLL is being studied and also a phase I study is ongoing for the treatment of autoimmune thrombocytopenic purpura.

AME-133 (LY2469298)

The production of this antibody is based on the fact that there is a strong correlation between FcγRIII (CD16)

polymorphisms and MoAb efficacy.[75,76] AME-133 is a type I, humanised IgG1 MoAb. It consists of a human germline framework region in which CDRs were inserted. CDRs were synthesised using a mutagenesis procedure by targeted insertion of synthetic oligonucleotide pools and their selection is based on enhanced MoAb affinity for CD20. In addition, the Fc region was also modified by targeting the constant region with synthetic oligonucleotides. This resulted in an antibody with enhanced affinity for human FcγRIII and with an enhanced ADCC activity as compared with rituximab. The clinical efficacy of AME-133 is currently being studied in a phase I/II trial for the treatment of NHL. No clinical data are available yet.

MURINE ANTIBODY (TYPE II)

Tositumomab

Tositumomab (B1) is a murine IgG2a lambda MoAb. Ionising radiation therapy with covalently linked Iodine-131 to tositumomab is successfully used for the treatment of patients with follicular and transformed NHL who failed or relapsed from prior rituximab treatment and standard chemotherapy.[77] Without the conjugation of an ionising agent, tositumomab also has a direct toxic effect. *In vitro* data show that tositumomab is far more efficient in inducing apoptosis and murine models show that tositumomab can prolong the survival of mice injected with Daudi lymphoma cells, in the absence of complement.[9] In addition, preclinical studies demonstrate that tositumomab is more efficient in depleting B cells than rituximab.[78] In patients, the direct effect of tositumomab alone is not clear. It is administrated often as a predose before the isotope-labelled tositumomab. This pre-dose was shown to exert a tumour-reducing effect, but on the contrary slowed down the effect of tositumomab linked with Iodine-131.[77] These results suggest the need for humanised B1-like antibodies for CDC-independent treatment of B-cell malignancies.

HUMANISED ANTIBODY (TYPE II)

GA-101 (RO5072759).

GA-101 is a humanised type II anti-CD20 MoAb. GA-101 is generated by grafting CDR sequences of the B-ly1 anti-CD20 MoAb on framework regions of fully human IgG1-kappa germline sequences. Different elbow hinge sequences in the variable region were optimised for optimal induction of apoptosis. In addition, the Fc region has been glycoengineered, which results in a 50-fold higher affinity to human FcγRIII receptors.[79] In cynomolgus monkeys, GA101 was shown to have a superior efficacy for B-cell depletion in the tissues as compared with rituximab.[80] Currently ongoing phase I and II clinical studies will demonstrate the efficacy of GA-101 and its unique property to enhance ADCC and apoptosis of B cells. The first clinical data in a rituximab-pretreated patient population showed a favourable toxicity profile and an overall response rate of 58%.[81]

DISCUSSION

Although CD20-targeted therapy with rituximab has greatly enhanced the outcome of patients with B-cell malignancies, resistance to rituximab is still a major problem, resulting in non-response and early relapse of disease (*figure 4*). Second- and third-generation anti-CD20 MoAbs have been developed to overcome resistance to rituximab. To assess the additional value of new antibodies, two approaches are recognised, i.e. to show superior efficacy if compared head-to-head with rituximab or to yield significant responses in rituximab-refractory NHL patients. Resistance is determined by a complex combination of the three mechanisms of action of rituximab (CDC, ADCC and apoptosis) and a patient-specific microenvironment of the lymphoma. B-cell depletion studies in monkeys and mice have also demonstrated that distinct subtypes of B cells in the lymph nodes exert different mechanisms of cell-specific resistance.[58,59] Therefore, the combination of each patient and each lymphoma subtype may have its unique mechanism of resistance. Understanding all these factors that contribute to resistance may eventually lead to an individual-patient-based anti-CD20 therapy.

REFERENCES

1. Stashenko P, Nadler LM, Hardy R, Schlossman SF. Characterization of a human B lymphocyte-specific antigen. J Immunol. 1980;125(4):1678-85.
2. Stashenko P, Nadler LM, Hardy R, Schlossman SF. Expression of cell surface markers after human B lymphocyte activation. Proc Natl Acad Sci U S A. 1981;78(6):3848-52.
3. Nadler LM, Korsmeyer SJ, Anderson KC, et al. B cell origin of non-T cell acute lymphoblastic leukemia. A model for discrete stages of neoplastic and normal pre-B cell differentiation. J Clin Invest. 1984;74(2):332-40.
4. Anderson KC, Bates MP, Slaughenhoupt BL, Pinkus GS, Schlossman SF, Nadler LM. Expression of human B cell-associated antigens on leukemias and lymphomas: a model of human B cell differentiation. Blood. 1984;63(6):1424-33.
5. Einfeld DA, Brown JP, Valentine MA, Clark EA, Ledbetter JA. Molecular cloning of the human B cell CD20 receptor predicts a hydrophobic protein with multiple transmembrane domains. EMBO J. 1988;7(3):711-7.
6. Press OW, Appelbaum F, Ledbetter JA, et al. Monoclonal antibody 1F5 (anti-CD20) serotherapy of human B cell lymphomas. Blood. 1987;69(2):584-91.
7. Deans JP, Li H, Polyak MJ. CD20-mediated apoptosis: signalling through lipid rafts. Immunology. 2002;107(2):176-82.
8. Polyak MJ, Deans JP. Alanine-170 and proline-172 are critical determinants for extracellular CD20 epitopes; heterogeneity in the fine specificity of CD20 monoclonal antibodies is defined by additional requirements imposed by both amino acid sequence and quaternary structure. Blood. 2002;99(9):3256-62.

9. Cragg MS, Glennie MJ. Antibody specificity controls in vivo effector mechanisms of anti-CD20 reagents. Blood. 2004;103(7):2738-43.

10. Teeling JL, Mackus WJ, Wiegman LJ, et al. The biological activity of human CD20 monoclonal antibodies is linked to unique epitopes on CD20. J Immunol. 2006;177(1):362-71.

11. Cartron G, Watier H, Golay J, Solal-Celigny P. From the bench to the bedside: ways to improve rituximab efficacy. Blood. 2004;104(9):2635-42.

12. Walshe CA, Beers SA, French RR, et al. Induction of cytosolic calcium flux by CD20 is dependent upon B Cell antigen receptor signaling. J Biol Chem. 2008;283(25):16971-84.

13. Reff ME, Carner K, Chambers KS, et al. Depletion of B cells in vivo by a chimeric mouse human monoclonal antibody to CD20. Blood. 1994;83(2):435-45.

14. Chan HT, Hughes D, French RR, et al. CD20-induced lymphoma cell death is independent of both caspases and its redistribution into triton X-100 insoluble membrane rafts. Cancer Res. 2003;63(17):5480-9.

15. Manches O, Lui G, Chaperot L, et al. In vitro mechanisms of action of rituximab on primary non-Hodgkin lymphomas. Blood. 2003;101(3):949-54.

16. Byrd JC, Kitada S, Flinn IW, et al. The mechanism of tumor cell clearance by rituximab in vivo in patients with B-cell chronic lymphocytic leukemia: evidence of caspase activation and apoptosis induction. Blood. 2002;99(3):1038-43.

17. Van der Kolk LE, Evers LM, Omene C, et al. CD20-induced B cell death can bypass mitochondria and caspase activation. Leukemia. 2002;16(9):1735-44.

18. Idusogie EE, Presta LG, Gazzano-Santoro H, et al. Mapping of the C1q binding site on rituxan, a chimeric antibody with a human IgG1 Fc. J Immunol. 2000;164(8):4178-84.

19. Cragg MS, Morgan SM, Chan HT, et al. Complement-mediated lysis by anti-CD20 mAb correlates with segregation into lipid rafts. Blood. 2003;101(3):1045-52.

20. Golay J, Zaffaroni L, Vaccari T, et al. Biologic response of B lymphoma cells to anti-CD20 monoclonal antibody rituximab in vitro: CD55 and CD59 regulate complement-mediated cell lysis. Blood. 2000;95(12):3900-8.

21. Weng WK, Levy R. Expression of complement inhibitors CD46, CD55, and CD59 on tumor cells does not predict clinical outcome after rituximab treatment in follicular non-Hodgkin lymphoma. Blood. 2001;98(5):1352-7.

22. Bellosillo B, Villamor N, Lopez-Guillermo A, et al. Complement-mediated cell death induced by rituximab in B-cell lymphoproliferative disorders is mediated in vitro by a caspase-independent mechanism involving the generation of reactive oxygen species. Blood. 2001;98(9):2771-7.

23. Golay J, Lazzari M, Facchinetti V, et al. CD20 levels determine the in vitro susceptibility to rituximab and complement of B-cell chronic lymphocytic leukemia: further regulation by CD55 and CD59. Blood. 2001;98(12):3383-9.

24. Harjunpaa A, Junnikkala S, Meri S. Rituximab (anti-CD20) therapy of B-cell lymphomas: direct complement killing is superior to cellular effector mechanisms. Scand J Immunol. 2000;51(6):634-41.

25. Dall'Ozzo S, Tartas S, Paintaud G, et al. Rituximab-dependent cytotoxicity by natural killer cells: influence of FCGR3A polymorphism on the concentration-effect relationship. Cancer Res. 2004;64(13):4664-9.

26. Cartron G, Dacheux L, Salles G, et al. Therapeutic activity of humanized anti-CD20 monoclonal antibody and polymorphism in IgG Fc receptor FcgammaRIIIa gene. Blood. 2002;99(3):754-8.

27. Uchida J, Hamaguchi Y, Oliver JA, et al. The innate mononuclear phagocyte network depletes B lymphocytes through Fc receptor-dependent mechanisms during anti-CD20 antibody immunotherapy. J Exp Med. 2004;199(12):1659-69.

28. Clynes RA, Towers TL, Presta LG, Ravetch JV. Inhibitory Fc receptors modulate in vivo cytoxicity against tumor targets. Nat Med. 2000;6(4):443-6.

29. Di Gaetano N, Cittera E, Nota R, et al. Complement activation determines the therapeutic activity of rituximab in vivo. J Immunol. 2003;171(3):1581-7.

30. Golay J, Cittera E, Di Gaetano N, et al. The role of complement in the therapeutic activity of rituximab in a murine B lymphoma model homing in lymph nodes. Haematologica. 2006;91(2):176-83.

31. Kennedy AD, Beum PV, Solga MD, et al. Rituximab infusion promotes rapid complement depletion and acute CD20 loss in chronic lymphocytic leukemia. J Immunol. 2004;172(5):3280-8.

32. Van der Kolk LE, Grillo-Lopez AJ, Baars JW, Hack CE, van Oers MH. Complement activation plays a key role in the side-effects of rituximab treatment. Br J Haematol. 2001;115(4):807-11.

33. Koene HR, Kleijer M, Algra J, Roos D, von dem Borne AE, de Haas M. Fc gammaRIIIa-158V/F polymorphism influences the binding of IgG by natural killer cell Fc gammaRIIIa, independently of the Fc gammaRIIIa-48L/R/H phenotype. Blood. 1997;90(3):1109-14.

34. Wu J, Edberg JC, Redecha PB, et al. A novel polymorphism of FcgammaRIIIa (CD16) alters receptor function and predisposes to autoimmune disease. J Clin Invest. 1997;100(5):1059-70.

35. Maloney DG, Liles TM, Czerwinski DK, et al. Phase I clinical trial using escalating single-dose infusion of chimeric anti-CD20 monoclonal antibody (IDEC-C2B8) in patients with recurrent B-cell lymphoma. Blood. 1994;84(8):2457-66.

36. Grillo-Lopez AJ, White CA, Varns C, et al. Overview of the clinical development of rituximab: first monoclonal antibody approved for the treatment of lymphoma. Semin Oncol. 1999;26(5 Suppl 14):66-73.

37. Feugier P, van Hoof A, Sebban C, et al. Long-term results of the R-CHOP study in the treatment of elderly patients with diffuse large B-cell lymphoma: a study by the Groupe d'Etude des Lymphomes de l'Adulte. J Clin Oncol. 2005;23(18):4117-26.

38. Forstpointner R, Dreyling M, Repp R, et al. The addition of rituximab to a combination of fludarabine, cyclophosphamide, mitoxantrone (FCM) significantly increases the response rate and prolongs survival as compared with FCM alone in patients with relapsed and refractory follicular and mantle cell lymphomas: results of a prospective randomized study of the German Low-Grade Lymphoma Study Group. Blood. 2004;104(10):3064-71.

39. Habermann TM, Weller EA, Morrison VA, et al. Rituximab-CHOP versus CHOP alone or with maintenance rituximab in older patients with diffuse large B-cell lymphoma. J Clin Oncol. 2006;24(19):3121-7.

40. Herold M, Haas A, Srock S, et al. Rituximab added to first-line mitoxantrone, chlorambucil, and prednisolone chemotherapy followed by interferon maintenance prolongs survival in patients with advanced follicular lymphoma: an East German Study Group Hematology and Oncology Study. J Clin Oncol. 2007;25(15):1986-92.

41. Hiddemann W, Kneba M, Dreyling M, et al. Frontline therapy with rituximab added to the combination of cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP) significantly improves the outcome for patients with advanced-stage follicular lymphoma compared with therapy with CHOP alone: results of a prospective randomized study of the German Low-Grade Lymphoma Study Group. Blood. 2005;106(12):3725-32.

42. Lenz G, Dreyling M, Hoster E, et al. Immunochemotherapy with rituximab and cyclophosphamide, doxorubicin, vincristine, and prednisone significantly improves response and time to treatment failure, but not long-term outcome in patients with previously untreated mantle cell lymphoma: results of a prospective randomized trial of the German Low Grade Lymphoma Study Group (GLSG). J Clin Oncol. 2005;23(9):1984-92.

43. Marcus R, Imrie K, Solal-Celigny P, et al. Phase III study of R-CVP compared with cyclophosphamide, vincristine, and prednisone alone in patients with previously untreated advanced follicular lymphoma. J Clin Oncol. 2008;26(28):4579-86.

44. Pfreundschuh M, Trumper L, Osterborg A, et al. CHOP-like chemotherapy plus rituximab versus CHOP-like chemotherapy alone in young patients with good-prognosis diffuse large-B-cell lymphoma: a randomised controlled trial by the MabThera International Trial (MInT) Group. Lancet Oncol. 2006;7(5):379-91.

45. Salles G, Mounier N, de Guibert S, et al. Rituximab combined with chemotherapy and interferon in follicular lymphoma patients: results of the GELA-GOELAMS FL2000 study. Blood. 2008;112(13):4824-31.

46. Van Oers MH, Klasa R, Marcus RE, et al. Rituximab maintenance improves clinical outcome of relapsed/resistant follicular non-Hodgkin lymphoma in patients both with and without rituximab during induction: results of a prospective randomized phase 3 intergroup trial. Blood. 2006;108(10):3295-301.

47. Hainsworth JD, Litchy S, Shaffer DW, Lackey VL, Grimaldi M, Greco FA. Maximizing therapeutic benefit of rituximab: maintenance therapy versus re-treatment at progression in patients with indolent non-Hodgkin's lymphoma--a randomized phase II trial of the Minnie Pearl Cancer Research Network. J Clin Oncol. 2005;23(6):1088-95.

48. Marcus R, Hagenbeek A. The therapeutic use of rituximab in non-Hodgkin's lymphoma. Eur J Haematol Suppl. 2007;(67):5-14.

49. Hagenbeek A. Maintenance or Eradication of Residual Disease in Indolent Lymphoma: Where Do We Stand? (Editorial) J Clin Oncol. 2009;27(10):1540-2.

50. Hochster H, Weller E, Gascoyne RD, et al. Maintenance Rituximab After Cyclophosphamide, Vincristine, and Prednisone Prolongs Progression-Free Survival in Advanced Indolent Lymphoma: Results of the Randomized Phase III ECOG1496 Study. J Clin Oncol. 2009;27(10):1607-14.

51. Browning JL. B cells move to centre stage: novel opportunities for autoimmune disease treatment. Nat Rev Drug Discov. 2006;5(7):564-76.

52. Taylor RP, Lindorfer MA. Drug insight: the mechanism of action of rituximab in autoimmune disease--the immune complex decoy hypothesis. Nat Clin Pract Rheumatol. 2007;3(2):86-95.

53. Edwards JC, Cambridge G. B-cell targeting in rheumatoid arthritis and other autoimmune diseases. Nat Rev Immunol. 2006;6(5):394-403.

54. Ratanatharathorn V, Ayash L, Reynolds C, et al. Treatment of chronic graft-versus-host disease with anti-CD20 chimeric monoclonal antibody. Biol Blood. Marrow Transplant 2003;9(8):505-11.

55. Canninga-van Dijk MR, van der Straaten HM, Fijnheer R, Sanders CJ, van den Tweel JG, Verdonck LF. Anti-CD20 monoclonal antibody treatment in 6 patients with therapy-refractory chronic graft-versus-host disease. Blood. 2004;104(8):2603-6.

56. Cutler C, Miklos D, Kim HT, et al. Rituximab for steroid-refractory chronic graft-versus-host disease. Blood. 2006;108(2):756-62.

57. Carella AM, Biasco S, Nati S, Congiu A, Lerma E. Rituximab is effective for extensive steroid-refractory chronic graft-vs.-host-disease. Leuk Lymphoma. 2007;48(3):623-4.

58. Gong Q, Ou Q, Ye S, et al. Importance of cellular microenvironment and circulatory dynamics in B cell immunotherapy. J Immunol. 2005;174(2):817-26.

59. Vugmeyster Y, Beyer J, Howell K, et al. Depletion of B cells by a humanized anti-CD20 antibody PRO70769 in Macaca fascicularis. J Immunother. 2005;28(3):212-9.

60. Jazirehi AR, Bonavida B. Cellular and molecular signal transduction pathways modulated by rituximab (rituxan, anti-CD20 mAb) in non-Hodgkin's lymphoma: implications in chemosensitization and therapeutic intervention. Oncogene. 2005;24(13):2121-43.

61. Askew D, Chu RS, Krieg AM, Harding CV. CpG DNA induces maturation of dendritic cells with distinct effects on nascent and recycling MHC-II antigen-processing mechanisms. J Immunol. 2000;165(12):6889-95.

62. Friedberg JW, Neuberg D, Gribben JG, et al. Combination immunotherapy with rituximab and interleukin 2 in patients with relapsed or refractory follicular non-Hodgkin's lymphoma. Br J Haematol. 2002;117(4):828-34.

63. Hacker G, Redecke V, Hacker H. Activation of the immune system by bacterial CpG-DNA. Immunology 2002;105(3):245-51.

64. Teeling JL, French RR, Cragg MS, et al. Characterization of new human CD20 monoclonal antibodies with potent cytolytic activity against non-Hodgkin lymphomas. Blood. 2004;104(6):1793-800.

65. Coiffier B, Lepretre S, Pedersen LM, et al. Safety and efficacy of ofatumumab, a fully human monoclonal anti-CD20 antibody, in patients with relapsed or refractory B-cell chronic lymphocytic leukemia: a phase 1-2 study. Blood. 2008;111(3):1094-100.

66. Hagenbeek A, Gadeberg O, Johnson P, et al. First clinical use of ofatumumab, a novel fully human anti-CD20 monoclonal antibody in relapsed or refractory follicular lymphoma: results of a phase I/II trial. Blood. 2008;111(12):5486-95.

67. Morschhauser F, Marlton P, Vitolo U, et al. Interim Results of a Phase I/II Study of Ocrelizumab, a New Humanised Anti-CD20 Antibody in Patients with Relapsed/Refractory Follicular Non-Hodgkin's Lymphoma. ASH Annual Meeting Abstracts 2007;110(11):645.

68. Genovese MC, Kaine JL, Lowenstein MB, et al. Ocrelizumab, a humanized anti-CD20 monoclonal antibody, in the treatment of patients with rheumatoid arthritis: A phase I/II randomized, blinded, placebo-controlled, dose-ranging study. Arthritis Rheum. 2008;58(9):2652-61.

69. Maloney DG. Follicular NHL: From antibodies and vaccines to graft-versus-lymphoma effects. Hematology Am Soc Hematol Educ Program. 2007. p.226-32.

70. Goldenberg DM, Rossi EA, Stein R, et al. Properties and structure-function relationships of veltuzumab (hA20), a humanized anti-CD20 monoclonal antibody. Blood. 2009;113(5):1062-70.

71. Stein R, Qu Z, Chen S, et al. Characterization of a new humanized anti-CD20 monoclonal antibody, IMMU-106, and Its use in combination with the humanized anti-CD22 antibody, epratuzumab, for the therapy of non-Hodgkin's lymphoma. Clin Cancer Res. 2004;10(8):2868-78.

72. Morschhauser F, Leonard JP, Coiffier B, et al. Initial safety and efficacy results of a second-generation humanized anti-CD20 antibody, IMMU-106 (hA20), in Non-Hodgkin's Lymphoma. ASH Annual Meeting Abstracts. 2005;106(11):2428.

73. Morshhauser F, Leonard JP, Coiffier B, et al. Phase I/II results of a second-generation humanized anti-CD20 antibody, IMMU-106 (hA20), in NHL. J Clin Oncol. (Meeting Abstracts). 2006;24(18 suppl):7530.

74. Morschhauser F, Leonard JP, Fayad L, et al. Low doses of humanized anti-CD20 antibody, IMMU-106 (hA20), in refractory or recurrent NHL: Phase I/II results. J Clin Oncol. (Meeting Abstracts) 2007;25(18 suppl):8032.

75. Weiner G, Bowles J, Link B, Campbell M, Wooldridge J, Breitmeyer J. Anti-CD20 monoclonal antibody (mAb) with enhanced affinity for CD16 activates NK cells at lower concentrations and more effectively than rituximab (R). ASH Annual Meeting Abstracts. 2005;106(11):348.

76. Bowles JA, Wang SY, Link BK, et al. Anti-CD20 monoclonal antibody with enhanced affinity for CD16 activates NK cells at lower concentrations and more effectively than rituximab. Blood. 2006;108(8):2648-54.

77. Sharkey RM, Press OW, Goldenberg DM. A re-examination of radioimmunotherapy in the treatment of non-Hodgkin lymphoma: Prospects for dual-targeted antibody/radioantibody therapy. Blood. 2009, Epub ahead of print.

78. Beers SA, Chan CH, James S, et al. Type II (tositumomab) anti-CD20 monoclonal antibody out performs type I (rituximab-like) reagents in B-cell depletion regardless of complement activation. Blood. 2008;112(10):4170-7.

79. Umana P, Moessner E, Bruenker P, et al. Novel 3rd Generation Humanized type II CD20 antibody with glycoengineered Fc and modified elbow hinge for enhanced ADCC and superior apoptosis induction. ASH Annual Meeting Abstracts. 2006;108(11):229.

80. Ferrara C, Brunker P, Suter T, Moser S, Puntener U, Umana P. Modulation of therapeutic antibody effector functions by glycosylation engineering: influence of Golgi enzyme localization domain and co-expression of heterologous beta1, 4-N-acetylglucosaminyltransferase III and Golgi alpha-mannosidase II. Biotechnol Bioeng. 2006;93(5):851-61.

81. Salles G, Morschhauser F, Cartron G, et al. A Phase I/II Study of RO5072759 (GA101) in Patients with Relapsed/Refractory CD20+ Malignant Disease. ASH Annual Meeting Abstracts. 2008;112(11):234.

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REVIEW

Investigating obscure gastrointestinal bleeding: capsule endoscopy or double balloon enteroscopy?

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ABSTRACT

The possibility to visualise the small bowel has dramatically improved with the introduction of capsule endoscopy (CE) and double balloon enteroscopy (DBE). CE and DBE have become standard practice in investigating suspected diseases of the small bowel. An important reason to perform small bowel investigations is obscure gastrointestinal bleeding. To investigate obscure gastrointestinal bleeding, some advocate performing CE while others recommend DBE. In this systematic review, we provide an overview of studies in which patients with obscure gastrointestinal bleeding underwent both CE and DBE. These data show that CE and DBE have comparable diagnostic yields in the evaluation of obscure gastrointestinal bleeding of 50 to 60%. Therapeutic interventions using DBE were performed in 11 to 57% of cases. In most studies, there was good concordance between the two procedures but both techniques can be falsely negative. Given its safety, patient tolerability and ability to view the entire small bowel, CE can be recommended as the first investigation for obscure gastrointestinal bleeding, if necessary, followed by DBE. Finally, we provide an algorithm with practical guidelines for the evaluation of obscure gastrointestinal bleeding.

KEYWORDS

Capsule endoscopy, double balloon endoscopy, double balloon enteroscopy, gastrointestinal bleeding

INTRODUCTION

Obscure gastrointestinal bleeding is defined as bleeding from the digestive tract that persists or recurs without an obvious aetiology after a normal oesophagogastroduodenoscopy and colonoscopy.[1] It can be categorised into overt and occult obscure gastrointestinal bleeding based on the presence or absence of clinically evident bleeding. Approximately 5% of patients presenting with gastrointestinal bleeding have no identified source on upper endoscopy and colonoscopy.[1] The cause of obscure gastrointestinal bleeding is usually a lesion located in the small bowel, but also includes lesions that were overlooked during conventional endoscopy, either because of intermittent bleeding or truly missed lesions. An often occurring dilemma in obscure gastrointestinal bleeding is whether to undertake invasive investigations or to take a conservative supportive approach (stopping NSAIDs, supplementing iron, or blood transfusion). The investigation of obscure gastrointestinal bleeding has been revolutionised by the introduction of capsule endoscopy (CE) and double balloon enteroscopy (DBE).[2] Until recently, erythrocyte scintigraphies and angiography were proposed for patients with obscure gastrointestinal bleeding and *active* bleeding, and repeat endoscopies, push-enteroscopy, enteroclysis and small bowel series were recommended in patients with obscure gastrointestinal bleeding and *occult* bleeding.[3] Over the last years, CE has proven to be superior to all of these diagnostic modalities in the evaluation of obscure gastrointestinal bleeding.[4-9] In addition, CE has a high negative predictive value. An important limitation of CE is the inability to obtain histology and to perform therapeutic interventions. A technique that has proven to be of complementary value is DBE. This method, introduced in 2001, is based on the combined use of a balloon-loaded enteroscope and a similarly balloon-loaded overtube.[10] Alternately inflating and deflating the balloons

and straightening the endoscope with the overtube achieves a stepwise progression of the enteroscope throughout the small bowel. DBE can be carried out through the antegrade (oral) or the retrograde (anal) route. With a combined antegrade and retrograde approach a complete small bowel examination can be achieved in up to 86% of patients.[11,12] Endoscopic interventions such as mucosal biopsy, argon plasma coagulation, polypectomy and balloon dilation can be performed. However, DBE is an invasive and time-consuming procedure and there is a considerable risk of complications such as pancreatitis or perforations, especially in therapeutic procedures.[13]

An important question for the clinician is how to proceed in the evaluation of obscure gastrointestinal bleeding after normal initial investigations. It is unclear how the new diagnostic and therapeutic strategies should be incorporated in our current armamentarium. Following a normal gastroduodenoscopy and colonoscopy, should the next step be CE or DBE? To answer this dilemma, we performed a systematic literature search on studies in which CE was compared with DBE in patients with obscure gastrointestinal bleeding.

METHODS

A systematic literature PubMed search was performed using the search terms 'capsule endoscopy' and 'double balloon enteroscopy', 'double balloon endoscopy' or 'push-and-pull enteroscopy'. Only articles in which patients with obscure gastrointestinal bleeding had undergone both techniques, and of whom information regarding the findings was provided, were included. Only full-text articles in the English language published between 2000 and 31 December 2008 were included. Reference lists of identified articles were reviewed.

RESULTS

Our results retrieved nine articles, which are summarised in *table 1*.[14-23] Seven of these articles were prospective studies. We also included two retrospective studies, because of their large number of patients. The number of patients in these studies varied between 13 and 74. Mean age of patients was around 60 years in almost all studies. Most studies included both patients with obscure-overt and obscure-occult gastrointestinal bleeding.

Technical characteristics

In all studies except one, DBE was performed following CE; Matsumoto *et al.* used the reverse order of procedures.[14] In one study, all patients underwent both an antegrade as well as a retrograde DBE procedure,[20] whereas in the other studies the DBE strategy varied. In three of these studies, the antegrade or retrograde approach of DBE was chosen based on the time a lesion was seen on CE in relation to the small-bowel transit time of the capsule.[18,19,23] Two studies chose the route of DBE depending on the findings of CE without providing further details[22] or depending on the medical history.[16] One study chose the antegrade route of DBE in all cases, followed by the alternate approach if considered necessary.[15] Only one study attempted complete small bowel examination with both antegrade and retrograde DBE in all patients.[20] In many studies, the decision to perform an additional DBE using the alternate route was made after considering several factors, including the results of the initial procedure, clinical indication and patient consent.[15,16,18,19] Only two studies had a single-blinded design, i.e. the endoscopist performing the DBE was unaware of the results of the CE.[16,21]

Diagnostic yields of CE and DBE

The diagnostic yields of CE and DBE for obscure gastrointestinal bleeding varied between 38 and 83%

Table 1. *Overview of studies on capsule endoscopy (CE) and double balloon enteroscopy (DBE) in obscure gastrointestinal bleeding*

Author (reference)	n	Age (mean)	Design	Overt/occult	Diagnostic yield CE (%)	Diagnostic yield DBE (%)	Concordance (%)
Matsumoto[14]	13	48	Prospective	13/0	38	46	92
Hadithi[15]	35	63	Prospective	22/13	80	60	74
Nakamura[16]	32	59	Prospective, single-blinded*	32/0	59	43	29
Ohmiya[17]	74	Data missing	Retrospective	Data missing	50	53	73
Kaffes[18]	60	62	Prospective	34/26	83	75	65
Fujimori[19]	45	60	Prospective	Data missing	40	50	89
Kamalaporn[20]	51	64	Prospective	14/37	Data missing	Data missing	Data missing
Kameda[21]	32	62	Prospective, single-blinded	26/6	72	66	50
Arakawa[22]	74	Data missing	Retrospective	Data missing	54	64	80

*The endoscopist performing the double-balloon enteroscopy was unaware of the result of the preceding.

for CE, and between 43 and 75% for DBE. Considering all studies together, the most frequent diagnosis was angiodysplasias, followed by tumours and ulcerations/erosions. The diagnostic yield was higher in overt gastrointestinal bleeding than in occult gastrointestinal bleeding.[22] The diagnostic yields of both DBE and video capsule endoscopy (VCE) for ongoing overt bleeding were significantly higher than those of previous overt and occult bleeding (87 *vs* 52% using DBE, and 88 *vs* 48% using CE).[22] Therapeutic interventions using DBE were performed in 11 to 57% of cases. These included electrocoagulation of angiodysplasias and radiation enteritis, applying haemo-clips in Dielafoy's lesions, endoscopic mucosal resections of polyps and balloon dilation of strictures.

Concordance between CE and DBE

In most studies, DBE confirmed the findings of CE in the majority of cases. The concordance between findings of CE with those of DBE varied between 29 and 92% (*table 1*). However, in almost every study, several lesions that were detected by DBE had been missed by CE, and vice versa. Lesions missed by CE but identified by DBE included angiodysplasias,[15,17,19,21] ulcers,[17,18,21] small bowel diverticula,[16,19,21] gastrointestinal stromal tumour (GIST),[17,19] malignant lymphoma,[19] leiomysarcoma,[19] enteric tuberculosis,[19] varices[17] and colorectal cancer.[16] In the largest series published so far, in 162 patients with obscure gastrointestinal bleeding, of whom 74 underwent both VCE and DBE, Arakawa found 11 DBE positive cases where VCE was normal.[22] These concerned cases of varices in a Roux-en-Y loop, varices elsewhere in the small bowel, angiodysplasias, a Dieulafoy's lesion, a GIST, a Meckel's diverticulum, a lipoma and a colon carcinoma.

Similarly, there were lesions detected by CE that were not confirmed at DBE. These included angiodysplasias,[15,17,19,21,22] GIST,[17,22] a submucosal tumour,[14,16] polyps,[15] erosions[21] and varices.[17]

Finally, in some studies, CE identified lesions that were within the reach of conventional endoscopy. These included cases of colorectal cancer,[22] duodenal varices,[22] gastric antral vascular ectasia,[19] colonic diverticula[19] and oesophageal varices.[19]

Completion rates and complications

The entire small intestine was more often visualised by CE compared with DBE in most studies.[14,16,17,21] In the study by Arakawa *et al.*, complete small bowel investigation was achieved by DBE and CE with similar success rates (70 and 68%, respectively).[22] However, in this study, total enteroscopy by DBE combining the oral and anal route was attempted in only about half of the cases.[22]

In all studies, complication rates were low. Capsule retention occurred in up to 5% of cases.[22] In most of these cases, the lodged capsule could be removed by DBE, thereby preventing surgery. Minor complications after DBE included abdominal pain, nausea, a painful throat or mucosal injury due to contact with the overtube.[17,19,21] In the largest study, major complications for DBE included pancreatitis (1.7%) and perforations (0.8%).[17] DBE was considered more painful than CE by patients.[15]

Finally, it must be noted that a small number of patients did not undergo DBE at all due to severe cardiopulmonary comorbidity or thrombocytopenia.[17,21]

Outcome and follow-up

All but two studies included a follow-up period. The mean duration of follow-up varied from 5 to 19 months.[15-19,21,22] Re-bleeding rates were calculated in most of these studies and varied from 5 to 20%.

In the largest series published to date, none of the cases with normal findings on CE and/or DBE experienced re-bleeding.[22] Contradictory results were obtained by Fujimori *et al.*, who found that the re-bleeding rate was 5% in patients with positive diagnoses on CE and/or DBE, and 12% in normal cases.[19]

Small-bowel vascular lesions seem to be more prone to re-bleeding than small-bowel nonvascular diseases.[22] This has also been observed by others.[17] The presence of comorbidity, especially portal hypertensive disease and chronic renal failure and severe anaemia at presentation were factors associated with an increased risk of re-bleeding.[22]

After combined use of CE and DBE, blood transfusions were needed during the follow-up period in 0 to 20% of cases.[22] In one study, the number of patients requiring blood transfusions decreased from 57% before small bowel investigations to 17% after combining CE and DBE.[18]

DISCUSSION

In this paper, we reviewed the use of CE and DBE in the evaluation of obscure gastrointestinal bleeding. We found a wide variety in reported diagnostic yields of CE and DBE, which can be explained by several factors. First, different definitions of diagnostic yield existed. In some studies, every abnormality detected by CE or DBE was included in the diagnostic yield, whereas in others only possible sources of bleeding were considered diagnostic. In addition, the timing of CE has shown to be of importance. The yield of CE in patients with obscure gastrointestinal bleeding was 91% if performed within two weeks after the initial bleeding, compared with 34% in patients undergoing CE thereafter.[23,24] Next, several studies indicate that the diagnostic yields of CE and DBE are higher in obscure-overt than in obscure-occult bleeding. Given that the proportion of patients with obscure-overt and obscure-occult bleeding differed between studies, this

may have contributed to the variety in reported diagnostic yields. It must also be realised that in some studies, DBE was performed with the combination of the antegrade and retrograde approach, whereas in others only one approach was chosen, leading to major differences in the proportion of complete small bowel examination with DBE between studies. However, taking all data together, the diagnostic yield seems comparable between CE and DBE for the evaluation of obscure gastrointestinal bleeding.

The major drawback of CE is the inability to obtain histological samples or perform therapeutic interventions. The role of DBE is a complementary, therapeutic one, providing endoscopic therapy of bleeding sites in the small bowel. The percentage of cases in which therapeutic interventions using DBE were performed, the therapeutic rate, varied between 11 and 57%. This variation may be due to different definitions of therapeutic procedures. In most studies, this was defined as the proportion of cases in which endoscopic intervention was performed. In other studies, establishing a histopathological diagnosis or marking tumours or diverticula for surgery were also considered therapeutic procedures.

Given the comparable diagnostic yield of CE and DBE in the evaluation of obscure gastrointestinal bleeding, how should these investigations be incorporated in clinical practice? CE is relatively patient-friendly, minimally invasive, safe and usually performed on an outpatient basis. DBE is relatively labour intensive, usually involves one endoscopist and two nurses, requires the use of sedation, is more painful and invasive for the patient and has a larger risk of complications compared with CE. With respect to the cost-effectiveness of these procedures, two studies are available.[25,26] Both reports used models comparing five different management strategies of obscure gastrointestinal bleeding including CE and DBE, although these strategies were not exactly similar in the two studies. Nevertheless, both reports indicate that DBE is the most cost-effective approach for the evaluation of obscure gastrointestinal bleeding.[25,26] However, in both papers it is suggested that CE-guided DBE may be associated with better long-term outcomes because of the potential for fewer complications and decreased utilisation of endoscopic resources. This concept of CE as a way to select patients for DBE, and helping to direct whether the oral or anal approach should be used, has been studied in clinical practice.[27-29] Such a strategy has shown to generate a diagnostic yield of DBE of up to 83%[29] and a therapeutic yield of up to 69%.[29] With this strategy, CE is used for the initial diagnosis and DBE for histopathological confirmation of the diagnosis and, if technically possible, endoscopic therapy. One could consider deviating from this strategy in emergent cases with massive bleeding, where DBE should be selected over CE, to prevent delay in endoscopic therapy.

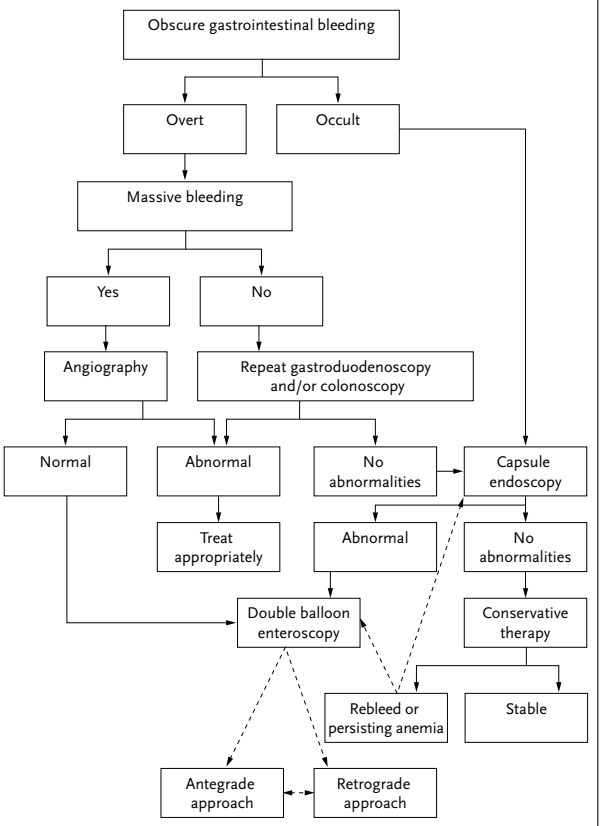
Another important question is how patients with a normal CE are best managed. Most studies indicate that re-bleeding rates and need for transfusions are low after normal CE.[30] Consensus emerges that patients with obscure-occult GI bleeding and a normal CE are probably best managed conservatively without further investigations.[31] Examples of conservative management are a 'wait and see' policy, cessation of NSAIDs, iron supplementation or blood transfusions. Nevertheless, if a patient has repeated overt bleeding and/or continues to be transfusion dependent, two options seem reasonable. One could repeat a CE procedure or perform a DBE. In a study in 24 patients with obscure gastrointestinal bleeding and a normal CE, repeat CE revealed abnormal findings in 75% of cases.[32] In another series of 20 patients, a second CE procedure revealed significant pathology in 35% of cases.[33] Alternatively, DBE could be performed after an initial normal CE. It seems reasonable to start with the antegrade approach, given that the depth of insertion is larger compared with the retrograde approach, and the fact that the majority of abnormalities are located in the proximal small bowel. Indeed, such an approach in four patients with obscure gastrointestinal bleeding and normal CE revealed a diagnosis in all cases.[34]

In *figure 1*, we propose an algorithm incorporating CE and DBE in the evaluation of obscure gastrointestinal bleeding. In patients with massive overt bleeding, a CT angiography or conventional angiography should be considered. Especially in patients with obscure-overt bleeding, we suggest repeating conventional endoscopies. As the next step, we recommend CE as the preferred diagnostic procedure in obscure gastrointestinal bleeding, based on its safety, patient tolerance and ability to view the entire small bowel. DBE should be considered in patients with a positive finding on CE requiring endoscopic evaluation and in cases with massive bleeding with a normal CT angiography. The route of insertion for DBE should be guided by the medical history or the findings of CE. If needed, the alternative route may be chosen. In patients in whom small intestinal bleeding is suspected despite a normal CE, for example those with unexplained refractory or recurring anaemia, a repeat CE procedure could be considered or, alternatively, a DBE.

CONCLUSION

The possibility of visualisation of the small bowel has dramatically improved with the introduction of CE and DBE and they have rapidly become standard practice in investigating diseases of the small bowel. The procedures can be considered complementary rather than competing techniques. In suspected small bowel bleeding, CE should be used for the initial diagnosis and, if necessary, DBE for histopathological confirmation of the diagnosis and, if technically possible, endoscopic therapy.

Figure 1. *Algorithm with guidelines on the use of capsule endoscopy and double balloon enteroscopy for the evaluation of obscure gastrointestinal bleeding*



Dotted lines indicate alternative strategies.

REFERENCES

1. Raju GS, Gerson L, Das A, Lewis B. American Gastroenterological Association. American Gastroenterological Association (AGA) Institute medical position statement on obscure gastrointestinal bleeding. Gastroenterology. 2007;133:1694-6.
2. Westerhof J, Koornstra JJ, Weersma RK. Capsule endoscopy: a review from the clinician's perspectives. Minerva Gastroenterol Dietol. 2008;54:189-207.
3. American Gastroenterological Association medical position statement. evaluation and management of occult and obscure gastrointestinal bleeding. Gastroenterology. 2000;118:197-201.
4. Triester SL, Leighton JA, Leontiadis GI, et al. A meta-analysis of the yield of capsule endoscopy compared with other diagnostic modalities in patients with obscure gastrointestinal bleeding. Am J Gastroenterol. 2005;100:2407-18.
5. De Leusse A, Vahedi K, Edery J, et al. Capsule endoscopy or push enteroscopy for first-line exploration of obscure gastrointestinal bleeding? Gastroenterology. 2007;132:855-62.
6. Saperas E, Dot J, Videla S, et al. Capsule endoscopy versus computed tomographic or standard angiography for the diagnosis of obscure gastrointestinal bleeding. Am J Gastroenterol. 2007;102:731-7.
7. Carey EJ, Leighton JA, Heigh RI, et al. A single-center experience of 260 consecutive patients undergoing capsule endoscopy for obscure gastrointestinal bleeding. Am J Gastroenterol. 2007;102:89-95.
8. Pennazio M, Santucci R, Rondonotti E, et al. Outcome of patients with obscure gastrointestinal bleeding after capsule endoscopy: report of 100 consecutive cases. Gastroenterology. 2004;126:643-53.
9. Ge ZZ, Chen HY, Gao YJ, Hu YB, Xiao SD. Best candidates for capsule endoscopy for obscure gastrointestinal bleeding. J Gastroenterol Hepatol. 2007;22:2076-80.
10. Yamamoto H, Sekine Y, Sato Y, et al. Total enteroscopy with a nonsurgical steerable double-balloon method. Gastrointest Endosc. 2001;53:216-20.
11. Heine GD, Hadithi M, Groenen MJ, Kuipers EJ, Jacobs MA, Mulder CJ. Double-balloon enteroscopy: indications, diagnostic yield, and complications in a series of 275 patients with suspected small-bowel disease. Endoscopy. 2006;38:42-8.
12. Zhong J, Ma T, Zhang C, et al. A retrospective study of the application on double-balloon enteroscopy in 378 patients with suspected small-bowel diseases. Endoscopy. 2007;39:208-15.
13. Mensink PB, Haringsma J, Kucharzik T, et al. Complications of double balloon enteroscopy: a multicenter survey. Endoscopy. 2007;39:613-5.
14. Matsumoto T, Esaki M, Moriyama T, Nakamura S, Iida M. Comparison of capsule endoscopy and enteroscopy with the double-balloon method in patients with obscure bleeding and polyposis. Endoscopy. 2005;37:827-32.
15. Hadithi M, Heine GD, Jacobs MA, van Bodegraven AA, Mulder CJ. A prospective study comparing video capsule endoscopy with double-balloon enteroscopy in patients with obscure gastrointestinal bleeding. Am J Gastroenterol. 2006;101:52-7.
16. Nakamura M, Niwa Y, Ohmiya N, et al. Preliminary comparison of capsule endoscopy and double-balloon enteroscopy in patients with suspected small-bowel bleeding. Endoscopy. 2006;38:59-66.
17. Ohmiya N, Yano T, Yamamoto H, et al. Diagnosis and treatment of obscure GI bleeding at double balloon endoscopy. Gastrointest Endosc. 2007;66(3 Suppl):S72-7.
18. Kaffes AJ, Siah C, Koo JH. Clinical outcomes after double-balloon enteroscopy in patients with obscure GI bleeding and a positive capsule endoscopy. Gastrointest Endosc. 2007;66:304-9.
19. Fujimori S, Seo T, Gudis K, et al. Diagnosis and treatment of obscure gastrointestinal bleeding using combined capsule endoscopy and double balloon endoscopy: 1-year follow-up study. Endoscopy 2007;39:1053-8.
20. Kameda N, Higuchi K, Shiba M, et al. A prospective, single-blind trial comparing wireless capsule endoscopy and double-balloon enteroscopy in patients with obscure gastrointestinal bleeding. J Gastroenterol. 2008;43:434-40.
21. Kamalaporn P, Cho S, Basset N, et al. Double-balloon enteroscopy following capsule endoscopy in the management of obscure gastrointestinal bleeding: outcome of a combined approach. Can J Gastroenterol. 2008;22:491-5.
22. Arakawa D, Ohmiya N, Nakamura M, et al. Outcome after enteroscopy for patients with obscure GI bleeding: diagnostic comparison between double-balloon endoscopy and videocapsule endoscopy. Gastrointest Endosc. 2009;69:866-74.
23. Bresci G, Parisi G, Bertoni M, Tumino E, Capria A. The role of video capsule endoscopy for evaluating obscure gastrointestinal bleeding: usefulness of early use. J Gastroenterol. 2005;40:256-9.
24. Hartmann D, Schmidt H, Bolz G, et al. A prospective two-center study comparing wireless capsule endoscopy with intraoperative enteroscopy in patients with obscure GI bleeding. Gastrointest Endosc. 2005;61:826-32.
25. Somsouk M, Gralnek IM, Inadomi JM. Management of obscure occult gastrointestinal bleeding: a cost-minimization analysis. Clin Gastroenterol Hepatol. 2008;6:661-70.
26. Gerson L, Kamal A. Cost-effectiveness analysis of management strategies for obscure GI bleeding. Gastrointest Endosc. 2008;68:920-36.
27. Gay G, Delvaux M, Fassler I. Outcome of capsule endoscopy in determining indication and route for push-and-pull enteroscopy. Endoscopy. 2006;38:49-58.
28. Leighton JA, Sharma VK, Hentz JG, et al. Capsule endoscopy versus push enteroscopy for evaluation of obscure gastrointestinal bleeding with 1-year outcomes. Dig Dis Sci. 2006;51:891-9.

29. Vilmann P, Jensen T, Hendel J. Double balloon endoscopy in obscure GI bleeding: the Danish experience. Gastrointest Endosc. 2007;66 (3 Suppl):S63-5.

30. Lai LH, Wong GL, Chow DK, Lau JY, Sung JJ, Leung WK. Long-term follow-up of patients with obscure gastrointestinal bleeding after negative capsule endoscopy. Am J Gastroenterol. 2006;101:1224-8.

31. Mergener K, Ponchon T, Gralnek I, et al. Literature review and recommendations for clinical application of small-bowel capsule endoscopy, based on a panel discussion by international experts. Consensus statements for small-bowel capsule endoscopy, 2006/2007. Endoscopy. 2007;39:895-909.

32. Jones BH, Fleischer DE, Sharma VK, et al. Yield of repeat wireless video capsule endoscopy in patients with obscure gastrointestinal bleeding. Am J Gastroenterol. 2005;100:1058-64.

33. Bar-Meir S, Eliakim R, Nadler M, et al. Second capsule endoscopy for patients with severe iron deficiency anemia. Gastrointest Endosc. 2004;60:711-3.

34. Chong AK, Chin BW, Meredith CG. Clinically significant small-bowel pathology identified by double-balloon enteroscopy but missed by capsule endoscopy. Gastrointest Endosc. 2006;64:445-9.

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REVIEW

Cell-derived microvesicles and cancer

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ABSTRACT

Blood and other body fluids contain cell-derived microvesicles. The presence of microvesicles in cancer patients was already noticed in the late 1970s. Since then, the prothrombotic state in cancer patients has invariably been associated with the presence of such microvesicles. More recently, a growing body of evidence supports an important contribution of microvesicles to cancer cell survival, invasiveness and metastases. Here, we will present an overview of the many contributions of microvesicles to cancer development and progression. In addition, their role in risk stratification and treatment of cancer patients is discussed.

KEYWORDS

Anti-cancer treatment, cancer, exosomes, microparticles, microvesicles

INTRODUCTION

Compared with healthy controls, blood from cancer patients contains elevated levels of cell-derived microvesicles. What are these microvesicles and why are their levels elevated in cancer patients?

Types of microvesicles

Human body fluids contain two different types of cell-derived microvesicles: microparticles and exosomes. Eukaryotic cells, including blood cells, endothelial cells and cancer cells, release *microparticles* by budding off parts of their outer cell membrane. Based on electron microscopy, microparticles range in size from 100 nm to 1.0 μm.[1] *Exosomes* arise from endosomes, which are initially formed by plasma membrane invagination. Endosomes release vesicles into their lumen, 'intraluminal vesicles'. Endosomes containing 'intraluminal vesicles' are called multivesicular bodies (MVBs). Finally, when MVB membranes fuse with the plasma membrane, these 'intraluminal vesicles' become secreted and are then called exosomes. Exosomes range in size from 30 to 100 nm and all cell types containing MVBs can be expected to secrete exosomes. Such cell types include haematopoietic cells, cancer cells, and epithelial cells.[2] At present, there is no generally accepted definition of microparticles and exosomes. Not only theoretical issues but especially methodological problems hamper the achievement of consensus. In this review, we will use the general term microvesicles.

General effects of microvesicles

Microvesicles are involved in many (patho) physiological processes in the human body (*table 1*). Membranes of microvesicles contain phospholipids and proteins that often originate from membrane lipid rafts of the parental cell, including functional transmembrane receptors such as tissue factor (TF). Furthermore, intracellular proteins, second messengers and genetic material can be enclosed and specifically sorted into microvesicles. As a consequence of sorting, the functional properties and biological role of microvesicles may differ from their parental cells.[3]

Microvesicles interact with cells by binding to cell-type specific adhesion receptors. After this initial interaction, membranes of microvesicles may fuse with the plasma membrane of the target cell, thereby transferring receptors that can induce cell signalling or even transformation, genetic information and second messengers.[4] Microvesicles are not only involved in intercellular communication, but also in other processes including regulation of programmed cell death, modulation of the immune response, inflammation, angiogenesis and coagulation.[5-7]

Table 1. *Effects of cancer cell derived microvesicles*

Effect	Role of microvesicles	References
Improves cellular survival	Removal apoptosis inducing proteins	5
	Chemotherapy resistance	17, 18
Escape immune surveillance	Complement resistance	20-22
	T-cell suppression	23-28
	Mimic environment	13,29,30
Environmental degradation	Transport active matrix metalloproteinases	31-34
Angiogenesis	Activation coagulation system: fibrin matrix formation and PAR signalling by thrombin formation	13,38,40
	Intercellular transfer mRNA coding for growth factors	45,46
Metastasising	Intercellular transfer oncogenes	47-49

PAR=protease activated receptor.

The release of microparticles is a physiological phenomenon. All sorts of biochemical triggers induce release of microvesicles, such as cytokines and chemotherapeutics, as do physical triggers such as hypoxia and shear stress. In diseases, aberrant levels of microvesicles are observed, and their numbers, cellular origin and composition are disease (state) dependent.

Microvesicles in cancer patients

The presence of microvesicles in cancer patients was already noticed in the late 1970s.[8] The underlying mechanism leading to the release of microvesicles from cancer cells, however, is still unknown. In a mouse model, the loss of the tumour suppressor gene p53 leads to an increased release of TF-bearing microvesicles, indicating involvement of p53 in this process.[9]

Blood from cancer patients contains not only microvesicles from cancer cells but especially high levels of procoagulant platelet-derived microvesicles. The procoagulant state of cancer patients has at least partly been attributed to these microvesicles.[10,11] Recent studies have shown that cancer patients with venous thromboembolism have higher levels of TF-bearing microvesicles compared with cancer patients without thrombosis.[12,13] In our opinion, this procoagulant phenotype of microvesicles is merely a side effect of a more important role they may have in cancer patients, i.e. by facilitating cancer progression. This review summarises the effects of cancer cell-derived microvesicles in cancer biology. Finally, the possible value of these vesicles in clinical practice will be discussed.

ROLE OF MICROVESICLES IN CANCER PROGRESSION

Cellular survival

Escape from apoptosis

Cells release microvesicles as a protective mechanism against *intracellular* stress. In nucleated mammalian cells, caspase 3 is one of the main executioner enzymes of apoptosis. Microvesicles containing substantial quantities of caspase 3 are present in conditioned medium of viable cell cultures,[14,15] but caspase 3 is not detectable within the cells from which these microvesicles originate. Various investigators have postulated that cells may escape from apoptosis by releasing caspase 3-containing microvesicles, thus preventing intracellular accumulation of the potentially dangerous caspase 3. Recently, this hypothesis was strengthened by the observation that cells indeed accumulate caspase 3 and undergo apoptosis when the release of microvesicles is inhibited.[5] Thus, the release of caspase 3-containing microvesicles contributes to cellular survival. In addition, caspase 3 itself is also involved in the release of microvesicles. MCF-7 cells, a human breast cancer cell line lacking caspase 3, do not release any or hardly any microvesicles. Their ability to release microvesicles, however, can be restored by transfection with functional caspase 3.[16] Since these microvesicles also contain caspase 3, it appears that caspase 3 contributes to its own removal (A.N. Böing, unpublished observation).

A second example, illustrating how the release of cancer cell-derived microvesicles contributes to cellular survival, comes from studies demonstrating an association between their release and multidrug resistance. Shedden and colleagues, who quantified membrane shedding-related gene expression, observed that chemo-insensitive cancer cell lines express more membrane shedding-related genes compared with chemo-sensitive cells. Furthermore, the microvesicles contained high levels of the chemotherapeutic agent doxorubicin.[17] The most convincing evidence comes from a study by Safeaei and colleagues, who demonstrated that microvesicles of cisplatin-insensitive cancer cells contained 2.6-fold more cisplatin than microvesicles released from the cisplatin-sensitive cells.[18]

Escape from immune surveillance

In a pioneering study published in 1988, Sims and co-workers showed that complement activation triggers the release of microvesicles. When human platelets were incubated with a lytic concentration of the complement C5b-9 complex,

platelets simply survived by releasing C5b-9-enriched microvesicles. This mechanism was called 'complement resistance', and this release can be considered a form of protection against *external* stress.[19] Similarly, cancer cells use the release of microvesicles to escape from complement-induced lysis.[20,21] A recent study showed that cancer cells can inactivate the complement complexes by shedding vesicles containing the complement inhibitor membrane cofactor protein CD46, which promotes inactivation of complement C4b and C3b. Liberation of CD46 minimalises inflammation in the microenvironment. Although a solid tumour is well protected from complement attacks, micro tumours are attacked by the complement system.[22]

A more indirect way to improve survival of cancer cells is by suppressing the immune response, i.e. via the release of microvesicles bearing immune modulatory molecules. Microvesicles from various cancer cells expose Fas ligand (FasL, CD95L), a ligand of the death receptor Fas (CD95), which induces T-cell apoptosis and diminishes the function of adaptive immune cells.[23,24] Kim and colleagues showed a modest correlation between lymph node infiltration and tumour burden and the numbers of circulating FasL-exposing microvesicles in blood from patients with oral squamous cell cancer.[25] Microvesicles from lymphoblastoma cells exposed latent membrane protein-1 (LMP-1), another immune suppressing transmembrane protein, thereby inhibiting leucocyte proliferation. This finding may explain the observed inhibition of T-cell proliferation in patients with Epstein-Barr virus associated tumours.[26,27] Microvesicles not only suppress effector T lymphocytes but also target antigen-presenting cells, the latter also known as dendritic cells. Valenti *et al.* showed that cancer cell-derived microvesicles are able to fuse with plasma membranes of monocytes, thereby impairing their differentiation to antigen-presenting cells.[28] Finally, cancer cells may hide from the immune system by mimicking the host environment. In a study by Tesselaar *et al.*, a low number of circulating microvesicles were present in cancer patients that stained for MUC1, a cancer cell antigen, and glycoprotein IIIa (integrin β_3), which is mainly present on platelets and platelet-derived microvesicles. Based on these data, they suggested that such microvesicles are released after fusion of microvesicles from malignant epithelial cells with platelets.[13] Alternatively, platelet-derived microvesicles were shown to transfer integrins to breast and lung cancer cells.[29,30] Thus, cancer cells can fuse with non-cancer cell-derived microvesicles, thereby receiving lipids and membrane-specific proteins which may help to escape from immune surveillance. *Figure 1A* summarises the effects of microvesicles on cellular survival.

Invasive growth and metastasising

Environmental degradation

Degradation of the extracellular matrix (ECM) is essential for tumour growth.[31] Microvesicles expose and contain proteases, including matrix metalloproteinase (MMP)-2 and MMP-9 and its zymogens, and urokinase-type plasminogen activator (uPA). MMPs degrade basement membrane collagens, whereas uPA catalyses the conversion of plasminogen into plasmin. Plasmin, a serine protease, degrades numerous components of the ECM, including fibrin, and activates MMP zymogens. Ginestra *et al.* analysed the content of microvesicles in ascites from 33 women with different gynaecological pathologies, including benign ovarian lesions, ovarian carcinomas, and endometrial carcinomas. They showed that ascites from the cancer patients contained higher numbers of microvesicles compared with ascites from women with benign disease. Microvesicles from patients with benign serous cysts had only minimal lytic activity, whereas those from cancer patient ascites contained active MMPs.[32] Similarly, the malignant potential of tumours was associated with the MMP-2 activity of microvesicles.[33] Graves *et al.*, who evaluated microvesicles in women with early-stage and late-stage ovarian carcinoma, reported increased numbers of microvesicles in late stage ascites and showed that MMP-2, MMP-9 and uPA activities are primarily concentrated within microvesicles. Inhibition of MMP-2, MMP-9 or uPA nearly abolished the ability of these microvesicles to support tumour invasiveness, which underlines the relevance of this pathway, at least *in vitro*.[34] The increased invasiveness of cancer cells by microvesicle formation is shown in *figure 1B*.

Angiogenesis

Fibrin, the insoluble end product of coagulation, plays an important role in tumour growth. Tumour cells can be coated with fibrin to escape from immune detection and attacks, and the fibrin matrix supports outgrowth of new blood vessels. One of the general effects of microvesicles is their support of coagulation.[35-37] Especially in cancer patients, TF-bearing microvesicles are present in the peripheral blood, albeit that the cellular origin of such microvesicles is still disputed.[38-40] A part of the TF-bearing microvesicles is likely to originate from cancer cells and probably contributes to thrombus formation equally to leucocyte-derived microvesicles, which may also expose procoagulant TF. TF-bearing microvesicles can be captured and trapped by activated platelets at the site of a wound, thereby delivering and accumulating their procoagulant TF at the site of vascular damage.[11,41,42] Furthermore, TF-bearing microvesicles may fuse with (membranes of) activated platelets, thereby transferring TF to the platelet membrane, which can then not only propagate but also initiate coagulation.[3] *Figure 1C* shows the contribution of microvesicles to fibrin formation.

The procoagulant effect of microvesicles also indirectly leads to the release of growth factors. Thrombin

activates cells via cleavage of protease-activated receptors (PARs), and this activation results in release of vascular endothelial growth factor (VEGF).[43,44] Finally, platelet-derived microvesicles stimulate mRNA expression of pro-angiogenic factors in cancer cells,[29] and cancer cell-derived microvesicles contain mRNA encoding growth factors such as VEGF and hepatocyte growth factor. Baj *et al.* showed that such vesicles fuse with monocytes, transferring their nucleic acids and inducing production of growth factors.[45,46] *Figure 1D* shows the influence of cancer cell-derived microvesicles on angiogenesis.

Metastasising

Cancer cell-derived microvesicles contribute to horizontal propagation of oncogenes and their associated transforming phenotype. Recently, Newadi *et al.* demonstrated the intercellular transfer of the truncated oncogenic form of the epidermal growth factor receptor (EGFRvIII) from glioma cancer cells to glioma cells lacking this receptor. After this transfer, the recipient cells became transformed and showed characteristic EGFRvIII-dependent changes in expression levels of target genes.[47] Although not studied yet, a similar intercellular transfer of other mutant oncogenes, such as MET and HER-2, may be a general mechanism operative in different tumour types which cause cancer growth at distant sites.

DNA-containing microvesicles from apoptotic cells ('apoptotic bodies') were shown to transfer DNA to other cells. In that study, apoptotic bodies from cancer cells triggered the expression of oncogenes in fibroblasts *in vitro*. After injecting these cells to SCID mice, tumours expressing the oncogene were observed. Thus, also the genetic information necessary for transformation and cells may by functionally transferred between cells by cancer cell-derived microvesicles.[48]

Skog *et al.* showed that glioblastoma cancer cells release microvesicles containing mRNA, microRNA and angiogenic growth factors. After transfer of vesicular RNA by fusion of the microvesicles with endothelial cells, the mRNA was translated into functional pro-angiogenic proteins thereby promoting angiogenesis. Cells with low levels of mRNAs produced microvesicles with high levels of mRNA in a constant distribution. This supports the hypothesis that the enrichment of microvesicles with mRNA and intracellular proteins is a selective process.[49]

Whether or not microvesicles promote mobilisation of tumour cells, however, has not been extensively studied. Lymphogenous spread could be enhanced by the immune-suppressive effects of cancer cell-derived microvesicles.[25] Activation of platelets by TF-bearing microvesicles is probably helpful in the haematological spread of cancer cells, since activated platelets expose the adhesion receptor P-selectin and cancer cells expose the corresponding P-selectin ligands, such as P-selectin glycoprotein (PSGL) and Sialyl Lewis. As a consequence, the cancer cells will be surrounded by platelets and/or P-selectin-bearing microvesicles, thus protecting cancer cells from immune surveillance and facilitating their binding to the vessel wall.[39,50] The procoagulant properties of cancer cell-derived microvesicles may further support intravascular fibrin formation, which will facilitate adherence of cancer cells to the vessel wall. *Figure 1E* presents the contribution of microvesicles to cancer cell migration.

FUTURE APPLICATIONS IN CANCER THERAPY

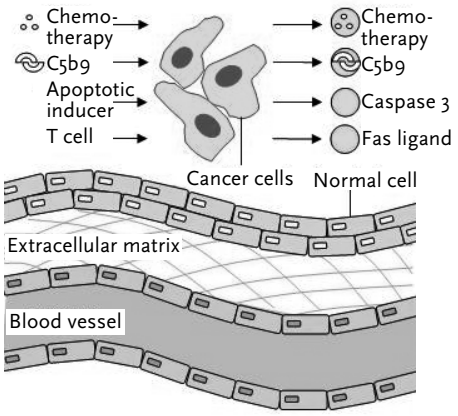
Anti-cancer treatment

Cancer cell-derived microvesicles have been used as adjuvant anti-cancer treatment. As described above, they have immunosuppressive activity due to functional alterations induced in T cells, ranging from apoptosis to defects in T-cell function.[23-25,51] However, cancer cell-derived microvesicles may also facilitate immune attacks.[2,52-59] Wolfers *et al.* showed that cancer cell-derived microvesicles transferred tumour antigens to antigen-presenting cells, which in turn triggered a T-cell-dependent anti-tumour response.[59] In addition, antigen-presenting cells were capable of producing microvesicles that primed cytotoxic T lymphocytes *in vivo* and even eradicated or suppressed growth of murine tumours. These autologous denditric cell-derived microvesicles have been tested in phase I clinical trials in patients with metastatic melanoma,[60] advanced non-small cell lung cancer[61] and colorectal cancer.[62] All studies concluded that this therapy is beneficial and safe, with some patients experiencing long-term stability of disease. Currently, several studies are ongoing to optimise this autologous anti-cancer immunotherapy.[57,63,64]

Release of microvesicles itself could be an interesting target of anti-cancer therapy, i.e. by counteracting the beneficial effects of vesicle release on cellular survival or tumour growth. Some currently used chemotherapeutics impair, at least partially, the underlying mechanisms of microvesicle release, e.g. drugs targeting at Rho-associated coiled coil-containing protein kinases (ROCK).[65] ROCK-I and II are both serine-threonine kinases which not only affect cell morphology, migration and adherence, but also markedly contribute to release of microvesicles.[15,66] Rattan and colleagues showed that inhibition of the Rho/Rock pathway resulted in smaller tumour mass in patients with glioblastioma.[65] Because the release of microvesicles by cancer cells influences many processes associated with tumour growth, inhibition of microvesicle release is a potential target in anti-cancer treatment.

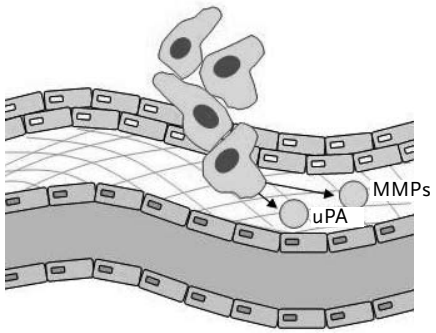
Figure 1. *The role of cancer cell derived microvesicles in cancer progression*

A) Cellular survival



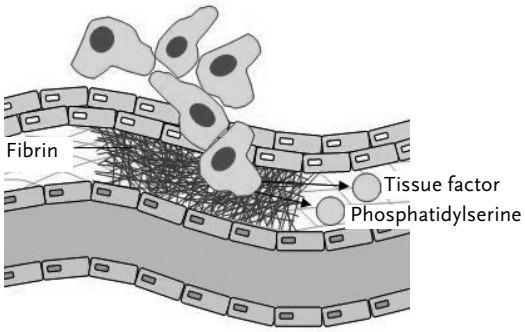
Cancer cells escape from internal (caspase 3) and external (chemotherapy, complement C5b9 complex, immune attack) stress by releasing microvesicles either containing (caspase 3, chemotherapy) or exposing C5b9 and Fas ligand. Thus, the release of microvesicles contributes to cellular survival.

B) Invasiveness



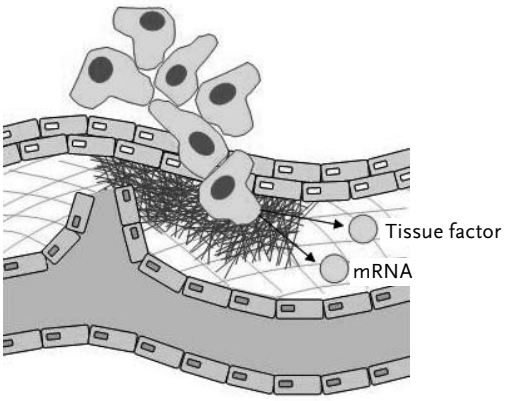
Microvesicles expose and contain several proteases, including matrix metalloproteinase (MMP)-2 and MMP-9 and its zymogens, and urokinase-type plasminogen activator (uPA). By degrading the extra cellular matrix, these enzymes facilitate cancer invasiveness.

C) Fibrin formation



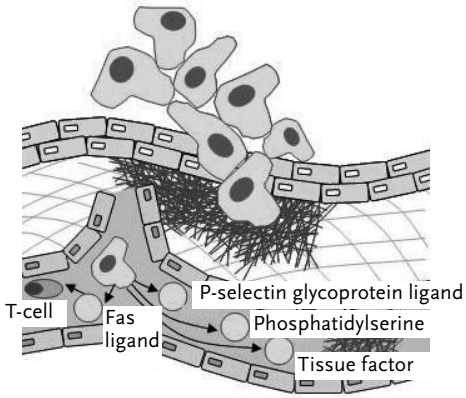
The membrane of microvesicles facilitates and initiates intravascular coagulation by exposing phosphatidylserine and tissue factor, respectively. Fibrin protects the tumour against immune attacks and forms a matrix to support angiogenesis.

D) Angiogenesis



Cancer cells induce angiogenesis by releasing microvesicles containing mRNA encoding growth factors and by exposure of tissue factor. Tissue factor not only initiates coagulation, as shown in figure 1B, but also plays a critical role in angiogenesis. Activation of the cytoplasmatic tail of tissue factor and subsequent downstream signalling events induce angiogenesis. Furthermore, thrombin, the final enzyme of the coagulation cascade, cleaves several protease-activated receptors, which in turn trigger angiogenesis.

E) Metastasising



Fas ligand exposing microvesicles enhance lymph node infiltration by killing T cells. Procoagulant microvesicles facilitate intravascular fibrin formation, thus enhancing haematological spread. P-selectin glycoprotein ligand-1 bearing cancer cell-derived microvesicles contribute to clot formation by binding to P-selectin-exposing (activated) platelets.

Measurement of protein composition of microvesicles may be useful to monitor the efficacy of anti-cancer treatment. Clayton *et al.* exposed B-lymphoblastoid cell lines to external stress, i.e. 42 °C for three hours.[67] Although the number of released microvesicles was comparable with control cells, the protein composition was markedly different. Stressed cells produced microvesicles containing relatively high quantities of heat shock proteins. Since heat shock proteins form complexes with proteins containing one or more production errors, their increased presence within microvesicles could help to maintain cellular homeostasis. Thus, possibly also the protein composition of cancer-cell derived microvesicles may directly reflect the effects of anti-cancer treatment, and could be an early and noninvasive biomarker to assess the effectiveness of anti-cancer therapy.

Risk stratification

Diagnosis

Tumour-specific markers, such as mucin in adenocarcinomas, exposed on circulating microvesicles, may be useful in the early detection of cancer. In a pilot study by Smalley *et al.*, microvesicles were isolated from urine of healthy individuals and patients with bladder cancer. Eight proteins were found to be elevated in microvesicles from cancer patients compared with controls.[68] Thus, the protein composition of such microvesicles can potentially be used in the early detection of bladder cancer. Similarly, cancer-specific mRNA can be used as a marker for detection of cancer. In the study by Skog *et al.*, microvesicles were purified from serum samples from glioblastoma patients. A glioblastoma-specific mutation was observed in almost 50% of the samples, which was comparable to the percentage of this mutant in glioblastoma patients.[49] Cancer-specific microRNA was also observed in exosomes purified from plasma samples of patients with ovarian cancer. No differences in microRNA profiles were observed between early and advanced diseased patients whereas patients with benign ovarian disease and healthy women did not express these microRNA profiles. Therefore, the authors suggest that microRNA profiles could be used in patients with a high risk for ovarian cancer.[69]

Prognosis

Different studies have evaluated the association between the level of microvesicles and survival of cancer patients. In the study by Tesselaar and colleagues, patients with both high microvesicle-associated procoagulant TF activity and epithelial mucin (MUC1) had a lower survival rate at three to nine months follow-up compared with patients with low TF activity and no MUC1 expression. After adjustment for other prognostic factors, the likelihood of survival for an individual with both membrane proteins present on circulating microvesicles was 0.42 (95% CI: 0.19 to 0.94).[13] In a prospective, nonrandomised single-centre study in hormone refractory prostate cancer patients, the impact of platelet-derived microvesicles on overall survival was assessed in 43 patients before starting chemotherapy. The overall survival was significantly shorter in patients with platelet-derived microvesicles above a certain cut-off level than in patients with values below that level.[70] Kim *et al.* performed a study in 109 patients with gastric cancer and in 29 healthy controls. Plasma levels of platelet-derived microvesicles were significantly higher in the patients than in controls, and the levels were significantly higher in patients with stage IV disease than those in patients with stage I or stage II/III without a significant difference in platelet number. Platelet-derived microvesicles predicted distant metastasis with a sensitivity and specificity of 93.3 and 91.1%, respectively.[71] Thus, microvesicles may be used as a predictor of disease stage and survival in cancer patients.

Another potential application of microvesicles, especially those bearing TF, is the prediction of venous thromboembolism.[12,13,72] Although cancer patients have four to fivefold higher risk to develop venous thromboembolism, there are currently no clinical or laboratory criteria to decide which patients warrant primary thromboprophylaxis.[73,74] Ongoing studies are evaluating the potential of (tissue factor bearing) microvesicles levels as a marker to decide about the appropriateness of primary thromboprophylaxis.

CONCLUSION

It is now generally accepted that cell-derived microvesicles are involved in (patho) physiological processes in humans. This review supports the concept that cancer cell-derived microvesicles play an important role in cancer biology. This field requires further investigation, and additional studies are needed to establish their potential relevance as novel biomarkers in the detection of cancer and their relevance as a new target in anti-cancer therapy.

REFERENCES

1. Piccin A, Murphy WG, Smith OP. Circulating microparticles: pathophysiology and clinical implications. Blood Rev. 2007;21(3):157-71.
2. Thery C, Zitvogel L, Amigorena S. Exosomes: composition, biogenesis and function. Nat Rev Immunol. 2002;2(8):569-79.
3. Del Conde I, Shrimpton CN, Thiagarajan P, Lopez JA. Tissue-factor-bearing microvesicles arise from lipid rafts and fuse with activated platelets to initiate coagulation. Blood. 2005;106(5):1604-11.
4. Hunter MP, Ismail N, Zhang X, et al. Detection of microRNA expression in human peripheral blood microvesicles. PLoS ONE. 2008;3(11):e3694.

5. Hussein MN, Boing AN, Sturk A, Hau CM, Nieuwland R. Inhibition of microparticle release triggers endothelial cell apoptosis and detachment. Thromb Haemost. 2007;98(5):1096-107.

6. Furie B, Furie BC. Mechanisms of thrombus formation. N Engl J Med. 2008;359(9):938-49.

7. Distler JH, Pisetsky DS, Huber LC, Kalden JR, Gay S, Distler O. Microparticles as regulators of inflammation: novel players of cellular crosstalk in the rheumatic diseases. Arthritis Rheum. 2005;52(11):3337-48.

8. Friend C, Marovitz W, Henie G, et al. Observations on cell lines derived from a patient with Hodgkin's disease. Cancer Res. 1978;38(8):2581-91.

9. Yu X, Riley T, Levine AJ. The regulation of the endosomal compartment by p53 the tumor suppressor gene. FEBS J. 2009;276(8):2201-12.

10. Callander N, Rapaport SI. Trousseau's syndrome. West J Med. 1993;158(4):364-71.

11. Furie B, Furie BC. Thrombus formation in vivo. J Clin Invest. 2005;115(12):3355-62.

12. Zwicker JI, Kos CA, Johnston KA, Liebman HA, Furie BC, Furie B. Cancer-associated thrombosis: tissue factor-bearing microparticles are associated with an increased risk of venous thromboembolic events in cancer patients. XXIst congress of the international society on thrombosis and haemostasis. Geneva. J Thromb Haemost. 2007;5(suppl 1):O-M-005.

13. Tesselaar ME, Romijn FP, van der Linden IK, Prins FA, Bertina RM, Osanto S. Microparticle-associated tissue factor activity: a link between cancer and thrombosis? J Thromb Haemost. 2007;5(3):520-7.

14. Boing AN, Hau CM, Sturk A, Nieuwland R. Platelet microparticles contain active caspase 3. Platelets. 2008;19(2):96-103.

15. Sapet C, Simoncini S, Loriod B, et al. Thrombin-induced endothelial microparticle generation: identification of a novel pathway involving ROCK-II activation by caspase-2. Blood. 2006;108(6):1868-76.

16. Janicke RU, Sprengart ML, Wati MR, Porter AG. Caspase-3 is required for DNA fragmentation and morphological changes associated with apoptosis. J Biol Chem. 1998;273(16):9357-60.

17. Shedden K, Xie XT, Chandaroy P, Chang YT, Rosania GR. Expulsion of small molecules in vesicles shed by cancer cells: association with gene expression and chemosensitivity profiles. Cancer Res. 2003;63(15):4331-7.

18. Safaei R, Larson BJ, Cheng TC, et al. Abnormal lysosomal trafficking and enhanced exosomal export of cisplatin in drug-resistant human ovarian carcinoma cells. Mol Cancer Ther. 2005;4(10):1595-604.

19. Sims PJ, Faioni EM, Wiedmer T, Shattil SJ. Complement proteins C5b-9 cause release of membrane vesicles from the platelet surface that are enriched in the membrane receptor for coagulation factor Va and express prothrombinase activity. J Biol Chem. 1988;263(34):18205-12.

20. Whitlow MB, Klein LM. Response of SCC-12F, a human squamous cell carcinoma cell line, to complement attack. J Invest Dermatol. 1997;109(1):39-45.

21. Carney DF, Hammer CH, Shin ML. Elimination of terminal complement complexes in the plasma membrane of nucleated cells: influence of extracellular Ca2+ and association with cellular Ca2+. J Immunol. 1986;137(1):263-70.

22. Hakulinen J, Junnikkala S, Sorsa T, Meri S. Complement inhibitor membrane cofactor protein (MCP; CD46) is constitutively shed from cancer cell membranes in vesicles and converted by a metalloproteinase to a functionally active soluble form. Eur J Immunol. 2004;34(9):2620-9.

23. Andreola G, Rivoltini L, Castelli C, et al. Induction of lymphocyte apoptosis by tumor cell secretion of FasL-bearing microvesicles. J Exp Med. 2002;195(10):1303-16.

24. Huber V, Fais S, Iero M, et al. Human colorectal cancer cells induce T-cell death through release of proapoptotic microvesicles: role in immune escape. Gastroenterology. 2005;128(7):1796-804.

25. Kim JW, Wieckowski E, Taylor DD, Reichert TE, Watkins S, Whiteside TL. Fas ligand-positive membranous vesicles isolated from sera of patients with oral cancer induce apoptosis of activated T lymphocytes. Clin Cancer Res. 2005;11(3):1010-20.

26. Keryer-Bibens C, Pioche-Durieu C, Villemant C, et al. Exosomes released by EBV-infected nasopharyngeal carcinoma cells convey the viral latent membrane protein 1 and the immunomodulatory protein galectin 9. BMC Cancer. 2006;6:283.

27. Flanagan J, Middeldorp J, Sculley T. Localization of the Epstein-Barr virus protein LMP 1 to exosomes. J Gen Virol. 2003;84(Pt 7):1871-9.

28. Valenti R, Huber V, Filipazzi P, et al. Human tumor-released microvesicles promote the differentiation of myeloid cells with transforming growth factor-beta-mediated suppressive activity on T lymphocytes. Cancer Res. 2006;66(18):9290-8.

29. Janowska-Wieczorek A, Wysoczynski M, Kijowski J, et al. Microvesicles derived from activated platelets induce metastasis and angiogenesis in lung cancer. Int J Cancer. 2005;113(5):752-60.

30. Janowska-Wieczorek A, Marquez-Curtis LA, Wysoczynski M, Ratajczak MZ. Enhancing effect of platelet-derived microvesicles on the invasive potential of breast cancer cells. Transfusion. 2006;46(7):1199-209.

31. Hotary KB, Allen ED, Brooks PC, Datta NS, Long MW, Weiss SJ. Membrane type I matrix metalloproteinase usurps tumor growth control imposed by the three-dimensional extracellular matrix. Cell. 2003;114(1):33-45.

32. Ginestra A, Miceli D, Dolo V, Romano FM, Vittorelli ML. Membrane vesicles in ovarian cancer fluids: a new potential marker. Anticancer Res. 1999;19(4C):3439-45.

33. Ginestra A, La P, Saladino F, Cassara D, Nagase H, Vittorelli ML. The amount and proteolytic content of vesicles shed by human cancer cell lines correlates with their in vitro invasiveness. Anticancer Res. 1998;18(5A):3433-7.

34. Graves LE, Ariztia EV, Navari JR, Matzel HJ, Stack MS, Fishman DA. Proinvasive properties of ovarian cancer ascites-derived membrane vesicles. Cancer Res. 2004;64(19):7045-9.

35. Berckmans RJ, Neiuwland R, Boing AN, Romijn FP, Hack CE, Sturk A. Cell-derived microparticles circulate in healthy humans and support low grade thrombin generation. Thromb Haemost. 2001;85(4):639-46.

36. Sinauridze EI, Kireev DA, Popenko NY, et al. Platelet microparticle membranes have 50- to 100-fold higher specific procoagulant activity than activated platelets. Thromb Haemost. 2007;97(3):425-34.

37. Gilbert GE, Sims PJ, Wiedmer T, Furie B, Furie BC, Shattil SJ. Platelet-derived microparticles express high affinity receptors for factor VIII. J Biol Chem. 1991;266(26):17261-8.

38. Rauch U, Antoniak S. Tissue factor-positive microparticles in blood associated with coagulopathy in cancer. Thromb Haemost. 2007;97(1):9-10.

39. Polgar J, Matuskova J, Wagner DD. The P-selectin, tissue factor, coagulation triad. J Thromb Haemost. 2005;3(8):1590-6.

40. Hron G, Kollars M, Weber H, et al. Tissue factor-positive microparticles: cellular origin and association with coagulation activation in patients with colorectal cancer. Thromb Haemost. 2007;97(1):119-23.

41. Satta N, Toti F, Feugeas O, et al. Monocyte vesiculation is a possible mechanism for dissemination of membrane-associated procoagulant activities and adhesion molecules after stimulation by lipopolysaccharide. J Immunol. 1994;153(7):3245-55.

42. Nieuwland R, Berckmans RJ, McGregor S, et al. Cellular origin and procoagulant properties of microparticles in meningococcal sepsis. Blood. 2000;95(3):930-5.

43. Rickles FR, Patierno S, Fernandez PM. Tissue factor, thrombin, and cancer. Chest. 2003;124(3 Suppl):S58-68.

44. Rak J, Milsom C, May L, Klement P, Yu J. Tissue factor in cancer and angiogenesis: the molecular link between genetic tumor progression, tumor neovascularization, and cancer coagulopathy. Semin Thromb Hemost. 2006;32(1):54-70.

45. Baj-Krzyworzeka M, Szatanek R, Weglarczyk K, et al. Tumour-derived microvesicles carry several surface determinants and mRNA of tumour cells and transfer some of these determinants to monocytes. Cancer Immunol Immunother. 2006;55(7):808-18.

46. Baj-Krzyworzeka M, Szatanek R, Weglarczyk K, Baran J, Zembala M. Tumour-derived microvesicles modulate biological activity of human monocytes. Immunol Lett. 2007;113(2):76-82.

47. Al-Nedawi K, Meehan B, Micallef J, et al. Intercellular transfer of the oncogenic receptor EGFRvIII by microvesicles derived from tumour cells. Nat Cell Biol. 2008;10(5):619-24.

48. Bergsmedh A, Szeles A, Henriksson M, et al. Horizontal transfer of oncogenes by uptake of apoptotic bodies. Proc Natl Acad Sci U S A. 2001;98(11):6407-11.

49. Skog J, Wurdinger T, van Rijn S, et al. Glioblastoma microvesicles transport RNA and proteins that promote tumour growth and provide diagnostic biomarkers. Nat Cell Biol. 2008;10(12):1470-6.

50. Falati S, Liu Q, Gross P, et al. Accumulation of tissue factor into developing thrombi in vivo is dependent upon microparticle P-selectin glycoprotein ligand 1 and platelet P-selectin. J Exp Med. 2003;197(11):1585-98.

51. Taylor DD, Gercel-Taylor C, Lyons KS, Stanson J, Whiteside TL. T-cell apoptosis and suppression of T-cell receptor/CD3-zeta by Fas ligand-containing membrane vesicles shed from ovarian tumors. Clin Cancer Res. 2003;9(14):5113-9.

52. Kovar M, Boyman O, Shen X, Hwang I, Kohler R, Sprent J. Direct stimulation of T cells by membrane vesicles from antigen-presenting cells. Proc Natl Acad Sci U S A. 2006;103(31):11671-6.

53. Hwang I, Shen X, Sprent J. Direct stimulation of naive T cells by membrane vesicles from antigen-presenting cells: distinct roles for CD54 and B7 molecules. Proc Natl Acad Sci U S A. 2003;100(11):6670-5.

54. Utsugi-Kobukai S, Fujimaki H, Hotta C, Nakazawa M, Minami M. MHC class I-mediated exogenous antigen presentation by exosomes secreted from immature and mature bone marrow derived dendritic cells. Immunol Lett. 2003;89(2-3):125-31.

55. Andre F, Chaput N, Schartz NE, et al. Exosomes as potent cell-free peptide-based vaccine. I. Dendritic cell-derived exosomes transfer functional MHC class I/peptide complexes to dendritic cells. J Immunol. 2004;172(4):2126-36.

56. Andre F, Schartz NE, Movassagh M, et al. Malignant effusions and immunogenic tumour-derived exosomes. Lancet. 2002;360(9329):295-305.

57. Chaput N, Schartz NE, Andre F, et al. Exosomes as potent cell-free peptide-based vaccine. II. Exosomes in CpG adjuvants efficiently prime naive Tc1 lymphocytes leading to tumor rejection. J Immunol. 2004;172(4):2137-46.

58. Zitvogel L, Regnault A, Lozier A, et al. Eradication of established murine tumors using a novel cell-free vaccine: dendritic cell-derived exosomes. Nat Med. 1998;4(5):594-600.

59. Wolfers J, Lozier A, Raposo G, et al. Tumor-derived exosomes are a source of shared tumor rejection antigens for CTL cross-priming. Nat Med. 2001;7(3):297-303.

60. Escudier B, Dorval T, Chaput N, et al. Vaccination of metastatic melanoma patients with autologous dendritic cell (DC) derived-exosomes: results of the first phase I clinical trial. J Transl Med. 2005;3(1):10.

61. Morse MA, Garst J, Osada T, et al. A phase I study of dexosome immunotherapy in patients with advanced non-small cell lung cancer. J Transl Med. 2005;3(1):9.

62. Dai S, Wei D, Wu Z, et al. Phase I clinical trial of autologous ascites-derived exosomes combined with GM-CSF for colorectal cancer. Mol Ther. 2008;16(4):782-90.

63. Chaput N, Flament C, Viaud S, et al. Dendritic cell derived-exosomes: biology and clinical implementations. J Leukoc Biol. 2006;80(3):471-8.

64. Delcayre A, Shu H, Le Pecq JB. Dendritic cell-derived exosomes in cancer immunotherapy: exploiting nature's antigen delivery pathway. Expert Rev Anticancer Ther. 2005;5(3):537-47.

65. Rattan R, Giri S, Singh AK, Singh I. Rho/ROCK pathway as a target of tumor therapy. J Neurosci Res. 2006;83(2):243-55.

66. Thornberry NA, Lazebnik Y. Caspases: enemies within. Science. 1998;281(5381):1312-6.

67. Clayton A, Turkes A, Navabi H, Mason MD, Tabi Z. Induction of heat shock proteins in B-cell exosomes. J Cell Sci. 2005;118(Pt 16):3631-8.

68. Smalley DM, Sheman NE, Nelson K, Theodorescu D. Isolation and identification of potential urinary microparticle biomarkers of bladder cancer. J Proteome Res. 2008;7(5):2088-96.

69. Taylor DD, Gercel-Taylor C. MicroRNA signatures of tumor-derived exosomes as diagnostic biomarkers of ovarian cancer. Gynecol Oncol. 2008;110(1):13-21.

70. Helley D, Banu E, Bouziane A, et al. Platelet microparticles: a potential predictive factor of survival in hormone-refractory prostate cancer patients treated with docetaxel-based chemotherapy. Eur Urol. 2009;55(4):28-35.

71. Kim HK, Song KS, Park YS, et al. Elevated levels of circulating platelet microparticles, VEGF, IL-6 and RANTES in patients with gastric cancer: possible role of a metastasis predictor. Eur J Cancer. 2003;39(2):184-91.

72. Rectenwald JE, Myers DD Jr, Hawley AE, et al. D-dimer, P-selectin, and microparticles: novel markers to predict deep venous thrombosis. A pilot study. Thromb Haemost. 2005;94(6):1312-7.

73. Heit JA, Silverstein MD, Mohr DN, Petterson TM, O'Fallon WM, Melton LJ, III. Risk factors for deep vein thrombosis and pulmonary embolism: a population-based case-control study. Arch Intern Med. 2000;160(6):809-15.

74. Khorana AA, Kuderer NM, Culakova E, Lyman GH, Francis CW. Development and validation of a predictive model for chemotherapy-associated thrombosis. Blood. 2008;111(10):4902-7.

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ORIGINAL ARTICLE

Evaluation of Endocrine Tests. D: the prolonged fasting test for insulinoma

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ABSTRACT

Objective: To establish the diagnostic performance of the prolonged fasting test in patients suspected of insulinoma.
Methods: We included all patients who were referred to our department between August 1995 and August 2006 with a clinical suspicion of insulinoma. Insulinoma was diagnosed by a positive Whipple's triad during the prolonged fast in combination with an insulin/C-peptide ratio below 1. The presence of insulinoma was confirmed by histopathological data, which was considered the golden standard. If the prolonged fast was negative, long-term follow-up was obtained.
Results: Ten patients had a positive Whipple's triad during the prolonged fast: eight had a histologically proven insulinoma, and two had factitious hypoglycaemia (insulin/C-peptide ratio >1.0) One additional patient likely had an insulinoma, but the Whipple's triad remained absent at up to 56 hours of fasting. Follow-up (median 53 months (3 to 142) in 76% of patients with a negative fasting test revealed no missed cases of insulinoma. During the prolonged fast the glucose, insulin and C-peptide concentrations overlapped in patients with and without insulinoma.
Conclusion: In our centre, the prolonged fasting test defined as a positive Whipple's triad in combination with an insulin/C-peptide ratio <1 had a sensitivity of 88.9% and a specificity of 100% for the diagnosis of insulinoma.

KEYWORDS

Insulinoma, prolonged fast, Whipple's triad

INTRODUCTION

In patients with a clinical suspicion of endogenous hyperinsulinism a prolonged fast is the recommended first test in the diagnostic work-up. The golden standard for a positive fasting test is the presence of a Whipple's triad and an insulin/C-peptide ratio below 1.[1] The Whipple's triad is positive if biochemical hypoglycaemia is accompanied by neuroglycopenic symptoms, with disappearance of these symptoms after correction of the hypoglycaemia. To differentiate between factitious hypoglycaemia and insulinoma the insulin/C-peptide ratio should be determined at the time point when Whipple's triad is positive. In the case of insulinoma, the ratio has been reported to be lower than 1.0.[1] To further substantiate endogenous hyperinsulinism the presence of sulphonylurea derivatives in serum should be excluded. Our aim was to report our experience with the prolonged fasting test in a group of patients with clinical suspicion of insulinoma referred to our hospital.

MATERIALS AND METHODS

Patients

We included all patients referred to our department between August 1995 and August 2006 because of clinical suspicion of insulinoma. They all were subjected to a supervised prolonged fasting test. When neuroglycopenic signs and symptoms occurred during the fast in the presence of hypoglycaemia (venous glucose below 2.8 mmol/l),[2] a blood sample was taken for determination of the insulin/C-peptide ratio and for the analysis of the presence of sulphonylurea derivatives. In patients meeting the Whipple's triad, factitious hypoglycaemia was diagnosed by an insulin/C-peptide ratio exceeding 1.0 or by the presence of sulphonylurea derivatives. In patients meeting the criteria of Whipple in whom factitious hypoglycaemia had been excluded, abdominal imaging was performed and a definitive diagnosis of insulinoma was sought by pancreas surgery. In patients not meeting the Whipple's triad during

the fast, insulinoma was excluded by extended follow-up. If our patients were no longer visiting our outpatient clinic, we contacted their general practitioners by telephone to look for evidence that insulinoma had been diagnosed during the follow-up. If no information could be obtained, the follow-up period was arbitrarily set at three months.

Prolonged fasting test

Patients were admitted to the Department of Internal Medicine of the Academic Medical Centre, Amsterdam. The first blood sample was withdrawn at midnight, marking the beginning of the prolonged fasting test. Patients were allowed to drink only calorie-free drinks. Blood samples were routinely taken at 8.00 am and 8.00 pm for assay of glucose, insulin and C-peptide. The fast was stopped after 80 hours. If neuroglycopenic symptoms occurred, a venous blood sample was drawn. If the glucose was below 2.8 mmol/l, 50 ml glucose 50% was administered intravenously and the response of the patient was recorded after 15 minutes.

Analytical methods

Insulin was assayed until March 2004 with a RIA (Pharmacia Diagnostics, Uppsala, Sweden) and thereafter on an Immulite system (Siemens Healthcare Diagnostics B.V., Breda, the Netherlands). Both assays had an inter-assay coefficient of variation of 7% at 60 pmol/l and 4.5% at 120 pmol/l and a detection limit of 15 pmol/l. There was no systematic difference in insulin measurements between both assays. Cross-reactivity of pro-insulin in the Pharmacia assay was 25% and on the Immulite system 8%, and there was no cross-reactivity with the C-peptide in either assay.

C-peptide was measured with a RIA (RIA-coat C-peptid, Byk Sangtec Diagnostica, Dietzenbach, Germany). The inter-assay coefficient of variation was 9% at 100 pmol/l and 7% at 500 pmol/l. From April 2004, C-peptide was determined with a RIA of Linco (St. Charles, USA). The inter-assay coefficient of variation was 6% at 100 pmol/l and 4% at 500 pmol/l. All results were transformed to the Linco assay (Linco = 0.8 x Byk-Sangtec). The detection limit was 50 pmol/l. Cross-reactivity of pro-insulin was <4% in the Linco assay and 78.5% in the Byk assay.

Glucose was measured using the hexokinase method on a Hitachi Modular P800 system (Roche Diagnostics, Almere, the Netherlands).

Statistical analysis

Values below the detection limit of the assays were included in the analyses as having a value of 50% of the detection limit. Normality was tested by the Kolmogorov-Smirnov test. Differences in demographic characteristics, glucose, insulin and C-peptide were evaluated using the Student t test and nonparametric tests where appropriate. P values of less than 0.05 were considered statistically significant.

All statistical analyses were performed using the Statistical Package of Social Sciences and Problem Solutions (SPSS version 16.0).

RESULTS

A total of 82 prolonged fasting tests were performed. We excluded one patient who underwent the prolonged fasting test after previous resection of an insulinoma. Among the remaining 81 patients, ten had a positive Whipple's triad: two patients had a factitious hypoglycaemia (insulin/C-peptide ratio >1.0; sulphonylurea derivatives in serum were absent), and eight had an insulinoma (insulin/C-peptide ratio <1.0) (*table 1*). Among the insulinoma patients, the Whipple's triad had occurred within 48 hours in all except one. In that patient neuroglycopenic symptoms developed after 67 hours of fasting; after 56 hours the glucose level was below 2.5 mmol/l, but the insulin (18 pmol/l) and C-peptide (220 pmol/l) were still not suppressed.

Table 1. *Time in hours of prolonged fast at which a positive Whipple's triad was observed*

Patient number	Time hours	Glucose mmol/l	Insulin pmol/l	C-peptide pmol/l	Insulin/C-peptide ratio	Localisation insulinoma
1	5	2.2	71	1150	0.06	Tail, 1.2 cm
2	17	2.2	304	1500	0.2	Tail, 1.9 cm
3	19	2.1	64	500	0.13	Tail, 1.2 cm
4	21.5	1.3	255	713	0.36	Head
5	34	1.9	105	712	0.15	Head, 1.2 cm
6	39	1.8	220	1048	0.21	Corpus/tail
7	44	2.2	80	432	0.19	Tail, 1.5 cm
8	67	1.9	17	190	0.09	Tail, 1.4 and 0.6 cm
10	32	2.8	420	40	10.5	-
11	64	2.7	280	40	7	-

Due to insulinoma in patients 1 to 8 and to factitious hyperinsulinaemia patients in 10 and 11; patient 9 is not mentioned because of a negative Whipple's triad.

Definitive proof of insulinoma in these eight patients was obtained from histopathology of the surgical specimen (*table 1*). One additional case of insulinoma was found in a patient (no. 9) in whom no neuroglycopenic symptoms occurred during the prolonged fast, which for unknown reasons was discontinued after 56 hours. During the fast the lowest measured glucose concentration was 1.9 mmol/l with an insulin level of 32 pmol/l and a C-peptide level of 460 pmol/l (insulin/C-peptide ratio 0.07). Because of repeated findings of low glucose and elevated insulin concentrations on follow-up, imaging studies were performed because of the persistent clinical suspicion of endogenous hyperinsulinaemia. Computed tomography and endoscopic ultrasonography did not show any abnormality of the pancreas. However, positron emission tomography (PET) with fluorine-18 L-3,4- dihydroxyphenylalanine (^{18}F-DOPA) revealed elevated uptake in the head of the pancreas. Surgery was declined by the patient, excluding any histopathological confirmation. We consider the result of the prolonged fast in this patient as a false-negative.

Follow-up was completed in 76% of all the patients with a negative Whipple's triad during the prolonged fast. Evidence had not emerged in any of these patients for the existence of an insulinoma during follow-up (median duration 53 months, range 3 to 142). One patient died due to ruptured aneurysm of the abdominal aorta. There was a tendency for insulinoma patients to be older, more often female, and to have a higher BMI than non-insulinoma patients (*table 2*).

Table 2. *Patient characteristics*

	Insulinoma	Non-insulinoma	Significance p value
N	9	72	
Age (years)	66 (28-83)	43(16-86)	0.06
Women (%)	66.7	59.7	0.69
BMI	25.4 (19.7-29.7)	22.6 (17.7-39.6)	0.29

Values are given as median and range

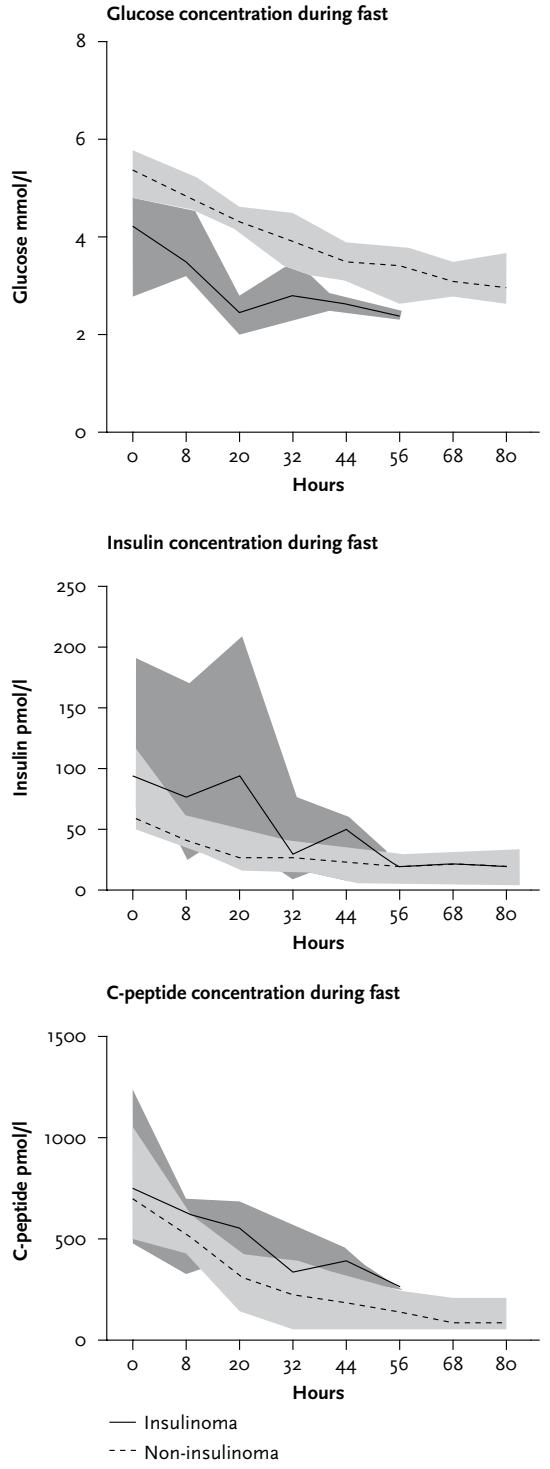
In our further analysis of venous glucose, insulin and C-peptide concentrations during the prolonged fast we excluded five patients in the non-insulinoma group, i.e., the two patients with factitious hypoglycaemia, and three patients because of missing values. Another two patients were analysed only until the time point they had obviously eaten something during the fast, as evident from elevated glucose, insulin and C-peptide levels at t=44 and t=68 hours, respectively. The time course of the biochemical parameters during the prolonged fast in the nine insulinoma and the 67 non-insulinoma patients is given in *table 3* and *figure 1*. There was a considerable overlap between insulinoma and non-insulinoma patients, although mean glucose levels were lower and insulin and C-peptide levels were higher in the insulinoma patients. Consequently, the prolonged fasting test defined as a positive Whipple's triad in combination

Table 3. *Glucose, insulin and C-peptide concentrations during a prolonged fast in insulinoma (I) (n=9) versus non-insulinoma (NI) patients (n=67)*

Hours of fasting	Glucose mmol/l		Insulin pmol/l		C-peptide pmol/l	
	I	NI	I	NI	I	NI
T=0	3.7	5.3	100	57	880	660
	2.9-5.7	4.9-5.7	76-144	41-105	519-1235	496-1048
p value	0.037		0.078		0.665	
T=8	3.5	5.0	66	40	560	488
	3.2-4.6	4.7-5.2	32-153	34.5-60	356-674	381-625
p value	0.001		0.133		0.572	
T=20	2.4	4.4	60	25	488	296
	2.1-2.7	4.1-4.7	35-184	16-35	408-672	152-400
p value	0.000		0.005		0.015	
T=32	2.7	4.2	32	21	344	230
	2.0-3.2	3.7-4.6	11-68	7.5-36	276-500	140-368
p value	0.001		0.394		0.131	
T=44	2.4	3.7	38	20	336	180
	2.1-2.6	3.2-4.1	27-60	7.5-30.8	280-448	40-264
p value	0.023		0.052		0.036	
T=56	2.2	3.4	25	20	350	138
	2.0-2.4	3.0-3.9	18-32	7.5-28	220-480	40-252
p value	0.022		0.416		0.103	
T=68		3.3		16		112
		2.9-3.6		7.5-28		40-200
T=80		3.2		20		128
		2.7-3.7		7.5-30		40-192

Values are given as median and interquartile range

Figure 1. *Glucose, insulin and C-peptide levels during the prolonged fast in nine insulinoma and in 67 non-insulinoma patients*



Values are given as median plus minimum and maximum range.

with an insulin/C-peptide ratio <1 had a sensitivity of 88.9% and a specificity of 100% for the diagnosis insulinoma.

We made two additional observations in the non-insulinoma group. First, 36 patients recognised their symptoms during the prolonged fast, but the Whipple's triad was absent and their glucose, insulin and C-peptide levels were higher than in the insulinoma patients (*table 4*). Second, glucose levels ≤2.5 mmol/l during the fast were observed at n=29 time points in patients without endogenous hyperinsulinaemia and without symptoms (*table 5*).

Table 4. *Plasma concentrations of glucose, insulin and C-peptide at time of symptoms in the non-insulinoma group compared with patients with insulinoma (n=8)*

	Insulinoma	Non-insulinoma	p value
N	**8**	**36**	
Glucose (mmol/l)	1.9 (1.3-2.2)	4.0 (2.5-5.9)	<0.001
Insulin (pmol/l)	93 (17.0-304.0)	29.0 (7.5-124.0)	0.003
C-peptide (pmol/l)	712.5 (190-1500.0)	210 (40.0-680.0)	0.001
Time (hours)	28 (5.0-67.0)	32.0 (4.5-76.0)	0.73

Time is defined as number of hours until Whipple's triad in the insulinoma group or symptoms in the non-insulinoma group. Values are given as median and range (minimum and maximum)

Table 5. *Patients in the non-insulinoma group with glucose concentrations ≤2.5 mmol/l*

Number of patients	Glucose levels mmol/l	Insulin levels pmol/l	C-peptide levels pmol/l
11	2.5	7.5-40	40-240
4	2.4	7.5-28	40-190
3	2.3	7.5-17	40-260
3	2.2	7.5-38	40-328
4	2.1	7.5-27	40-336
1	2.0	32	480
2	1.9	7.5-32	80-368
1	1.8	7.5	150

DISCUSSION

We diagnosed an insulinoma in nine of 81 patients who underwent a prolonged fasting test because of clinical suspicion of endogenous hyperinsulinaemia. The diagnosis of insulinoma was confirmed by surgical pathology in eight patients and is highly likely in the ninth patient. In view of our extended follow-up it seems unlikely that we have missed any further cases of insulinoma. The nine insulinoma patients were slightly older, more often female and tended to have a higher BMI than non-insulinoma patients, which is in accordance with the

existing literature.[3-5] The incidence of insulinoma in our referral hospital is about one case per year.

Our data demonstrate that even in a population with a high pre-test likelihood of endogenous hyperinsulinaemia, the prolonged fast confirmed the clinical suspicion in only eight out of 81 patients (10%) as indicated by a positive Whipple's triad and insulin/C-peptide ratio below 1. Two positive triads of Whipple but a ratio above 1 were caused by factitious hypoglycaemia. Neither of these patients admitted this diagnosis, but during their long-term follow-up no insulinoma was found. Of the nine insulinoma patients, the prolonged fast gave a positive result in eight and a negative result in one patient. The diagnostic accuracy of the prolonged fast for insulinoma in our series is high (sensitivity 89%, specificity 100%). It is well conceivable that the patient with a negative prolonged fast who appeared to have a very high suspicion of insulinoma would have been positive if the prolonged fast had not been stopped prematurely after 56 hours. In this context it is noteworthy that a positive Whipple's triad had occurred in seven out of nine insulinoma patients (78%) within 48 hours of fasting. In large series reported earlier a positive Whipple's triad of fasting had occurred after 48 hours in 95%[5] and 93%,[6] and after 72 hours in 99%.[6] These data are in keeping with our experience, and do not imply that we should change the duration of our test.

Could the accuracy of the prolonged fast be improved? Hirschberg *et al.*[5] observed that serum proinsulin concentrations are poorly suppressed during fasting in insulinoma patients in contrast with non-insulinoma patients; the sensitivity of proinsulin in his series was 90% at the end of 72 hours fast. Vezzozi *et al.*[4] reported that the best criterion for the presence of an insulinoma was a proinsulin concentration above 5 pmol/l if the serum glucose was below 2.5 mmol/l, reporting sensitivity and specificity figures reaching 100%. Others included the assessment of beta-hydroxybutyrate (BHOB) in order to shorten the 72-hour fast.[3] BHOB is suppressed in insulinoma patients but there is an almost linear rise in BHOB after 18 hours fasting in insulinoma patients. If the cut-off level of BHOB is set at 2.7 mmol/l, 74% of persons in the non-insulinoma group will have reached this cut-off before 72 hours of fasting. Recently, the Endocrine Society published a clinical practice guideline about evaluation and management of an adult hypoglycaemia. Their recommendation, in case of a positive Whipple's triad and other potential causes of an hypoglycaemia are excluded, is measurement of glucose, insulin, proinsulin, BHOB, insulin antibodies and derivatives of sulphonylurea.[6]

Glucose, insulin and C-peptide serum concentrations during fasting do not really discriminate between insulinoma and non-insulinoma patients (*figure 1*). However, the analysis of these parameters is an excellent tool to distinguish between insulinoma and factitious hypoglycaemia in patients with a positive Whipple's triad. In keeping with the present study, Service *et al.*[8] reported overlap in glucose concentrations between insulinoma and non-insulinoma patients. Wiesli *et al.*[9] did not find plasma glucose below 2.5 to be indicative for insulinoma. Our study supports these findings: in the non-insulinoma group 11 subjects had a plasma glucose concentration of 2.5 mmol/l while 18 subjects reached a plasma glucose level below 2.5 mmol/l during fasting (*table 5*). Service *et al.*[10] found C-peptide levels higher than 200 pmol/l in all insulinoma patients. In our study C-peptide concentrations were above 190 pmol/l during the Whipple's triad, and C-peptide levels were above 200 pmol/l in the non-insulinoma group if glucose was below 2.5 mmol/l in the absence of neuroglycopenic symptoms (*table 5*).

It is intriguing why some patients have a marked fall in their glucose levels well into the hypoglycaemic range in the absence of hyperinsulinaemia (*table 5*). Ten of these patients participated in an extended metabolic study using stable isotope techniques. During these measurements no hypoglycaemic events occurred and no abnormalities in fatty acid oxidation or in amino acid/organic acid metabolism were observed.[11]

In conclusion a positive prolonged fast defined as a positive Whipple's triad and an insulin/C-peptide ratio below 1 has an excellent discriminative value for the presence of endogenous hyperinsulinaemia. To detect the few patients that become hypoglycaemic after 48 hours, the fast has to be continued for three days.

REFERENCES

1. Lebowitz MR, Blumenthal SA. The molar ratio of insulin to C-peptide. Arch Intern Med. 1993;153:650-5.
2. Service FJ. Hypoglycemic disorders. New Engl J Med. 1995;332:1144-52.
3. Service FJ, O'Brien PC. Increasing Serum betahydroxybutyrate concentrations during the 72-hour fast: evidence against hyperinsulinemic hypoglycaemia. J Clin Endocrinol Metab. 2005;90:4555-8.
4. Vezzosi D, Bennet A, Fauvel J, Caron P. Insulin, C-peptide, proinsulin for the biochemical diagnosis of hypoglycaemia related to endogenous hyperinsulinism. Eur J Endocrinol. 2007;157:75-83.
5. Hirschberg B, Livi A, Bartlett DL, et al. Forty-Eight-Hour-Fast: The diagnostic test for insulinoma. J Clin Endocrinol Metab. 2000;85:3222-6.
6. Service FJ, Natt N. The prolonged fasting. J Clin Endocrinol Metab. 2000;85:3973-4.
7. Cryer PE, Axelrod L, Grossman AB, et al. Evaluation and management of adult hypoglycaemic disorders: An endocrine society clinical practice guideline. Clin Endocrinol Metab. 2009;94(3):709-28.
8. Service FJ. Diagnostic approach to adults with hypoglycemic disorders. Endocrinol Metab Clin North Am. 1999;28:519-32.
9. Wiesli P, Brändle M, Schwegler B, Lehmann R, Spinas GA, Schmid C. A plasma glucose concentration below 2.5 mmol/l is not an appropriate to end the 72-h fast. J Int Med. 2002;252:504-9.
10. Service FJ, O'Brien PC, McMahon MM, Kao PC. C-peptide during the prolonged fast in insulinoma. J Clin Endocrinol Metab. 1993;76:655-9.
11. Soeters MR, Huidekoper HH, Duran M, et al Hypoinsulinemic hypoglycaemia during fasting in adults: a Gaussian tale. Thesis University of Amsterdam MR Soeters 2008; p:124-38.

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ORIGINAL ARTICLE

Diabetes specialist nurse as main care provider for patients with type 2 diabetes

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ABSTRACT

Background: The objective of this study was to determine whether the management of type 2 diabetes (DM2) can be transferred from an internist to a nurse specialised in diabetes (NSD).

Methods: Ninety-three patients with DM2 referred by their general practitioner were randomised; 84 patients completed the study. The intervention group received care from an NSD who treated glycaemia, blood pressure and lipid profile by protocol. The control group received care from an internist. The primary endpoint was the main decrease in HbA1c. Secondary endpoints included blood pressure, lipid profile, healthcare costs, QOL, and patient satisfaction.

Results: HbA1c, total cholesterol, LDL cholesterol and cholesterol/HDL ratio decreased significantly in both study populations after a follow-up time of 12 months. Cholesterol/HDL ratio decreased by 0.4 and 0.9 in the NSD and control group respectively (p=0.034 for the difference between groups). The decreases (95% confidence interval) in systolic blood pressure were 8.6 mmHg (2.6, 14.7) in the NSD group and 4.0 mmHg (-0.9, 8.9) in the control group, without a significant difference between groups. After one year, 33.3% of the patients in the NSD group achieved an HbA1c level <7% compared with 2.2% at baseline (p=0.002). Healthcare costs were less and patient satisfaction with the NSDs was significantly better (p<0.001), while maintaining the same QOL.

Conclusion: NSDs using treatment protocols are able to provide effective care for patients with DM2, comparable with the care provided by an internist, with respect to clinical parameters, and superior with respect to healthcare costs and patient satisfaction.

KEYWORDS

Diabetes mellitus type 2, nurses, randomised controlled trial

INTRODUCTION

Type 2 diabetes is a chronic, progressive illness which causes considerable morbidity and premature mortality.[1,2] The worldwide prevalence of type 2 diabetes is high and is increasing steadily, also in the Netherlands.[3,4] The burden of type 2 diabetes on healthcare has also increased because of the intensified cardiovascular risk management being practised to prevent macrovascular morbidity and mortality in these patients.[5] In the treatment of type 2 diabetes, tight guidelines are increasingly recommended for optimising glycaemia, blood pressure and lipid profile.[6] Therefore, the burden of treatment has increased and will further increase per patient as well as per population with type 2 diabetes. In order to meet this problem, in the current study, we tested the hypothesis that well-defined routine aspects in diabetes care, previously only handled by medical doctors in the secondary care setting, may be safely transferred to supervised nurses specialised in diabetes (NSD), including the prescription of medication, resulting in at least the same quality of clinical care, healthcare costs, health-related quality of life (HRQOL), and patient satisfaction.

MATERIALS AND METHODS

The study population consisted of patients with type 2 diabetes who were referred by general practitioners to the

diabetes outpatient clinics of two different hospitals in the northeast region of the Netherlands: the Isala Clinics in Zwolle and the Bethesda General Hospital in Hoogeveen. Patients were referred between March 2002 and January 2004. An internist saw all patients prior to randomisation and excluded patients in whom treatable comorbidity was found, such as macroalbuminuria, serum creatinine level >135 mol/l, Cockcroft <50 ml/min, and/or alanine aminotransferase (ALAT) >120 U/l. Pregnant patients were also not eligible. The study was approved by the local Medical Ethics Committee of the Isala Clinics. All participants provided written informed consent. Overall, 95 patients were recruited and randomised (*figure 1*). Eighty-four subjects, 46 in the intervention group and 38 in the control group, completed the study and were included in the analysis. Both groups were comparable for age, type 2 diabetes duration, body mass index (BMI), blood pressure, HbA1c, and lipid profile (*table 1*). The gender distribution was slightly different in the two groups.

Figure 1. *Flow of patients through the study*

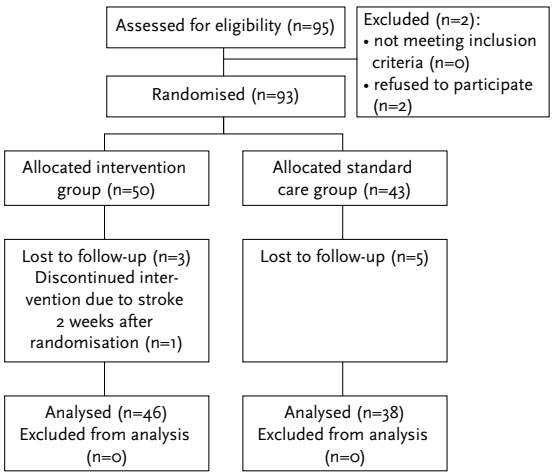

Table 1. *Baseline characteristics per group*

	NSD (n=46)	Standard care (n=38)
Gender (male)	43.5%	50.0%
Age (year) (mean ± SD)	63.1 ± 10.6	59.6 ± 10.6
Diabetes duration (year) (median (25-75%)	7.5 (4.0-13.0)	6.0 (3.5-10.5)
BMI (kg/m^2) (mean ± SD)	30.5 ± 5.6	30.1 ± 5.6
SBP (mmHg) (mean ± SD)	154.9 ± 23.3	156.3 ± 19.9
DBP (mmHg) (mean ± SD)	86.6 ± 10.9	85.6 ± 9.4
HbA1c (%) (mean ± SD)	8.9 ± 1.2	8.6 ± 1.3
Total cholesterol (mmol/l) (mean ± SD)	4.9 ± 0.8	5.2 ± 1.2
LDL cholesterol (mmol/l) (mean ± SD)	2.6 ± 0.9	2.7 ± 1.0
Cholesterol/HDL ratio (mean ± SD)	4.1 ± 1.2	4.4 ± 1.9

BMI = body mass index; SPB = systolic blood pressure; DBP = diastolic blood pressure; LDL = low-density lipoproteins; HDL = high-density lipoproteins.

Intervention and control group

The intervention group was primarily treated and educated by NSDs. NSDs were trained to follow a detailed treatment and management protocol aimed at optimising glycaemia, blood pressure, and lipid profile.[7] These protocols are based on the guidelines from the Dutch College of General Practitioners and those from the Dutch Diabetes Federation.[6,8] These protocols allowed the NSDs to prescribe medication and to order laboratory tests. They were permitted to initiate therapy with 14 different medications and to change dosages for a further 30. In some cases, the protocol indicated that consultation of an internist was necessary. All of the patients in the intervention group saw only one care provider (the NSD). The control group received standard care, in which an internist was responsible for treating type 2 diabetes and a 'standard' nurse specialised in diabetes was responsible for educating the patients.

Primary and secondary outcome measures

The primary endpoint was the mean decrease in HbA1c from baseline to one year after randomisation. Secondary endpoints were mean decrease in blood pressure, total cholesterol, LDL cholesterol and cholesterol/HDL ratio, proportion of patients achieving ranges of glycaemic control (HbA1c below 7 and 8.5%, respectively), of blood pressure (below 140/90 mmHg), and of lipid profile (individual target values according to the Dutch guidelines from 1999 to 2005 in which treatment is indicated in men between 50 and 70 years of age and women between 50 and 75 years of age with a 25% risk of developing cardiovascular disease in ten years) from baseline to one year after randomisation. Other secondary endpoints included measures of HRQOL, diabetes-related symptoms, patients' satisfaction, and healthcare consumption and costs (number of patient visits, number of contacts between NSD and internist, medication adjustments, costs of the prescribed medication, costs associated with requested lab work, and the costs of actual patient contact).

Measures

All subjects were seen prior to any intervention and before randomisation by an independent medical investigator and after six months (T6) and after 12 months (T12). These visits were planned independently from the visits to the care providers. At baseline, the duration of type 2 diabetes, any diabetes medication(s), general medication(s), and insulin-dose requirements were assessed. The patients were weighed dressed but without shoes. Height was measured without shoes, and blood pressure was measured with the patient in a sitting position. The mean of the two blood pressure readings was calculated. A calibrated and validated Omron M5-I (HEM-757) automatic blood pressure device was used to measure blood pressure.[9] HbA1c, serum total cholesterol, low-density lipoprotein (LDL), high-density

lipoprotein (HDL), triglycerides, ALAT, and creatinine levels were measured according to standard hospital procedures. HRQOL was assessed with the Short Form-36 questionnaire (SF-36). The SF-36 is a validated generic health-related quality-of-life questionnaire that includes both mental and physical factors.[10,11] To measure the presence and the perceived burden of diabetes-related symptoms, the revised version of the Type 2 Diabetes Symptom Checklist (DSC-type 2) was used.[12] Satisfaction with diabetes care was assessed using the Patients' Evaluation of the Quality of Diabetes Care (PEQD).[13]

Healthcare costs were determined at three levels: average cost per patient per month of the prescribed medication according to the prices as they are listed in the Dutch Pharmacotherapeutic Compass 2003 (analogous to the CPS in North America), the costs associated with requested lab work, based on the prices as listed in the Diagnostic Compass 2003, and the costs of actual patient contact which is calculated according to the salaries of the healthcare workers involved: internists € 60,00 per hour, and NSD and standard diabetes nurses € 23,00 per hour.[14,15]

Randomisation and power calculation

The patient population was randomised using non-transparent closed envelopes, with sequential numbers enclosed. Subjects with even numbers were assigned to the intervention group, and those with odd numbers were assigned to the control group. According to earlier study results, power analysis revealed that 81 patients would be required in order to have an 80% chance of detecting a significant (at the two-sided 5% level) 0.5% difference in mean HbA1c at T12 between the two groups, assuming a standard deviation of 0.75 and a 10% loss to follow-up.

Statistical analyses

Statistical analyses were performed using SPSS 11.0 for Windows. For longitudinal analyses we used the general linear model (GLM repeated measures) for continuous variables and the McNemar test for changes in dichotomous variables. To study changes in HRQOL, diabetes-related symptoms, and quality of diabetes care, we used the Mann-Whitney U tests for analyses between groups and the Wilcoxon signed-rank tests for changes from baseline within groups because of some skewed outcomes. To study changes in medical services and medication adjustments, we used Student's t-test for variables with a normal distribution, the Mann-Whitney U test for non-normal distributed variables, and the χ^2 test for categorical variables. All reported P values are two-tailed. To allow for multiple comparisons we adjusted the outcome analyses using the Bonferroni correction.

RESULTS

At T6 and T12, mean HbA1c, total cholesterol, LDL cholesterol and cholesterol/HDL ratio declined significantly in both groups (*table 2*). In the intervention group the systolic blood pressure was significantly lower at both T6 and T12, and the diastolic blood pressure was only significantly lower at T6. In the control group, the decreases

Table 2. *Mean change scores of outcome variables and percent of patients meeting outcome targets by treatment group*

		NSD			Standard care			p value†
		Difference between To-T6*	Difference between To-T12*		Difference between To-T6*	Difference between To-T12*		
SBP (mmHg)		-9.5 (-3.8, -15,2)	-8.6 (-2.6, -14.7)		-7.2 (-2.4, -12.1)	-4.0 (0.9, -8.9)		NS
DBP (mmHg)		-3.1 (-0.3, -5.9)	-1.4 (1.4, -4.1)		-1.0 (2.7, -4.8)	-2.4 (0.8, -4.9)		NS
BMI (kg/m²)		-0.1 (-0.5, 0.3)	0.4 (-0.1, 0.9)		0.1 (-0.3, 0.6)	0.2 (-0.3, 0.8)		NS
HbA1c (%)		-1.5 (-1.4, -1.9)	-1.5 (-1.0, -1.9)		-1.2 (-0.9, -1.6)	-0.9 (-0.5, -1.3)		NS
Total cholesterol (mmol/l)		-0.3 (-0.1, -0.6)	-0.4 (-0.2, -0.6)		-0.6 (-0.2, -1.1)	-0.9 (-0.5, -1.3)		NS
LDL cholesterol (mmol/l)		-0.1 (-0.3, 0)	-0.3 (-0.1, -0.5)		-0.3 (-0.6, 0.1)	-0.6 (-0.2, -0.9)		NS
Cholesterol/HDL		-0.3 (-0.1, -0.6)	-0.4 (-0.1, -0.6)		-0.7 (-0.4, -1.1)	-0.9 (-0.5, -1.4)		p=0.034‡
	Target	To	T12	p value	To	T12	p value	p value§
HbA1c	<7.0	2.2%	33.3%	p=0.002	10.5%	26.3%	NS	NS
	≤8.5	40.0%	93.3%	p<0.001	44.7%	81.6%	p<0.001	NS
SBP (mmHg)	<140	23.9%	32.6%	NS	23.7%	31.6%	NS	NS
DBP (mmHg)	<90	65.2%	63.0%	NS	65.8%	73.7%	NS	NS
BP (mmHg)	<140/90	21.7%	26.1%	NS	23.7%	23.7%	NS	NS
Dutch lipid profile	||	76.1%	91.3%	p=0.016	70.3%	91.9%	p=0.008	NS

SBP = systolic blood pressure; DBP = diastolic blood pressure, BMI = body mass index; LDL = low-density lipoproteins; HDL = high-density lipoproteins. *Mean with 95% CI. †Difference between NSD and standard care. ‡Difference between NSD and standard care in change between To and T12. §p value general linear model (GLM) between groups. || Individual target values according to Dutch guidelines from 1999-2005 in which treatment is indicated in men between 50 and 70 years and women between 50 and 75 years with a 25% chance of developing cardiovascular disease in ten years. During treatment, the target value for the cholesterol level is <5 mmol/l.

in blood pressure at both T6 and T12 were not significant. None of the differences between the two groups were significant except for the cholesterol/HDL ratio, which was lower in the control group. After one year, significantly more patients in the intervention group achieved the target HbA1c level of less than 7% compared with baseline. A majority of patients in both groups (93 and 82%) achieved an HbA1c <8.5% and the target for the lipid profile at T12 (91 and 92%). Between the groups, no differences were found in target levels.

Seventy-eight of the 84 patients completed the SF-36 and the Diabetes Symptom surveys at follow-up (T12), and 80 patients completed the satisfaction survey (data not shown). There were no differences in HRQOL or diabetes-related symptoms over time between the two groups. The patients' evaluations of care received from the NSD were significantly more positive than the evaluations reported by the control group (p<0.001). The total satisfaction sum score for the NSD was 73.9%, for the internist 53.3%, and for the 'standard' diabetes nurse 59.9%.

The use of medical services and the number of medication adjustments are presented in *table 3*. There was a significant difference in the number of visits between the two groups (lower in the NSD group) but not in the duration of the visits. In some cases, the protocol being followed by the NSDs indicated that consultation of an internist was necessary. In the intervention group, the total number of these consultations was 57, and the median number per patient was 1.0 (interquartile range: 0.0 to 2.0). The NSDs referred significantly more patients back to the GP within 12 months (38 patients (82.6%) *vs* 9 patients (23.7%); p<0.001). The NSDs only referred patients back to their GP when the treatment goals of glycaemic control, blood pressure and lipid profile had been met. The intensity of glucose-lowering therapy increased in both groups. Most patients were switched to insulin therapy. During the study period the NSD prescribed significantly more antihypertensive agents, and the internist prescribed significantly more cholesterol-lowering agents. The difference between the two groups was only significant for the cholesterol-lowering agents.

Table 3. *Medical utilisation, medication adjustments and healthcare costs*

	NSD			Standard care			p value
Number of visits ± SD	7.4 ± 3.0			9.8 ± 3.8 (total) 5.2 ± 1.4 (internist) 4.7 ± 3.3 (standard nurse)			p=0.002
Total duration of visits (minutes ± SD)	272.0 ± 120.5			249.2 ± 110.7 (total) 67.6 ± 17.5 (internist) 180.8 ± 104.8 (standard nurse)			
Number of consultations with internist (median (25-75%))	1.0 (0-2.0)			-			-
Percent of patients referred back to the GP <12 months	82.6%			23.7%			p<0.001
Percentage patients	**Baseline**	**T12**	**p value**	**Baseline**	**T12**	**p value**	**p value***
OHA without insulin	91.3%	34.8%	p<0.001	89.5%	34.2%	p<0.001	NS
Insulin without OHA	6.5%	19.6%	NS	2.6%	7.9%	NS	NS
Insulin with OHA	2.2%	45.7%	p<0.001	5.3%	57.9%	p<0.001	NS
AHA	67.4%	84.8%	p=0.016	55.3%	71.1%	NS	NS
CLA	45.7%	54.3%	NS	34.2%	68.4%	p<0.001	p=0.006
Total salary costs in euros (mean ± SD) [median, 25-75%]	114.6 ± 50.4 [101.0, 70.1-147.2] (total) 106.0 ± 46.9 [96.4, 68.7-140.2] (patient visits) 8.6 ± 10.1 [7.0, 0-13.9] (consultations with internist)*			138.3 ± 48.3[126.8, 96.8-175.2] (total) 67.9 ± 17.7 [60.0, 50.0-80.0] (internist) 70.5 ± 40.8 [58.5, 35.1-105.2] (standard DN)			p=0.032
Total lab costs in euros (mean ± SD) [median, 25-75%]	64.9 ± 34.5 [60.0, 36.3-82.4]			91.5 ± 36.7 [83.8, 73.0-116.6]			p=0.001
Medication costs per month	**Baseline**	**T12**	**p value**	**Baseline**	**T12**	**p value**	**p value***
Total costs (mean ± SD and median (25-75%))	57.5±44.3 50.5 (27.0-77.3)	136.3±91.9 110.2 (66.5-202.7)	p<0.001	49.6±39.4 38.6 (19.7-78.4)	149.0±94.4 136.0 (72.2-188.7)	p<0.001	NS
HA (mean ± SD and median (25-75%))	24.1±30.9 11.6 (8.9-26.9)	89.3±77.6 67.8 (17.9-163.6)	p<0.001	21.8±22.7 12.7 (8.9-27.1)	88.6±79.6 81.8 (12.5-133.0)	p<0.001	NS
AHA (mean ± SD and median (25-75%))	14.5±15.4 7.4 (0-27.6)	21.5±19.7 19.1 (4.8-32.4)	p=0.001	11.3±14.0 4.7 (0-19.1)	23.7±23.0 18.9 (0-39.3)	p<0.001	NS
CLM (mean ± SD and median (25-75%))	18.9±24.6 0 (0-48.6)	25.5±29.6 23.2 (0-51.3)	p=0.011	16.5±27.3 0 (0-30.8)	36.7±34.4 35.1 (0-48.6)	p<0.001	p=0.005

***p value GLM between groups. OHA = oral hypoglycaemic agents; HA = hypoglycaemic agents; AHA = antihypertensive agents; CLA = cholesterol-lowering agents.**

The costs for the consumption of medical services are also listed in *table 3*. It is clear from this table that the personnel costs and the costs associated with laboratory testing are significantly lower in the intervention group when compared with the control group ($p<0.001$). The average per month increase in medication costs are not significantly different between the two groups, except for the cost increase associated with cholesterol-lowering medications, which shows a greater increase in the control group ($p=0.005$).
As mentioned, the gender distribution was slightly different in the two groups. In order to investigate whether the results were applicable for both men and women, we performed additional analyses for these groups separately (data not shown). These analyses revealed that the results did not differ when stratifying for gender.

DISCUSSION

This is the first randomised controlled study in which the following two strategies of treatment in patients with type 2 diabetes have been compared in a secondary healthcare setting: the strategy with an almost complete shift of diabetes care from doctors to nurses versus the conservative strategy. The results of this study show that an NSD, following tight protocols, achieves results which are equal to those achieved by an internist working with a 'standard' nurse in the treatment of patients with type 2 diabetes without serious diabetic complications who have been referred by their general practitioners to the hospital. Both patient groups were successfully treated, considering the improvements in clinical parameters. Both groups showed comparable numbers of patients with values within the target range at one year after randomisation for: HbA1c, blood pressures, and lipid profiles. While the patients in the NSD group were more satisfied with the care they received than the patients in the control group, their HRQOL levels remained equal.
Healthcare costs appeared to be lower in the NSD group than in the control group. This study also shows that there is a time saving on the part of the internist. For each patient who is primarily treated by the NSD, the average time saving for the internist is 61.4 minutes (difference of the mean internist-patient contact time per patient between the groups minus difference of the mean consultation time between NSD and internist per patient between the groups). This means that the internist would be able to supervise the treatment of almost eleven patients by the NSD in the same amount of time that he or she would require to treat a single patient.
In this study patients treated by an internist achieved a lower cholesterol/HDL ratio. However, an equal number of patients with lipid profile within the target range were found in both groups after one year (91.3 and 91.9%). This means that internists prescribed more cholesterol-lowering medicines than was dictated to the NSDs by the protocol. One has to remember that the protocol used in this study is based on older guidelines. The treatment goals for the lipid profile in patients with type 2 diabetes have become more stringent nowadays. Another difference between both groups is the proportion of patients referred back to the general practitioner within 12 months. In our opinion, both differences are probably caused by strictly following the study protocol in the NSD group.
What is already known about this topic? A Cochrane review from 2003 looked at the effect of treatment by NSDs on the metabolic regulation of patients with diabetes.[16] Only six studies were included in this review including 1382 participants. The conclusion was that NSDs were better at regulating glucose in the short term (six months) but not in the long term (12 months). None of these studies systematically examined the effect of assigning the treatment of patients with type 2 diabetes to NSDs in a randomised controlled design. Three of the studies in the review included only patients with type 1 diabetes. In the other three studies, the nurse was only responsible for delivering treatment recommendations to the primary physician, without being responsible for treating the patient. All the studies up to 2002 were included in the Cochrane review. In addition to this review, we found six randomised studies in Medline, which were published between 2002 and 2009.[17-21] In three of these studies, nurses were assigned specific tasks in the treatment of patients with type 2 diabetes.
New *et al*. studied the effect of lipid lowering and antihypertensive treatment regimens by nurses in specialist nurse-led clinics according to protocols on treatment efficacy compared with that of standard regimens by physicians.[19] These specialist nurses titrated medications according to the local protocol, but did not initiate additional therapy when necessary. Overall, specialist nurse-led clinics were associated with a significant improvement in patients achieving the target.
The study by Taylor *et al*. examined the effects of assigning a nurse-care manager who, according to specific protocols, was permitted to titrate medications for glycaemic control, blood pressure and lipid profile.[20] Patients randomised to usual care were instructed to remain under the treatment of their primary care physician. The patient's primary care physician was called if a new medication was indicated. At one year, the mean reductions in HbAlc, total cholesterol, and LDL cholesterol were significantly greater for the intervention group compared with the usual care group.
The effect of antihypertensive treatment regimens by home care nurses who titrated drug regimen according to an algorithm, was investigated by Tobe *et al.*[21] After each medication change, follow-up was arranged with the patient's primary care physician. Participants assigned to the control group were treated by their primary care

physician. Both groups experienced a significant reduction in systolic blood pressure (between the groups not significant) and patients in the intervention group had a larger decrease in diastolic blood pressure over time than did those in the control group.

All of these studies were trying to determine if interventions involving a nurse would lead to improved care for the patient with diabetes. Our primary goal was not to improve the quality of care. We essentially questioned whether NSDs are able to offer diabetes care with maintenance of its actual quality and with relief of its increasing burden in a cost-effective way.

CONCLUSION

Standardised care, in patients without serious diabetic complications, delivered by a specially trained NSD is a good alternative to standard care by an internist, with comparable results after one year of treatment in treatment goals, and even better results in patient goals and goals for cost-effectiveness.

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REFERENCES

1. Kannel WB, McGee DL. Diabetes and cardiovascular disease. The Framingham study. JAMA. 1979;241:2035-8.
2. Nathan DM, Meigs J, Singer DE. The epidemiology of cardiovascular disease in type 2 diabetes mellitus: how sweet it is ... or is it? Lancet. 1997;350(Suppl 1):4-9.
3. Wild S, Roglic G, Green A, Sicree R, King H. Global prevalence of diabetes: estimates for the year 2000 and projection for 2030. Diabetes Care. 2004;27:2568-9.
4. Ubink-Veltmaat LJ, Bilo HJG, Groenier KH, Houweling ST, Rischen RO, Meyboom-de Jong B. Prevalence, incidence and mortality of type 2 diabetes revisited: a prospective population-based study in The Netherlands. Eur J Epidem. 2003;18:793-800.
5. Beckman JA, Creager MA, Libby P. Diabetes and atherosclerosis: epidemiology, pathophysiology, and management. JAMA. 2002;287:2570-81.
6. Rutten GEHM, Verhoeven S, Heine RJ, et al. NHG-Standaard Diabetes Type 2 (eerste herziening). Huisarts Wet. 1999;42:67-84.
7. Houweling ST, Kleefstra N, Verhoeven S, Ballegooie E van, Bilo HJG. Protocollaire diabeteszorg. Mogelijkheden voor taakdelegatie. Zwolle: Stichting Langerhans, 2004.
8. Richtlijnen en adviezen voor goede diabeteszorg. Leusden: Nederlandse Diabetes Federatie, 2000.
9. El Assaad MA, Topouchian JA, Asmar RG. Evaluation of two devices for self-measurement of blood pressure according to the international protocol: the Omron M5-I and the Omron 705IT. Blood Press Monit. 2003;8:127-33.
10. Ware JE Jr, Sherbourne CD. The MOS 36-item short-form health survey (SF-36). I. Conceptual framework and item selection. Med Care. 1992;30:473-83.
11. Aaronson NK, Muller M, Cohen PD, et al. Translation, validation, and norming of the Dutch language version of the SF-36 Health Survey in community and chronic disease populations. J Clin Epidemiol. 1998;51:1055-68.
12. Grootenhuis PA, Snoek FJ, Heine RJ, Bouter LM. Development of a type 2 diabetes symptom checklist: a measure of symptom severity. Diabet Med. 1994;11:253-61.
13. Pouwer F, Snoek FJ. Patients' Evaluation of the Quality of Diabetes Care (PEQD): development and validation of a new instrument. Qual Saf Healthcare. 2002;11:131-6.
14. Dutch Pharmacotherapeutic Compass 2003. Amstelveen: Healthcare Insurance Board (CVZ), 2003.
15. Dutch Diagnostic Compass 2003. Amstelveen: Healthcare Insurance Board (CVZ), 2003.
16. Loveman E, Royle P, Waugh N. Specialist nurses in diabetes mellitus. Cochrane Database Syst Rev. 2003;(2):CD003286.
17. Gary TL, Bone LR, Hill MN, et al. Randomized controlled trial of the effects of nurse case manager and community health worker interventions on risk factors for diabetes-related complications in urban African Americans. Prev Med. 2003;37:23-32.
18. Davidson MB. Effect of nurse-directed diabetes care in a minority population. Diabetes Care. 2003;26:2281-7.
19. New JP, Mason JM, Freemantle N, et al. Specialist nurse-led intervention to treat and control hypertension and hyperlipidemia in diabetes (SPLINT): a randomized controlled trial. Diabetes Care. 2003;26:2250-5.
20. Taylor CB, Miller NH, Reilly KR, et al. Evaluation of a nurse-care management system to improve outcomes in patients with complicated diabetes. Diabetes Care. 2003;26:1058-63.
21. Tobe SW, Pylypchuk G, Wentworth J, et al. Effect of nurse-directed hypertension treatment among First Nations people with existing hypertension and diabetes mellitus: the Diabetes Risk Evaluation and Microalbuminuria (DREAM 3) randomized controlled trial. Can Med Assoc J. 2006;174:1267-71.

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CASE REPORT

A rare case of heterotopic pancreas in the stomach which caused closed perforation

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ABSTRACT

Heterotopic pancreas is the presence of an abnormally located focus of normally developed pancreatic tissue outside the boundaries of the orthotopic pancreas, without anatomical or vascular connections. Heterotopic pancreas is usually found incidentally and is generally asymptomatic. However, the lesion can become symptomatic depending on the size, location and the pathological changes. We present the case of a 19-year-old female patient with a perforated heterotopic pancreas of the gastric antrum. There have been no reports describing perforated gastric heterotopic pancreas and it should always be considered in the differential diagnosis of gastric masses and acute abdomen.

KEYWORDS

Heterotopic pancreas, stomach, perforation

INTRODUCTION

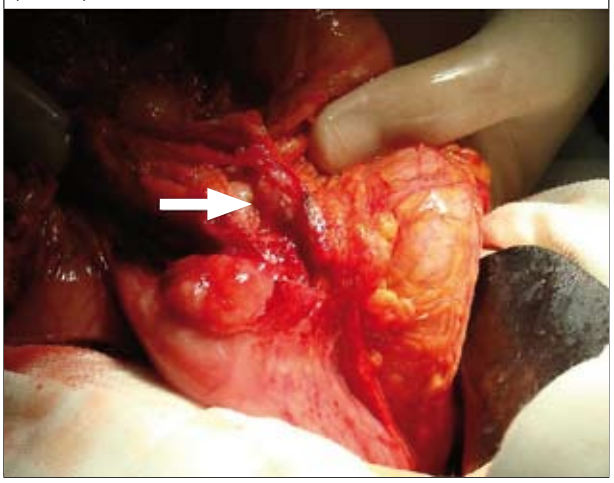
Heterotopic pancreas is the presence of an abnormally located focus of normally developed pancreatic tissue outside the boundaries of the orthotopic pancreas, without anatomical or vascular connections. The most common locations of heterotopic pancreas is the proximal gastrointestinal tract (70 to 90% of these lesions are detected in the stomach, duodenum and jejunum) but it may also be found anywhere in the gastrointestinal tract, pelvis and in the thorax.[1,2] Heterotopic pancreas is usually found incidentally and is generally asymptomatic. However, the lesion can become symptomatic depending on the size, location and the pathological changes. Gastric and duodenal obstruction, gastrointestinal bleeding due to ulceration, dyspeptic symptoms, acute and chronic pancreatitis, malignant change and cyst formation have also been reported due to heterotopic pancreas.[3]

We present the case of a 19-year-old female patient with a perforated heterotopic pancreas of gastric antrum. To the best of our knowledge, our patient has a very rare form of heterotopic pancreas not previously described.

CASE REPORT

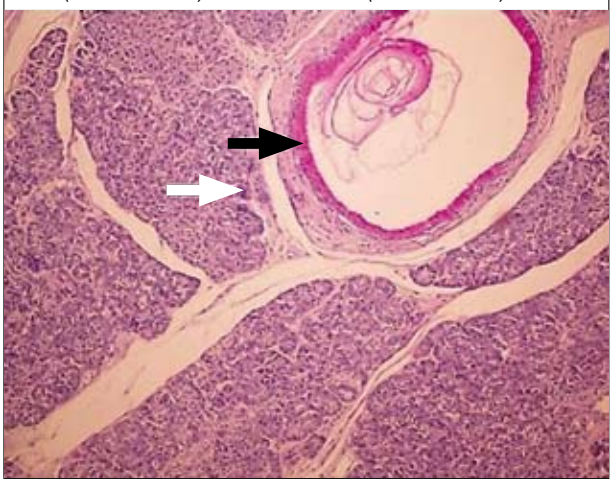
A 19-year-old woman was admitted to our hospital complaining of worsening epigastric pain, nausea and vomiting for two days. Her past medical history was unremarkable and she had no history of systemic disease, trauma or abdominal symptoms. On physical examination, there was tenderness and defence in the epigastric area. Vital signs were as follows: temperature 38.2°C, heart rate 118 beats /min, respiratory rate 22 breaths/min and blood pressure 95/45 mmHg. Plain chest and abdominal X-rays were unremarkable. Laboratory testing, including amylase at admission, were normal except for a leucocyte count of 13,200/mm^3 (89% polymorphonuclear) and C-reactive protein (CRP) 48 mg/dl. Abdominal ultrasonography revealed asymmetrically thickened gastric wall and an irregular, hypoechoic solid lesion of 32 x 25 mm with minimal free intra-abdominal fluid. Computed tomography was not performed and a decision was made to proceed with surgery with the clinical impression of perforated gastric ulcer or gastric cancer. On exploration through the midline excision, a yellow-coloured, centrally perforated mass about 3 x 3 cm in the gastric antrum, which was covered with omentum, was present (*figure 1*). Upon gastrotomy, intact gastric mucosa was seen and the mass was located intra-murally. No lymphadenopathy was noted and the other observations of the abdominal explorations were within normal limits. Distal gastrectomy

Figure 1. *Centrally perforated mass about 3 x 3 cm in the gastric antrum which was covered with omentum (arrow)*



and gastroduodenostomy was carried out under the clinical impression of gastrointestinal stromal tumour. The postoperative course was uneventful and she was discharged five days after operation. Macroscopically, the tumour measured 3.5 x 3 cm, was yellowish-white in colour, hard with an irregular margin and located intramurally. The cut surface was composed of greyish-brown necrotic tissue with a wide area of ulceration and perforation hole about 1 cm in diameter. Microscopic examination showed intramurally located dilated pancreatic ducts and acini and pyogenic inflammatory granulation tissue containing intense polymorphonuclear and mononuclear cell infiltration and wide ulceration. The overlying mucosa was intact and the diagnosis was gastric heterotopic pancreas without any evidence of malignancy or stromal tumour (*figure 2*).

Figure 2. *Histological examination of the resected specimen demonstrated pancreatic tissue with acinar cells (white arrow) and duct cells (black arrow)*



DISCUSSION

First described in 1729, heterotopic or ectopic pancreas is considered to be the most common congenital anomaly of the pancreas after divisum. The prevalence at autopsy has been reported to be between 0.55 and 13.7 %, and at laparatomy 0.2%.[4] Heterotopia can occur within any portion of the digestive tract having a propensity to be found in stomach and upper intestine as well as common bile duct, gallbladder, umbilicus, spleen and even within the fallopian tubes.[1,2] The aetiology of heterotopic pancreas is unknown. There are some theories involved in the aetiopathogenesis including separation of pancreatic tissue during embryonic rotation of the dorsal and the ventral pancreatic ducts and misplacement of buds of embryonic tissue as they penetrate into the bowel wall.[5]

The Heinrich classification system is frequently used to classify heterotopic pancreas: type 1 (contains acini, islets and duct), type 2 (acini and ducts, no islets) and type 3 (ducts alone). The present case belongs to the type 2 showing dilated pancreatic ducts and acini.[6]

Gastric heterotopic pancreas is located in the antrum in 85 to 95% of the cases and the involvement of the submucosal, muscularis and subserosal layer is 73, 17 and 10%, respectively. It is usually solitary, measuring 3 cm or less in diameter, although gastric lesions tend to be larger.[7] Because the findings on imaging studies (ultrasonography, endoscopic ultrasonography and computed tomography) are not specific for heterotopic pancreas, its preoperative definitive diagnosis is difficult. The definitive diagnosis of heterotopic pancreas is reached on histopathological examination. The endoscopic appearance is usually a small, centrally umbilicated, submucosal mass and the surface biopsies are usually normal because of the frequent submucosal localisation of the lesion.[8] Heterotopic pancreas is usually asymptomatic and found incidentally, but may become clinically evident depending on the size, location and the pathological changes. Although the real reason for perforation in our case is speculative, it was considered that necrotising inflammation in the heterotopic pancreas tissue resulted in ulceration and then perforation at the serosal site of gastric antrum.

CONCLUSION

To date, there have been no reports describing perforated heterotopic pancreas of the stomach. Therefore, the present case was considered to be a very rare case of this disorder. Heterotopic pancreas should always be considered in the differential diagnosis of gastric masses and acute abdomen.

REFERENCES

1. Kobayashi S, Okayama Y, Hayashi K, et al. Heterotopic pancreas in the stomach which caused obstructive stenosis in the duodenum. Intern Med. 2006;45:1137-41.
2. Inceoglu R, Dosluoglu HH, Kullu S, Ahiskali R, Doslu FA. An unusual cause of hydropic gallbladder and biliary colic--heterotopic pancreatic tissue in the cystic duct: report of a case and review of the literature. Surg Today. 1993;23:532-4.
3. Ormarsson OT, Gudmundsdottir I, Mårvik R. Diagnosis and treatment of gastric heterotopic pancreas. World J Surg. 2006;30:1682-9.
4. Song DE, Kwon Y, Kim KR, Oh ST, Kim JS Adenocarcinoma arising in gastric heterotopic pancreas: a case report. J Korean Med Sci. 2004;19:145-8.
5. Ayantunde AA, Pinder E, Heath DI. Symptomatic pyloric pancreatic heterotopia: report of three cases and review of the literature. Med Sci Monit. 2006;12:CS49-52.
6. Heinrich H. Ein Beitrag zur Histologie dessogen. Akzessorischen Pankreas. Virchows Arch. 1909;198:392-401.
7. Christodoulidis G, Zacharoulis D, Barbanis S, Katsogridakis E, Hatzitheofilou K. Heterotopic pancreas in the stomach: a case report and literature review. World J Gastroenterol. 2007;13:6098-100.
8. Hsia CY, Wu CW, Lui WY. Heterotopic pancreas: a difficult diagnosis. J Clin Gastroenterol. 1999;28:144-7.

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Abdominal pain, low grade fever and persistent shock

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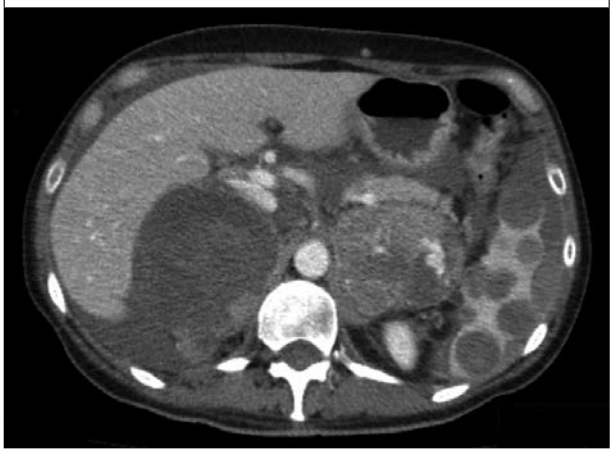
CASE REPORT

A 44-year-old female with a history of metastasised melanoma presented to the outpatients clinic with malaise, near collapse, nausea and abdominal pain radiating to the chest and back since three days. Physical examination revealed a pale woman with mild hypotension (95/50 mmHg), tachycardia (110 beats/min) and low-grade fever (38.2°C). Diffuse abdominal tenderness was present. Blood analysis showed haemoglobin 6.1 mmol/l, hyponatraemia 124 mmol/l and potassium 4.8 mmol/l. Cardiac enzymes and ECG were normal. However, after initial haemodynamic stability with saline infusion, our patient went into a persisting shock despite aggressive volume resuscitation. Subsequently, an abdominal computed tomography angiography was performed (*figure 1*).

Figure 1. *Abdominal computed tomography angiography*



WHAT IS YOUR DIAGNOSIS?

See page 292 for the answer to this photo quiz.

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PHOTO QUIZ

A woman with a painful hip

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CASE REPORT

A 25-year-old woman was seen on Christmas eve at the emergency department because of a subacute pain in the left leg of increasing severity. Two years previously, during pregnancy, she had presented with bilateral avascular necrosis of the femoral head and was diagnosed with sickle cell β-thalassaemia. Before this, she had never experienced any haemolytic crises and was therefore unaware of this diagnosis. Because of severe functional impairment, the orthopaedic surgeon was planning bilateral femoral head replacement. Apart from the pain in her left leg her history at presentation was unremarkable. She came to the emergency department because she suspected a sickle cell crisis. At physical examination she did not appear ill. Her BMI was 19 kg/m^2. She had no fever. The left leg was shortened and positioned in endorotation. Laboratory tests were normal apart from known haemolysis. Because of the increased pain the orthopaedic surgeon was consulted who ordered an X-ray (*figures 1* and *2*). She was admitted to the internal medicine ward and treated with hyperhydration and adequate pain medication. Several days later she was discharged.

WHAT IS YOUR DIAGNOSIS?

See page 295 for the answer to this photo quiz.

Figure 1. *Eight months prior to presentation*

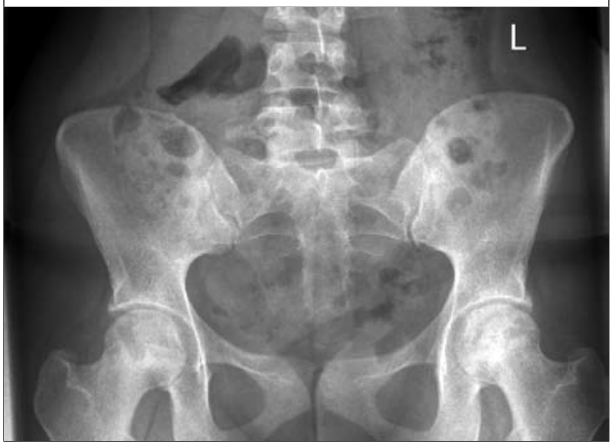
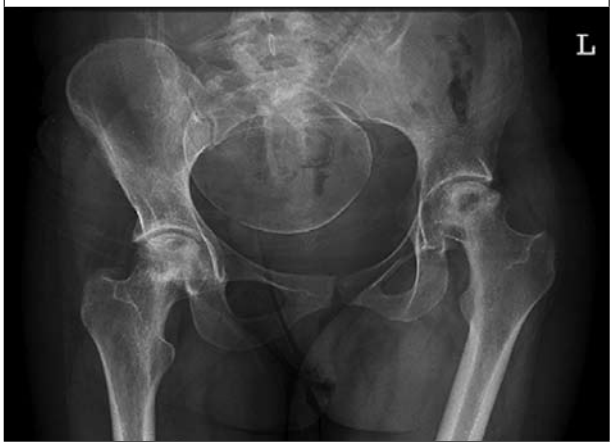


Figure 2. *At presentation*



Netherlands
The Journal of Medicine

PHOTO QUIZ

A patient with redness and swelling of his foot: rheumatoid arthritis?

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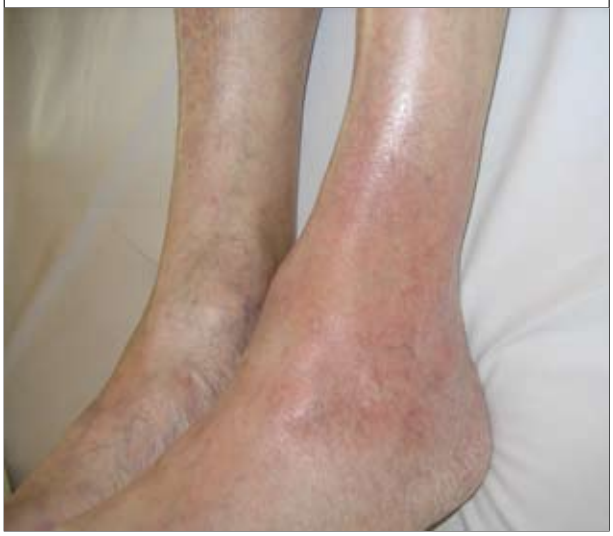
CASE REPORT

A 65-year-old man complained of swelling and pain of his left foot since one day (*figure 1*). He had a history of rheumatoid arthritis, for which he was taking azathioprine and oral corticosteroids. He had not experienced symptoms in this foot before, neither had he received local corticosteroid therapy. At the time of presentation he did not complain of pain in the other joints. Physical examination of all joints was normal. However, on the dorsum of the left foot there was localised redness of the skin with a palpable fluctuating mass. Ultrasound examination showed signs of tenosynovitis or peritendinitis, without signs of arthritis. Needle aspiration of the palpable mass revealed a red-yellow substance. Microscopic examination of the aspirated fluid is shown in *figure 2*.

Figure 1. *Left foot of the patient showing localised swelling and redness caused by the tenosynovitis or peritendinitis*



WHAT IS YOUR DIAGNOSIS?

See page 293 for the answer to this photo quiz.

Figure 2. *Microscopy of fluid obtained by fine needle aspiration (250x)*

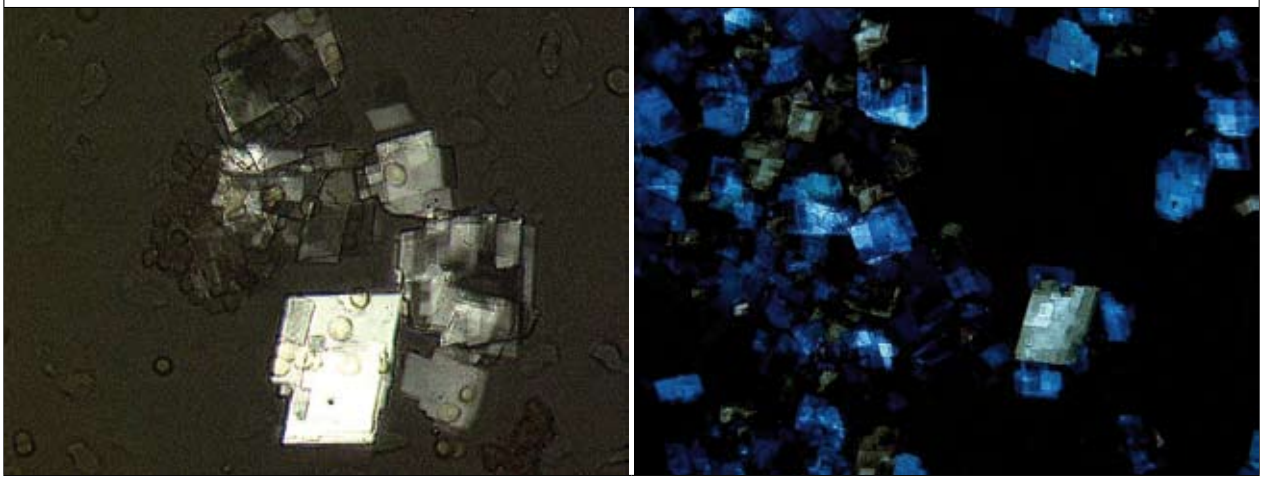


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PHOTO QUIZ

Turning green with shock

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CASE REPORT

A 65-year-old female rice farmer from north-east Thailand was brought to the emergency room by her family with complaints of fever and mental confusion since one day. At physical examination she was agitated and confused, hypotensive (80/40 mmHg), tachycardic (120 beats/min) and body temperature 38.5°C with warm peripheries. Otherwise, there were no findings suggestive of focal infection. Limited laboratory investigations were performed, which showed a normal leucocyte count, a mild anaemia and acute renal insufficiency (serum creatinine 150 µmol/l). After admission to the ward for intravenous fluid treatment, a urinary catheter was inserted and the patient was found to have green urine (*figure 1*).

Figure 1. *Green urine*

WHAT IS YOUR DIAGNOSIS?

See page 294 for the answer to this photo quiz.

ANSWER TO PHOTO QUIZ (PAGE 288)

ABDOMINAL PAIN, LOW GRADE FEVER AND PERSISTENT SHOCK

DIAGNOSIS

The abdominal computed tomography angiography (CTA) revealed active arterial bleeding from both large metastatic adrenal glands (8 cm) with old and fresh haematomas, rupturing into the peritoneal cavity, and metastases in the spleen. The presence of old and fresh haematomas suggested recurrent bleeding episodes, which had now been complicated by an acute event. The left adrenal artery was probably the culprit, because of the more pronounced contrast extravasation. The patient was treated with aggressive volume resuscitation, high-dose hydrocortisone (already given before ordering the CTA) and both adrenal arteries were successfully coiled. Surprisingly, CTA also revealed a large thrombus from the left hepatic vein up to the right atrium (not shown) for which no treatment was started. Nevertheless, our patient remained stable and was released from the hospital within two weeks. Because of the co-occurrence of hyponatraemia, hyperkalaemia and a low morning serum cortisol at time of presentation (0.228 µmol/l; ACTH not available), adrenal insufficiency was diagnosed and hormone replacement therapy was started. Her electrolyte disturbances disappeared subsequently.

Metastases in the adrenal gland are found in up to 50% of autopsied melanoma patients.[1] Bilateral adrenal masses larger than 5 cm in diameter on CT scan with irregular areas of necrosis or haemorrhage without lipomatous content are characteristic for malignant melanoma.[2] Patients may present with malaise, abdominal pain, flank pain or back pain. They may complain of nausea, vomiting or anorexia. In case of clinically overt adrenal insufficiency, hypotension, low grade fever, hyponatraemia and hyperkalaemia may be present. However, adrenal metastases do not usually cause hypocortisolism, because the adrenal cortex remains intact.[3] More often, patients have a partial adrenal insufficiency. Therefore, hydrocortisone replacement therapy should promptly be started in case of stress. However, symptoms are often attributed to the underlying malignancy, causing adrenal insufficiency to be overlooked.

Spontaneous arterial haemorrhage in the adrenal gland is a rare complication of metastatic disease.[3] Still, bleeding is no coincidence in malignant melanoma, but rather a characteristic of this tumour type. Although the usual treatment is adrenalectomy, bilateral adrenal melanoma metastases indicate advanced disease with a five-year survival rate of only 15%. Coiling may therefore be a good alternative to adrenalectomy.

REFERENCES

1. Dasgupta T, Brasfield R. Metastatic melanoma. A clinicopathological study. Cancer. 1964;17:1323-39.
2. Rajaratnam A, Waugh J. Adrenal metastases of malignant melanoma: Characteristic computed tomography appearances. Australas Radiol. 2005;49:325-9.
3. Seidenwurm D, Elmer E, Kaplan L, Williams E, Morris D, Hoffman A. Metastases to the adrenal glands and the development of Addison's disease. Cancer. 1984;54:552-7.

ANSWER TO PHOTO QUIZ (PAGE 290)

A PATIENT WITH REDNESS AND SWELLING OF HIS FOOT: RHEUMATOID ARTHRITIS?

DIAGNOSIS

Microscopic examination showed leucocytes and square-shaped ('plate-like') crystals. This shape is typical for cholesterol crystals. Cholesterol crystals are large, flat and rectangular with notched corners. Polarised light microscopy demonstrated that the crystals were moderately birefringent. Culture of the aspirated fluid was negative. Cholesterol concentration in the aspirated fluid was not measured. Serum total cholesterol, LDL cholesterol and triglycerides were normal. HDL cholesterol was slightly reduced (0.88 mmol/l, normal value >0.90 mmol/l). Apolipoprotein values were not determined. The patient had no cardiovascular symptoms, but received acenocoumarol because of recent venous thrombosis and pulmonary embolisms. The patient improved spontaneously.

Cholesterol crystals have been described to localise to arthritic fluid, bursal fluid and rheumatoid nodules of patients with rheumatoid arthritis. The pathophysiology of cholesterol crystal formation in these sites is not well known. Systemic causes that have been proposed are dyslipoproteinemia or formation of lipoprotein-directed antibodies, followed by deposition of antibody-antigen complexes in for example synovial fluids. However, deposits of cholesterol are usually considered to arise due to local factors, such as cell membrane degeneration, disturbed local cholesterol/lipoprotein metabolism and clearance or increased permeability to cholesterol because of chronic inflammation.

REFERENCES

1. Maldonado I, Eid H, Rodriguez GR, Rillo OL, Barcat JA, Reginato AJ. Rheumatoid nodulosis; is it a different subset of rheumatoid arthritis? J Clin Rheumatol. 2003;9:296-305.
2. Balint PV, Kane D, Sturrock RD. Case number 26: massive cholesterol crystal deposition: unusual location in rheumatoid arthritis. Ann Rheum Dis. 2003;62:512.
3. Jacobs JWG, Bijlsma JW. Diagnostic image (57). Tenosynovial swelling with cholesterol crystals in rheumatoid arthritis. Ned Tijdschrift Geneeskd. 2001;145:1886.
4. Gur H, Ehrenfeld M. The benign long term effect of cholesterol crystal synovial cysts. Clin Rheumatol. 1994;13:537-42.
5. Lazarevic MB, Skosey JL, Vitic J, Mladenovic, Myones BL, Popovic J, Swedler WI. Cholesterol crystals in synovial and bursal fluid. Semin Arthritis Rheum. 1993;2:99-103.

ANSWER TO PHOTO QUIZ (PAGE 291)

TURNING GREEN WITH SHOCK

DIAGNOSIS

The combination of discolouration of urine (blue-green), severe hypotension, and an altered consciousness level was suspected to be caused by intoxication with methylene blue. On specific questioning, her family reported her use of 'Marwitt's Wonder Kidney Pills', a non-prescription complementary medicine intended for patients with kidney problems, with methylene blue as one of the active ingredients (*figure 2*). It was unclear as to what dose and for how long she had used this drug. With only supportive treatment the patient made a quick and uneventful recovery and was discharged a few days after admission.

Methylene blue is used for the treatment of methaemoglobinaemia[1] as well as a dye for tests of surgical leaks and anastomoses and to stain the parathyroid glands in surgery. However, at high levels, a metabolite of methylene blue induces methaemoglobinaemia.[2] The discolouration noted in the urine is typical, and the intravenous administration of methylene blue is associated with apparent cyanosis and spuriously low oxygen saturations.[1-3] The acute ingestion of more than 4 mg/kg is potentially associated with serious toxicity. Treatment is generally supportive, although for acute ingestion, oral charcoal may prevent absorption. Exchange transfusion may be indicated if methaemoglobin levels are >70%. Overdosage is associated with nausea, abdominal pain, headache, sweating, hypotension, arrhythmias.[3] Caution is required in patients with glucose-6-phosphatase deficiency as methylene blue may cause severe haemolytic anaemia. In addition, methylene blue may also precipitate serotonergic syndrome in patients on SSRIs.[4]

Use of complementary 'herbal' medicines is common in Thailand. Adrenal crisis following discontinuation of herbal 'Yaa chud' or 'Yaa tom', containing dexamethasone, or precipitated by infections are a common problem (Cheng, personal experience). This case furthermore illustrates the potential disastrous side effect of the addition of methylene blue to non-prescription complementary medicine.

Figure 2. *Marwitt's Wonder Kidney Pills, a non-prescription complementary medicine intended for patients with kidney problems with methylene blue as one of the active ingredients*

REFERENCES

1. Strauch B, Buch W, Grey W, Laub D. Successful treatment of methemoglobinemia secondary to silver nitrate therapy. N Engl J Med. 1969:281:257.
2. Lamont AS, Roberts MS, Holdsworth DG, Atherton A, Shepherd JJ. Relationship between methaemoglobin production and methylene blue plasma concentrations under general anaesthesia. Anaesth Intensive Care. 1986;14:360.
3. Blass N, Fung D. Dyed but not dead--methylene blue overdose. Anesthesiology. 1976;45:458.
4. Khavandi A, Whitaker J, Gonna H. Serotonin toxicity precipitated by concomitant use of citalopram and methylene blue. Med J Aust. 2008;189:534.

ANSWER TO PHOTO QUIZ (PAGE 289)

A WOMAN WITH A PAINFUL HIP

DIAGNOSIS

The diagnosis is progression of bilateral avascular necrosis of the femoral head and advanced pregnancy. Unfortunately, the pregnancy was not seen by the orthopaedic surgeon, the resident internal medicine and the consulted resident radiology. Two weeks later she was seen again, this time because of a gastroenteritis. At physical examination she was found to be approximately 35 weeks pregnant. Because of deterioration of the foetal condition a caesarean section was performed the same evening without any complications. The birth weight of the baby was 2530 grams.

The patient was completely unaware of the pregnancy. Up until the last month she claimed to have had cyclic menstruation-like blood loss. Although the pathogenesis of this phenomenon is unknown, the prevalence of cyclic blood loss in pregnancy is estimated between 0.2 and 2.8%.[1,2] Factors that might have contributed to the 'denial' of pregnancy apart from the cyclic blood loss can be the fact that in sickle cell disease 21% of neonates are small for the gestational age.[3] Interestingly 70% of individuals presenting with denial of pregnancy report cyclic blood loss.[4] In contrast to general belief, no differences in demographics and/or education level were found compared with a control group.[1]

This case clearly demonstrates that one can very easily miss what one is not looking for.

REFERENCES

1. Wessel J, Endrikat J. Cyclic menstruation-like bleeding during denied pregnancy. Is there a particular hormonal cause? Gynecol Endocrinol. 2005;21:353-9.
2. Knörr K. Regelähnliche Blutungen in der Schwangerschaft und ihre forensischen Bedeutung. Zentralbl Gynäkol. 1953;75:1809-14.
3. Smith JA, Espeland M, Bellevue R, Bonds D, Brown AK, Koshy M. Pregnancy in sickle cell disease: experience of the Cooperative Study of Sickle Cell Disease. Obstet Gynecol. 1996;87:199-204.
4. Brezinka C, Huter O, Biebl W, Kinzl J. Denial of pregnancy: obstetrical aspects. J Psychosom Obstet Gynaecol. 1994;15:1-8.

Netherlands
The Journal of Medicine

SPECIAL REPORT

Current awareness of delirium in the intensive care unit: a postal survey in the Netherlands

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ABSTRACT

Background: Delirium in the ICU can compromise the recovery process, prolong ICU and hospital stay and increase mortality. Therefore, recognition of delirium is of utmost importance.

Methods: To ascertain current attitude pertaining to delirium in critically ill patients a simple questionnaire was sent to all intensive care units (ICUs) throughout the Netherlands.

Results: Seventy-five questionnaires were sent and 44 returned. A delirium protocol was present in the majority of cases (n=35, 80%), although implementation had occurred in only 22 ICUs (50%). The reported general incidence of delirium varied widely (<10-75%), but most participants thought it to occur in >25% of ventilated patients (n=33, 75%) and in patients older than 70 (n=38, 86%). Most participating centres reported that they could certainly (n=9, 20%) or most certainly (n=22, 50%) identify delirium. A geriatrician or a psychiatrist predominantly diagnosed delirium (n=30, 68%), while a diagnostic instrument such as the CAM-ICU was used in a minority of cases (n=11, 25%). A geriatrician or a psychiatrist was consulted when patients were agitated (n=40, 90%), or when routine pharmacological treatment had failed (n=40, 91%).

Conclusion: In the Netherlands, delirium is considered an important problem in the ICU, although its incidence is estimated to be low by the ICU team. The diagnosis of delirium is most frequently established by a geriatrician or psychiatrist after consultation, while diagnostic instruments are infrequently used. Efforts should be undertaken to implement delirium protocols and a routinely applied diagnostic instrument in the ICU.

KEYWORDS

Delirium, diagnostic instrument, CAM-ICU, ICU, postal survey

INTRODUCTION

Patients in the intensive care unit (ICU) are at increased risk for development of delirium. Factors such as age, multiple-system illnesses, comorbidities and the use of psychoactive medications all increase the risk.[1] Up to 60 to 80% of mechanically ventilated ICU patients eventually develop delirium.[2] During ICU stay, the presence of this syndrome is associated with a higher morbidity, such as a prolonged length of stay in the ICU, cognitive decline at follow-up months to years later, and even mortality.[2-11]

Delirium is defined as an acute change or fluctuation in mental status, combined with disorganised thinking or an altered level of consciousness.[12] However, the presence of these symptoms may be easily overlooked because of its fluctuating nature.[13] Pandharipande *et al.*[2] reported that as many as 32 to 66% of cases remain unrecognised by the managing physicians and nurses. In view of the high incidence and high mortality in combination with the under-recognition of delirium, a recent report of the Dutch Healthcare Inspectorate[14] recommended that care of patients with delirium should be markedly improved and also that assessment of delirium by validated diagnostic instruments should be part of routine management. Implementation of a diagnostic instrument will increase awareness of delirium and, hopefully, initiate a thorough search for an underlying cause or explanation to answer the

question why delirium symptoms are present in a patient and initiate treatment.
In this study we aimed to determine the current attitude towards delirium in ICUs in the Netherlands.

METHODS

In June 2007, an anonymous questionnaire was sent to the nursing staff of all non-paediatric ICUs with more than five beds suitable for mechanical ventilation in the Netherlands. The nurses were specifically asked in a covering letter to discuss the survey with the medical director and have him edit and/or complete the questionnaire. After three months all ICUs that had not yet responded to the questionnaire were contacted by telephone and if required, the questionnaire was sent a second time.

A questionnaire was developed containing four parts (see appendix A for details);

1. Demographic questions regarding the hospital and ICU settings.
2. Questions pertaining to the presence and implementation of some kind of delirium protocol.
3. Questions addressing the clinical importance of treatment of delirium judged by the ICU team.
4. Questions pertaining to the role of a geriatrician or psychiatrist in establishing the diagnosis delirium.

STATISTICAL ANALYSIS

Analysis was performed on anonymous data using the Statistical Package for the Social Sciences (SPSS) version 14 (Chicago IL, USA). Categorical data were presented in percentages. Data are presented in a descriptive way. Proportions were compared using χ^2 analysis or Fisher's exact test if applicable.

Results

Questionnaires were sent to 75 ICUs. A total of 38 ICUs spontaneously returned the questionnaire, with an additional six after contacting the non-responsive units by telephone. This resulted in a total of 44 returned questionnaires (59%). Four hospitals turned out to have less than five beds suitable for mechanical ventilation at the time of the survey, 29 hospitals had 5 to 15 beds, three hospitals had 15 to 20 beds and eight hospitals had more than 20 beds available for mechanical ventilation. Eight ICUs were located in academic hospitals and 36 ICUs were located in non-academic hospitals. All ICUs had a daily meeting with other disciplines.

Presence of a delirium protocol

A delirium protocol was present in the majority of hospitals (n=35, 80%). Seven out of the eight (88%) academic ICUs reported having a delirium protocol and 78% (28 out of 36) of the non-academic ICUs. However, practical implementation of a delirium protocol was reported in only 22 ICUs (50%). Although all ICUs with less than five beds reported the implementation of a delirium protocol, only one academic ICU (13%) and 16 non-academic ICUs (44%) implemented such a protocol routinely in daily care. No difference was found in implementation when academic and non-academic hospitals were compared. Treatment of delirium was judged clinically important by 72% of the ICU teams (*table 1*).

Clinical judgment of delirium by the ICU teams

The estimated incidence of delirium varied widely from less than 10% to 75%. When delirium is present, most participants estimated this to occur in more than 25% of ventilated patients (n=33, 75%) and in patients older than 70 (n=38, 86%) (*table 2*). Most centres reported that they could certainly (n=9, 20%) or most certainly (n=22, 50%) identify delirium (*table 3*). No difference was found with respect to the findings when academic and non-academic hospitals were compared. The diagnosis of delirium was

Table 1. *The presence, implementation and importance of a delirium protocol in the ICU*

		All centres (n=44)	Academic ICUs (n=8)	Non-academic ICUs (n=36)
Presence of a protocol (n,%)	Yes	35 (80%)	7 (88%)	28 (78%)
Implementation of a protocol (n, %)	Yes	11 (25%)	2 (25%)	9 (25%)
	No	17 (39%)	1 (13%)	16 (44%)
	Sometimes	7 (16%)	3 (38%)	4 (11%)
	Partly	4 (9%)	1 (13%)	3 (8%)
	Not known	5 (11%)	1 (13%)	4 (11%)
Importance of delirium, treatment and delirium ICU protocol by medical staff (n, %)	Very important	13 (30%)	4 (50%)	9 (25%)
	Important	19 (43%)	1 (13%)	18 (50%)
	Neutral	5 (11%)	2 (25%)	3 (8%)
	Not very important	3 (7%)	0 (0%)	3 (8%)
	Not important	4 (9%)	1 (13%)	3 (8%)

Table 2. *Incidence of delirium during ICU admittance*

		All centres (n=44)	Academic ICUs (n=8)	Non-academic ICUs (n=36)
Percentage of patients developing delirium during ICU admittance (n, %)	< 10%	4 (9%)	0 (0%)	4 (11%)
	10-25%	11 (25%)	0 (0%)	11 (31%)
	26-50%	10 (23%)	3 (38%)	7 (19%)
	51-75%	10 (23%)	3 (38%)	7 (19%)
	76-100%	2 (5%)	0 (0%)	2 (6%)
	No answer	7 (16%)	2 (25%)	5 (14%)
Percentage of mechanically ventilated patients developing delirium (n, %)	<10%	3 (7%)	0 (0%)	3 (8%)
	10-25%	2 (5%)	0 (0%)	2 (6%)
	26-50%	18 (41%)	3 (38%)	15 (42%)
	51-75%	7 (16%)	2 (25%)	5 (14%)
	76- 00%	7 (16%)	1 (13%)	6 (17%)
	No answer	7 (16%)	2 (25%)	5 (14%)

Table 3. *Recognition, diagnosing delirium and checking for delirium*

		All centres (n=44)	Academic ICUs (n=8)	Non-academic ICUs (n=36)
Recognition of delirium (n, %)	Certainly	9 (20%)	3 (38%)	6 (17%)
	Most certainly	22 (50%)	3 (38%)	19 (53%)
	Neutral	7 (16%)	0 (0%)	7 (19%)
	Not always	3 (7%)	1 (13%)	2 (6%)
	Totally not	1 (2%)	0 (0%)	1 (3%)
	No answer	2 (5%)	1 (13%)	1 (3%)
Diagnosing of delirium (n, %) (multiple answers possible)	Geriatrician/psychiatrist	30 (68%)	5 (63%)	25 (69%)
	ICU nurse	37 (84%)	8 (100%)	29 (81%)
	CAM-ICU	11 (25%)	1 (13%)	10 (28%)
	Clinical impression	25 (57%)	6 (75%)	19 (53%)
	Delirium-O-Meter	5 (11%)	2 (25%)	3 (8%)
	Trained ICU nurse	11 (25%)	4 (50%)	7 (19%)
	Other methods	9 (20%)	1 (13%)	8 (22%)
Checking for delirium (n, %)	Once a day	2 (5%)	0 (0%)	2 (6%)
	3 times a day	14 (32%)	3 (38%)	11 (31%)
	>3 times a day	8 (18%)	0 (0%)	8 (22%)
	No check	12 (27%)	3 (38%)	9 (25%)
	Don't know	1 (2%)	1 (13%)	0 (0%)
	No answer	7 (16%)	1 (13%)	6 (17%)

established by the attending nurses at the bedside in 84% of cases, whereas consultation of specially trained nurses was sought in 25% of cases, a geriatrician or psychiatrist in 68% of cases and using own experience in 57% of cases. A diagnostic instrument such as the Confusion Assessment Method for the ICU (CAM-ICU) was used in only a minority of cases (n=11, 25%). During the clinical judgement of delirium the degree of sedation was judged by using the Ramsay score (n=28, 64%) or the Richmond Agitation and Sedation Scale (RASS, n=9, 20%). The frequency of checking for the presence of delirium varied from never (n=12, 27%) to more than three times a day (n=8, 18%). This was not different in academic or non-academic hospitals. Delirium was predominantly treated with haloperidol (n=32, 73%), while non-pharmacological measures such as regulation of the sleep-wake cycle (n=25, 57%), incorporating the family in the ICU treatment (n=25, 57%), or improving the patient's feelings of safety (n=25, 57%) were also taken frequently (all measures taken together: n=25, 57%). Participants reported that delirium has a great impact on the duration of mechanical ventilation (n=39, 89%), the length of ICU stay (n=41, 93%), the mortality (n=32, 73%), the long-term cognitive function (n=17, 39%) and costs (n=41, 93%).

Role of a geriatrician or a psychiatrist in diagnosing delirium

A geriatrician was present in 30 hospitals, while he attended the daily ICU multidisciplinary meeting in only one hospital. A psychiatrist was present in all hospitals, but never attended the daily ICU multidisciplinary meeting. A geriatrician was consulted when patients were agitated (n=15, 34%) or when routine pharmacological treatment had failed (n=12, 27%). Worth mentioning is that 75% of the academic ICUs never consulted a geriatrician. A psychiatrist was consulted when patients were agitated (n=25, 57%) or when routine pharmacological treatment had failed (n=28, 64%) (*table 4*).

Table 4. *The role of a geriatrician or psychiatrist in ICU patients*

	All centres (n=44)	Academic ICUs (n=8)	Non-academic ICUs (n=36)
When is a geriatrician consulted (multiple answers possible)			
Every patient >70 year	2 (5%)	1 (13%)	1 (3%)
Agitated patients >70 years	9 (20%)	1 (13%)	8 (22%)
Agitated patients <70 years	6 (14%)	1 (13%)	5 (14%)
Whenever delirium was noticed using diagnostic instrument	3 (7%)	1 (13%)	2 (25%)
Patients with sleep disorders	2 (5%)	0 (0%)	2 (5%)
Routine pharmacological treatment fails	12 (27%)	2 (25%)	10 (28%)
Patient with cognitive disorders	8 (18%)	1 (13%)	7 (19%)
Never	17 (39%)	6 (75%)	11 (31%)
When is a psychiatrist consulted -(multiple answers possible)			
Every patient >70 years	1 (2%)	0 (0%)	1 (3%)
Agitated patients >70 years	13 (30%)	2 (25%)	11 (31%)
Agitated patients <70 years	12 (27%)	2 (25%)	10 (28%)
Whenever delirium was noticed using diagnostic instrument	8 (18%)	1 (13%)	7 (19%)
Patients with sleep disorders	5 (11%)	1 (13%)	4 (11%)
Routine pharmacological treatment fails	28 (64%)	5 (63%)	23 (64%)
Patient with cognitive disorders	9 (20%)	1 (13%)	8 (22%)
Never	13 (30%)	4 (50%)	9 (25%)

DISCUSSION

This survey suggests that in the Netherlands delirium is considered an important problem. The estimated incidence varied widely but was overall thought to be low. In addition, most ICUs reported that they could certainly or most certainly identify a delirium, although delirium was most frequently diagnosed by a geriatrician or psychiatrist after consultation. In addition, because psychiatrists are only consulted if the patient is hyperactive, the presence of delirium might be underestimated because hypoactive forms of delirium can easily be missed.

These results are remarkable because Pandharipande *et al.*[2] demonstrated that critically ill patients are at great risk for development of delirium and incidence appears to be as high as 80%. Consequently, the most important step in delirium management is early recognition. Incorporation of delirium assessment into clinical practice in the ICU using a validated tool may improve patient care and this is also recommended in the guidelines of the Society of Critical Care Medicine (SCCM).[15,16] In our survey most hospitals reported the presence of a delirium protocol. However, only a few Dutch ICU settings had really implemented the protocol into routine daily care. The Confusion Assessment Method for the ICU (CAM-ICU), which is a validated assessment tool for monitoring delirium in ICU patients,[1,2,17,18] was used in only a minority of cases (25%). Our results extend comparable screening data by van Eijk *et al.,* who demonstrated that only 14% of all Dutch ICUs routinely monitored ICU delirium, and only 7% used a validated instrument.[19] In contrast to their findings, the reported use of a validated instrument to detect delirium occurred most frequently in non-academic (28%) ICUs in comparison to its use in only one academic centre. However, the results are difficult to compare, since van Eijk *et al.* did not define the clear distinction between level 2 and 3 hospitals. Since level 3 hospitals included not only academic hospitals but also large teaching hospitals in their study, results may in fact be quite comparable to our data. Also, their study relates to a simple questionnaire by telephone yielding only very simple data and is therefore far from a complete survey, although this is claimed by the authors. Despite these differences, the results are comparable to our data, i.e. implementation of a validated tool to detect delirium is low in Dutch ICUs.

In our survey, delirium was predominantly treated with haloperidol and non-pharmacological measures were also taken frequently. Haloperidol is recommended by the SCCM guidelines as the drug of choice.[2] The use of haloperidol seems to be associated with lower mortality in patients who are mechanically ventilated for more than 48 hours,[3,20] although prospective data are still awaiting the results of ongoing trials.

Several limitations to our study should be mentioned. First, the response rate of participating centres was 59%. Although this is comparable to other previously performed surveys, this bares the question whether the results really reflect common attitude towards delirium in Dutch ICUs. However, we have no reason to think that responding centres represent a particular subset, also illustrated by the fact that the proportion of responding academic centres is comparable to responding non-academic centres. Also, we consider it hard to believe that implementation of validated delirium diagnostic instruments into daily critical care was considerably better in non-responding units when compared with ICUs that responded to our survey. Second, the results reflect awareness and approach to delirium in Dutch ICUs, which makes the translation to other countries and settings potentially difficult. However, the

lack of implementation of a validated delirium diagnostic instrument may be a phenomenon which applies in other countries as well.

CONCLUSION

Delirium is considered an important problem in Dutch ICUs, although its incidence is thought to be low. Diagnosis of delirium is most frequently established by a geriatrician or psychiatrist and a structural diagnostic instrument was used in only a few hospitals. Efforts should be undertaken by critical care nurses and physicians to implement a delirium protocol and a routinely applied diagnostic instrument into daily care to improve the recognition of delirium in ICU patients.

REFERENCES

1. Ely EW, Margolin R, Francis J, et al. Evaluation of delirium in critically ill patients: Validation of the Confusion Assessment Method for the Intensive Care Unit (CAM-ICU). Crit Care Med. 2001;29:1370-9.
2. Pandharipande P, Jackson J, Ely EW. Delirium. acute cognitive dysfunction in the critically ill. Curr Opin Crit Care. 2005;11:360-8.
3. Riekerk B, te Water W, Rommes JH, Spronk PE. Delier op de intensive care unit. Kritiek. 2007,5:3-11.
4. Gunther ML, Morandi A, Ely EW. Pathophysiology of delirium in the intensive care unit. Crit Care Clin. 2008,24:45-65.
5. Inouye SK, Schlesinger MJ, Lyndon TJ. Delirium. A symptom of how hospital care is failing older persons and a window to improve quality of hospital care. Am J Med. 1999,106:565-73.
6. Francis J, Martin D, Kapoor WN. A prospective study of delirium in hospitalized elderly. JAMA. 1990;263:1097-101.
7. Levkoff SE, Evans DA, Liptzin B, et al. Delirium: The occurrence and persistence of symptoms among elderly hospitilized patients. Arch Intern Med. 1992,152:334-40.
8. Williams-Russo P, Urquhart BL, Sharrock NE, Charloson ME. Post-operative delirium: Predictors and prognosis in elderly orthopaedic patients. J Am Geriatr Soc. 1992;40:759-67.
9. Legault SE, Joffe RT, Armstrong PW. Psychiatric morbidity during the early phase of coronary care for myocardial infarction: association with cardiac diagnosis and outcome. Can J Psychiatry. 1992,37:316-25.
10. Inouye SK, Rushing JT, Foreman MD, Palmer RM, Pompei P. Does delirium contribute to poor hospital outcomes? A three-site epidemiologic study. J Gen Intern Med. 1998;13:234-42.
11. Hebert PC, Drummond AJ, Singer J, Bernard GR, Russel JA. A simple multiple system organ failure scoring system predicts mortality of patients who have sepsis syndrome. Chest. 1993,104:230-5.
12. Thomason JW, Shintani A, Peterson JF, Pun BT, Jackson JC, Ely EW. Intensive care unit delirium is an independent predictor of longer hospital stay: a prospective analysis of 261 non-ventilated patients. Crit Care. 2005;9:R375-81.
13. De Jonghe JF, Kalisvaart KJ, Timmers JF, Kat MG, Jackson JC. Delirium-O-Meter: a nurses' rating scale for monitoring delirium severity in geriatric patients. Int Geriatr Psychiatry. 2005;20:1158-66.
14. http://www.minvws.nl/rapporten/cz/2005/de-oudere-patint-met-een-delirium-in-het-ziekenhuis.asp.
15. http:www.icudelirium.org/delirium/dl-treatment.html.
16. Jacobi J, Fraser GL, Coursin DB, et al. Clinical practice guidelines for the sustained use of sedatives and analgesics in the critically ill adult. Crit Care Med. 2002;30:119-44.
17. Pun BT, Cordon SM, Peterson JF, et al. Large-scale implementation of sedation and delirium monitoring in the intensive care unit: a report from two medical centers. Crit Care Med. 2005;33:1421-2.
18. Vreeswijk R, Toornvliet A, Honing M, et al. Validity of the Confusion Assessment Method for the Intensive Care Unit Dutch version (CAM-ICU). Neth J Crit Care. 2008;7:32-8.
19. Van Eijk MMJ, Kesecioglu J, Slooter AJC. Intensive care delirium monitoring and standardised treatment: a complete survey of Dutch intensive care units. Intensive Crit Care Nursing 2008;24:218-21.
20. Milbrandt EB, Kersten A, Kong L, et al. Haloperidol use is associated with lower hospital mortality in mechanically ventilated patients. Crit Care Med. 2005;33:226-9.

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SPECIAL REPORT

Second national serum bank for population-based seroprevalence studies in the Netherlands

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ABSTRACT

In 2006/2007 a large serum bank was established by means of a cross-sectional population-based study. This serum bank will be used to evaluate the Dutch national immunisation programme (NIP) by serosurveillance and additional immunological and epidemiological research. In this paper we describe the design of this population-based cross-sectional serosurvey and report the participation rates as well as general characteristics of the study population. A similar serum bank was collected in 1995/1996.

Dutch inhabitants (aged 0-79 years, men and women) were invited from 40 municipalities throughout the country and also from eight additional municipalities known with low vaccination coverage (LVC). An oversampling of the migrant population was performed.

Blood samples were obtained from all participants accompanied with extensive information on demographic and epidemiological data, such as vaccination history, risk factors and travelling.

In addition, sociodemographic data are available from individuals who declined to participate (non-response survey). Overall 33% of all invitees were included in this study. The serum bank comprises 6386 sera in the nationwide sample including the extra sample of immigrants (n=646) and 1518 sera from the LVC municipalities. The sera will be analysed for antibodies against all NIP antigens but will also be used for other infectious diseases research. Results of this second serosurveillance study will contribute to the discussion whether it is needed to reconsider the schedule and/or the vaccine components of the current National Immunisation Programme.

KEYWORDS

Immunisation, serosurveillance, serum bank, study design, the Netherlands, vaccines

INTRODUCTION

Vaccination of children through national immunisation programmes (NIP) has effectively reduced many of the target diseases and their complications. Despite lower disease incidence and smaller numbers of cases evaluation of the NIP is still essential. In this situation the role of serosurveillance, i.e. the assessment of antibody levels in serum in the evaluation of the NIP, becomes more evident. Groups with low immunity, e.g. as a result of waning of natural or vaccine-induced antibodies, could be identified. When necessary, vaccination strategies could be changed to prevent future epidemics of disease.

Although over the last decades the essential role of cellular immunity has become evident; for many pathogens antibody levels can be used as a surrogate for protection at the population level. Furthermore, even when correlates of protection are unclear (e.g. pertussis), insight into (the combination of) antibody levels, antibody affinity and response kinetics give an important indication about the protection and occurrence of infection.

In 2006/2007 a large study was conducted in the Netherlands to set up a population-based serum bank in which three additional substudies were incorporated (PIENTER 2). This is the second national Dutch serum bank, as a similar study was performed in 1995/1996 (PIENTER 1).[1] Results of this first study have been published.[2-6] The participation in the European Seroepidemiology Network (ESEN and ESEN2) enabled us

to compare our results internationally.[7-11] By repeating such studies we get insight into the changes of the population's immunity over time and in changes in infection pressure. Since the collection of the first serum bank in 1995, the NIP has changed (*table 1*). Schedules and vaccines used in the current National Immunisation Programme in the Netherlands can be found at http://www.rivm.nl/rvp/rijks_vp/vac_schema. An overview of the changes in recent years can be found in the annual report.[12]

In this paper we describe the design of this population-based cross-sectional serosurvey and report the participation rates as well as general characteristics of the study population. We focus on the improvements and additions implemented in the design and methods compared with the first PIENTER study.

Table 1. *Major changes in Dutch immunisation programme from 1997 onwards*

Year	Vaccination	Change in programme
1997	DTP-IPV-Hib	Schedule from 3,4,5 to 2,3,4 months
2001	aP	Introduction of booster at age of 4 years simultaneously administered with DT-IPV booster
2002	MenC	Introduction of conjugate MenC at age of 14 months simultaneously administered with MMR vaccine, catch-up for children up to 19 years
2005	aP	Change from whole cell to acellular vaccine in the first year of life
2006	Pneumo	Introduction of pneumococcal vaccination at 2,3,4, 11 months, simultaneously administered with DTPa-IPV-Hib (-HepB)
2009	HPV	Introduction for girls at age of 12 years with catch-up for girls up to 17 years

METHODS

The main objective of the PIENTER 2 study is to estimate age-specific seroprevalence to diseases included in the NIP for the following groups: general population, orthodox reformed individuals and non-Western immigrants. The participants were asked to donate blood and to complete a questionnaire.

Design of the study

General population

To assess the overall and age-specific seroprevalence for vaccine preventable diseases a nationwide sample was drawn in the Netherlands, using a two-stage cluster sampling technique. The study design was kept similar to that of the first serum bank in 1995/1996.[1] The Netherlands were first divided into five geographical regions of approximately equal population size. Within each of the five geographic regions, eight municipalities (e.g. clusters) were randomly drawn with a probability proportional to their size. Initially, in each of these 40 municipalities an age-stratified sample of 380 individuals was drawn from the population register. The age strata were 0 years, 1-4 years and thereafter intervals of five years 5-9, ..., 75-79 years. With exception of the first two age strata, 20 individuals per age stratum were sampled. In the first two age strata in each municipality, 40 individuals were sampled because of an expected lower response rate.[1] Due to the low response in certain age strata (0 and 20-39 years), the number of invited individuals was increased in these age strata during the study to 80 (infants) and 40 respectively. In total 17,223 individuals were invited in the nationwide sample.

Oversampling: communities with low vaccination coverage

Special attention was given to the group of orthodox reformed individuals who refuse vaccination for religious reasons and are socio-geographically clustered. The potential for epidemics of NIP diseases is high in this group because susceptibility levels increase as a result of absence of vaccine-induced antibodies, as was shown in the PIENTER-1 study.[1] Indeed, several outbreaks have occurred in these communities, namely polio type 3 in 1992/1993, measles in 1999/2000, rubella in 2004 and mumps in 2007/2008.[13-17] To assess overall and age-specific seroprevalence estimates in the low vaccination communities (LVCs) and in individuals who refuse vaccination based on religious grounds, an extra sample was taken in eight additional municipalities with low immunisation coverage. The vaccine coverage in these municipalities for three DTP-IPV immunisations in 2007 (birth cohort 2005) varied between 71 and 83%, which is considerably lower than the nationwide coverage (95%). Individuals were divided into three age strata: 0-9, 10-49 and 50-79 years. These age strata were chosen for the following reasons:

- Vaccinations in the NIP were given to children up to 9 years of age so within this group the effectiveness of the current NIP can be assessed.
- Individuals between 10 and 49 years could have participated in the NIP so that the effectiveness of the NIP in the longer term could be assessed.
- Individuals of 50 years and older did not participate in the NIP as no such programme was available at that time.

Antibodies measured in the group of participants of 50 years and older will mainly be derived upon natural infection. Similar to the nationwide sample an age-stratified sample of initially 380 individuals per municipality was

drawn. Also here the number of invited individuals was increased in certain age strata during the study. In total 4366 individuals were invited in the low immunisation coverage sample.

Additional aims in PIENTER 2 compared with the PIENTER 1 study

Oversampling: migrant population

Assessing the seroprevalence estimates in non-Western immigrants was added to the aims of the PIENTER 2 study. In the PIENTER 1 study it was shown that the number of non-Western immigrants was too low to reliably assess the seroprevalence in this group. This group has become a relatively large group in the Netherlands (11% of the total population in 2007, while 8% in 1996) and little is known about the immunity against vaccine preventable diseases. Van der Wal *et al.*[18] showed that in 2003 the vaccination coverage for DTP-IPV for the first-generation immigrants of 5-12 years of age born in Surinam, Morocco or Turkey and living in Amsterdam varied between 82 and 86%, which was lower than the average vaccination rate of 93%. Further, Pauw-Plomp *et al.*[19] showed that in 1984 the vaccination coverage for DTP-IPV for second generation immigrants of 1 to 14 years of age, whose mothers were born in Turkey or Morocco, was 41 and 43%, respectively, whereas 19 years later (in 2003) the vaccination rates were similar for 5-12 year old children with indigenous parents and children of immigrants.[18] It is unknown whether the seroprevalence in non-Western immigrants is currently different from the seroprevalence in the indigenous Dutch. For example, certain infectious diseases are still endemic in these non-Western countries, different immunisation schemes are used and frequent travelling to these countries takes place.

To assess the seroprevalence in non-Western immigrants extra individuals were invited from 12 municipalities of the nationwide sample. The number of migrant inhabitants, the distribution of country of births and the urbanisation degree were decisive factors in selecting the municipalities. The sampling of the individuals from the population register within each municipality was random, similar to the nationwide sample.

Twelve immigrant groups were distinguished by age group, country of birth and first or second generation (*table 2*). In total 2558 individuals were invited in this extra sample.

Table 2. *Immigrant groups were distinguished according to country of birth, age group and first and second generation*

Country of Birth	Age group	Generation
Morocco and Turkey	0-9 years	First generation
	0-9 years	Second generation
	10-49 years	First and second generation
	50-79 years	First and second generation
Suriname, Netherlands Antilles and Aruba	0-9 years	First generation
	0-9 years	Second generation
	10-49 years	First and second generation
	50-79 years	First and second generation
Other non-Western countries	0-9 years	First generation
	0-9 years	Second generation
	10-49 years	First and second generation
	50-79 years	First and second generation

Sampling additional objectives

Three additional studies were incorporated in this population-based serum collection, in contrast to the previous PIENTER project. The first additional study, which is part of the European modelling project Polymod, will provide insight into the spread of air-borne infections by estimating the number of social contacts between individuals by means of a diary.[20] About 1000 participants in the nationwide sample were randomly asked to complete the diary. The diary contained detailed questions on characteristics of social contacts with different individuals during one weekday including age, sex, location, duration, frequency and occurrence of physical contact.

The second additional study will provide insight into genetic differences between vaccine responders. For this purpose an extra blood sample or buccal swab for children less than five years old was taken for DNA isolation. The material will be used for research on genetic factors involved in cellular and humoral responses to infections, e.g. CD40, inducible co-stimulatory molecule (ICOS) or polymorphisms in Toll-like receptors.[21,22]

The third additional study aims to estimate the seroprevalence of food allergies by measuring IgE and assessing the suggested association of vaccination with (reported) allergies.[23] A special question on having disorders (e.g. COPD/asthma, eczema, hay fever, allergies and more specific certain food allergies) and whether these disorders were diagnosed by the general practitioner was included in the questionnaire.

Data collection

Data were collected from February 2006 to June 2007 in collaboration with the Public Health Service, an organisation well known to the local inhabitants. Each invited individual received a letter of invitation, along with a brochure containing information on the study, a questionnaire, an informed consent form, and a prescheduled appointment for blood donation. All invited individuals were asked to complete the questionnaire at home and to visit a clinic for the blood collection.

The questionnaire contained questions on demographic characteristics, vaccination history, health perception and diseases, activities possibly related to infectious diseases (e.g. travelling, profession, gardening), information related to sexually transmittable diseases for 15 to 79 year olds and opinion on vaccination-related topics for 0 to 14 year olds. The vaccination history of the participants was either checked by copying the vaccination certificates brought by the individuals to the clinic or a copy was retrieved from the regional vaccine administration offices archives.

Residents born in a foreign non-Western country received a letter of invitation in their own language (Turkish) or a partly translated letter in English, French and Arabian along with a Dutch version. To increase the response all invited individuals were approached by phone or mail one week before the consultation appointment. Individuals who refused to participate were asked to complete the questionnaire or as a second possibility to answer some questions for the non-response survey (by telephone or mail). Invitees who did not show up at one of the clinics were sent a reminder along with a non-response questionnaire. This questionnaire contained a limited number of questions on demographic characteristics, vaccination history and health perception. From all invited persons information on age, gender, residence, country of birth and country of birth of both parents was available from the population register. Participants were offered a gift voucher.

The study proposal was approved by the Medical Ethics Testing Committee of the foundation of therapeutic evaluation of medicines (METC-STEG) in Almere (clinical trial number: ISRCTN 20164309).

Serum processing and storage

A blood sample was taken at the clinic and transported to the laboratory the same day, where the blood samples were stored in a cold room (4°C) overnight. From adults a maximum of 22 ml blood was taken and depending on their age and the degree of discomfort, less blood (0.1 to 8 ml) was taken from children. The next day the blood was centrifuged (10 min at 2500 rpm) and divided into portions of 5 ml serum. One tube of serum per participant was thawed and aliquoted with a robot (Tecan 150) into 10 separate micronic blocks with different volumes and stored at -80°C until analysis. The other tubes with serum were stored at -80°C in different freezers. The blood samples and buccal swabs reserved for DNA isolation were stored at – 20°C.

Serology

With an NIP expanding over the past years and up till now consisting of 23 vaccine components involving 11 pathogens, it is crucial to have antibody determination techniques at one's disposal which are less serum consuming and take less analysis time than the already established methods such as ELISA. Multiplexing techniques (eg. Multiplex Immuno Assay, MIA with Luminex) are now state of the art in measuring large, population-based serum banks. Diphtheria, tetanus and pertussis (Pertussis toxin, Filamentous haemaglutin, Pertactin, Fimbriae 2/3) can be measured simultaneously with a small volume of serum.[24] Also the MIA determination of antibodies against *Neisseria* menigitidis type C (MenC), A, Y, W135 and *H. influenzae* type b (Hib) has been described.[25,26] This MIA technique has been described for other (candidate) NIP components such as human papilloma virus (HPV)[27,28] or is commercially available as a kit, e.g. mumps-measles-rubella-varicella or pneumococci. In the former population-based study this MIA technique was not yet available and most antibody levels were determined using the ELISA technique. Information on the functionality of the antibodies will be obtained using a neutralisation assay (NT) or serum bactericidal assay (SBA) similar to the former PIENTER study. Compared with the MIA, ELISA and especially the NT are labour intense techniques.

RESULTS

Serum bank

In total we have collected 6386 (32%) sera in the nationwide sample including the extra sample of immigrants (n=646, 25%) and 1518 (35%) sera in the low immunisation coverage sample. *Table 3* shows the participation and the obtained materials in the nationwide and in the low immunisation

Table 3. *Number of participants in the nationwide and low immunisation coverage sample, PIENTER 2 project 2006-2007, the Netherlands*

	Nationwide sample	Low immunisation coverage sample
	n (%)	n (%)
Total invited	19,781	4366
Materials obtained at clinic:		
• Blood and questionnaire	6351 (32.1%)	1517 (34.7%)
• Blood, no info questionnaire	35 (0.2%)	1 (0.02%)
• DNA	6207 (31.4%)	1469 (33.6%)
• Only questionnaire (visited consult)	135 (0.7%)	43 (1.0%)
• Only information population register	7 (0.04%)	
• Vaccination booklet	4583	932
• Diary	824	
Materials obtained otherwise:		
• Questionnaire (no consult visit)	1200 (6.1%)	354 (8.1%)
• Short questionnaire	1652 (8.4%)	450 (10.3%)
• Only information population register	10,402 (52.6%)	2001 (45.8%)

coverage sample. The number of persons who visited the clinic for blood collection (i.e. participants) was 8089. Almost all participants (95%) gave consent for DNA isolation. The vaccination history was confirmed for 68% (5515 of the 8089) of the total number of participants and for 4843 of the 6042 (80%) who were eligible for the vaccination programme. In total 824 of the 1162 (71%) diaries were completed by participants in the nationwide sample for the study on social contacts between individuals.

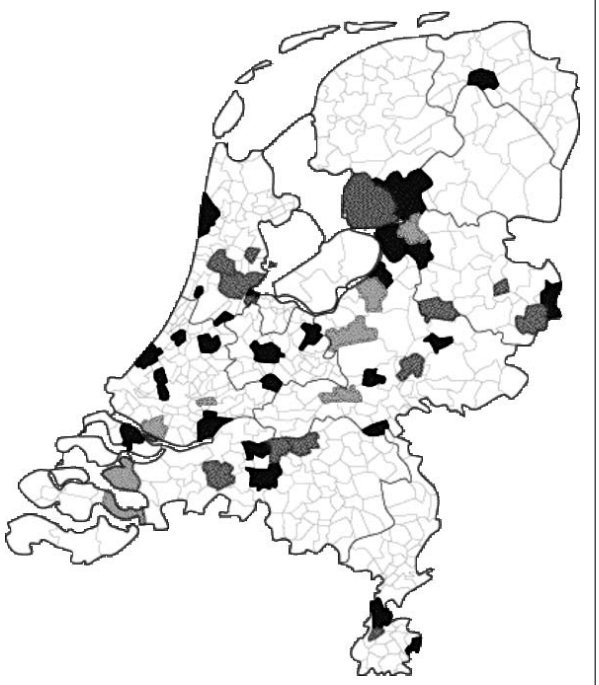
From the participants with a blood sample and a questionnaire, the highest response in the nationwide sample was seen in women aged 10-49 and 50-79 years and in the low immunisation coverage sample in women aged 10-49 years (*table 4*). Based on ethnicity the highest response was seen in indigenous Dutch and the lowest in individuals born in Morocco or Turkey. Overall, the response rate was higher in second-generation immigrants compared with first-generation immigrants.

In all age strata in the nationwide sample more than 260 participants are included (*figure 2*).

The number of participants in the age groups 1-4 and 5-9 years was highest (509 and 610 participants respectively) but note that the number of invited persons in the age group 0 and 1-4 years was twice as high as in the older age groups. The number of participants per age strata in the low vaccination coverage sample varied between 69 (35-39 years and 50-54 years) and 196 (1-4 years).

The median age in the nationwide sample was somewhat lower than in the low immunisation coverage sample and both were lower than the median age in the Dutch population, which is 38 (*table 5*; IQR: 19-54) (data, 1 January 2007, from the Central Bureau of Statistics (CBS). Further, 19% of the Dutch population considers themselves Protestant Christian, 28% Roman Catholic, 10% had another

Figure 1. *Selected municipalities in this study*



Black and dark grey municipalities are included in the nationwide sample, in the dark grey municipalities oversampling of immigrants also took place and the light grey municipalities are included in the low immunisation coverage sample.

Figure 2. *Number of participants per age stratum in the nationwide sample, stratified by gender*

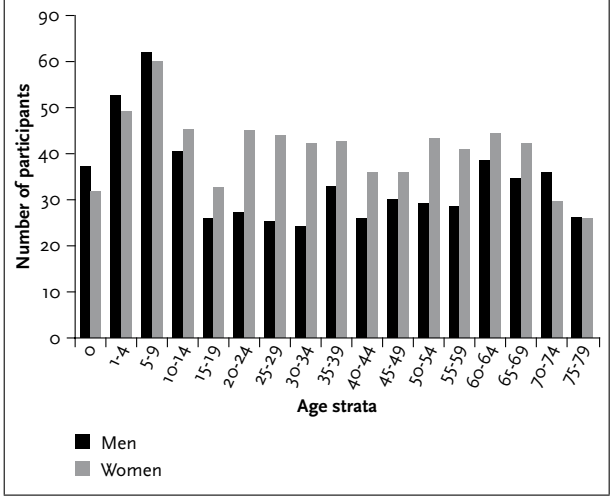


Table 4. *The response rate of participants with a blood sample and a questionnaire aged 0-79 years for age, gender, ethnicity and first and second generation in the nationwide sample and in the low immunisation coverage sample*

	Nationwide sample n (response rate %)	Low immunisation coverage sample n (response rate %)
Age and gender:		
• 0-9 years male/female	759 (27%)/705 (26%)	224 (32%)/188 (28%)
• 10-49 years male/female	1166 (26%)/1619 (38%)	290 (33%)/353 (44%)
• 50-79 years male/female	967 (36%)/1135 (38%)	220 (35%)/242 (35%)
Ethnicity:		
• Indigenous Dutch	4864 (36%)	1453 (35%)
• Morocco and Turkey	334 (21%)	3 (30%)
• Suriname and the Netherlands	352 (25 %)	3 (50%)
• Antilles and Aruba		
• Other non-Western countries	358 (23%)	11 (31%)
• Other Western countries	443 (29%)	47 (31%)
Generation:		
• First	799 (23%)	21 (26%)
• Second	688 (26%)	43 (36%)

Table 5. *Age and religion characteristics of participants with blood and a questionnaire aged 0-79 years in the nationwide sample and in the low immunisation coverage sample*

	Nationwide sample	Low immunisation coverage sample
	n (%)	n (%)
Median age (interquartile range)	34 (11-56) years	29 (9-55) years
Religion:		
• Protestant	1470 (23%)	1143 (76%)
• Roman Catholic	1806 (29%)	53 (4%)
• Other religion	812 (13%)	40 (3%)
• No religion	2215 (35%)	273 (18%)
Specific Protestant Christian religion:		
• Orthodox reformed	71 (5%)	326 (30%)
• Reformed bond	159 (12%)	294 (27%)
• Other specific Protestant Christian religion	1133 (83%)	450 (42%)

religion and 43% had no religion according to CBS, which is similar for the nationwide sample in our study. As expected, the percentage of individuals who consider themselves Protestant, orthodox reformed, and reformed bonders is much higher in the low immunisation coverage sample.

Seroprevalence estimates for all vaccine-preventable diseases in the NIP are part of this study: *Bordetella Pertussis* (Pertussis toxin, Filamentous haemaglutinin, Pertactin and Fimbriae2/3), diphtheria, tetanus, *Neisseria menigitidis* type C, A, Y, W135 and *H. influenzae* type b, *Streptococcus pneumoniae* (pneumo), mumps, measles, rubella, polio, hepatitis B, human papilloma and varicella viruses. Measuring antibody levels as a correlate of protection is generally accepted for several diseases and cut-off values are described. However, for other diseases (pertussis in particular) the correlate of protection and the antibody cut-off values are not established and research is ongoing.

The seroprevalence against other infectious diseases such as gastrointestinal infections (*Salmonella*, *Campylobacter*, *Noro* and hepatitis A), zoonotic infections (Q fever, *Toxoplasma*, *Toxocara*, *Echinococcus*, hepatitis E), vector borne infections (lyme, West Nile, dengue) or infections related to sexually transmitted diseases (herpes simplex virus, hepatitis C) can also be investigated.

DISCUSSION

Around ten years after the first serum bank, a second serum bank of the general Dutch population has been established with the aim to evaluate the Dutch national immunisation programme. The availability of a questionnaire and individual vaccination data is an important additional value for these population-based studies in contrast to a collection of residual sera.

Furthermore, oversampling of specific groups, such as orthodox reformed individuals who refuse vaccination on religious grounds (both studies) and immigrant groups (present study), enables us to study whether the protection against the target diseases of the NIP in these groups needs additional attention. In addition to the current target diseases, insight into the seroprevalence in the population against diseases for which a vaccine will be available in the near future, or already is available, i.e. varicella zoster and HPV, can be obtained.

In contrast to the first study, information on contact patterns of individuals in the population was collected in this study, which offers the unique opportunity to directly link the infection frequency according to age with age-specific contact patterns. Furthermore, additional studies on the incidence of allergies and genetic factors involved in vaccine responsiveness were incorporated in the study design.

The design is extensive and costly but efficient since a broad spectrum of study questions including both NIP and broader infectious diseases issues could be addressed. Also in relative terms, the costs of the study were only a very small proportion of the budget spent on the NIP in total.

Large serological studies have been done in other countries. The NHANES study in the United States is an important source of data for a variety of nutritional and health parameters[29,30] and the European Sero-Epidemiology Network (ESEN) aims at coordinating and harmonising serological surveillance in Europe. Some countries in the ESEN network obtained their serum bank through population-based random sampling whereas other countries could only collect residual sera. Although residual sera provide valuable information on immunity against infectious diseases[31] the sets of residual samples might not be representative for the general population and might lead to bias towards a more hospitalised or medical subpopulation.

In the first Dutch population study performed in 1995/1996 (PIENTER 1) the response rate was higher than in the present study (50 *vs* 33%). The designs were identical between the two studies to ensure maximal comparison of seroprevalence estimates and input for modelling analysis. There are several reasons which might explain the lower response rate in the current study. Most importantly, the study design included municipalities from which the participants were invited. Compared with 11-12 years ago the municipality boundaries in the Netherlands have become much larger, which means that invitees were expected to travel much further. In addition, most non-participating invitees stated that they were too busy and had no time to visit the clinic or did

not want to donate blood. Other recent large studies in the Netherlands reported a response rate of 26%[32] or 44%.[33] The slightly higher financial compensation and the individual limited health checks included in the Amsterdam study might have contributed to the higher response rate in Amsterdam.

The materials obtained in the PIENTER 2 study are well characterised and extensive information is available on the participants. However, although the design of the study ensures random selection of the participants, the response rate of 33% makes a non-response bias plausible. A thorough non-response analysis will therefore be performed by analysing the data from the non-response questionnaires and the data from the registers of the municipalities. Data from all Dutch inhabitants from the Central Bureau of Statistics will be used to interpolate the PIENTER 2 study to the general population. Part of the non-response bias in characteristics, which is most likely to be associated with immune status for vaccine-preventable disease (e.g. age, gender, country of birth), can be compensated by weighting the frequencies of seropositives to the Dutch population.

Antibody measurements and the corresponding analysis of the seroprevalences for all vaccine-preventable diseases in the NIP are in progress. Antibody levels above cut-off values are indicative for protection against disease. However, for some vaccines, vaccine-induced antibody levels will decrease within a few years after vaccination and eventually reach a pre-vaccination level.[34] Although antibody levels are low, protection based on memory immunity might be present. This indicates that serosurveillance based on the analysis of seroprevalence of antibodies alone will not provide a complete overview of or insight into immunity against infectious diseases. Further laboratory tests using functional assays (Opsonophagocytosis assay, NT and SBA) and better insight into more profound immunological processes such as memory immunity are essential to evaluate the present national immunisation programme completely.

CONCLUSION

We have established a new population-based serum bank together with additional information from questionnaire, vaccination booklet and diary. This material will primarily be used to evaluate the Dutch immunisation programme. Results of this second serosurveillance study might contribute to the discussion whether it is necessary to reconsider the schedule and/or the vaccine components of the current NIP. Certainly it will strengthen our insight into the Dutch immunisation programme and it will contribute to the confidence in the NIP and to the continuation of its success.

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REFERENCES

1. De Melker HE, Conyn-van Spaendonck MA. Immunosurveillance and the evaluation of national immunization programmes: a population-based approach. Epidemiol Infect. 1998;121:637-43.
2. Van den Hof S, de Melker HE, Berbers GA, der Kraak PH, Spaendonck MA. Antibodies to Haemophilus influenzae serotype b in the Netherlands a few years after the introduction of routine vaccination. Clin.Infect Dis. 2001;32(1):2-8.
3. De Melker HE, van den Hof S, Berbers GA, Conyn-van Spaendonck MA. Evaluation of the national immunisation programme in the Netherlands: immunity to diphtheria, tetanus, poliomyelitis, measles, mumps, rubella and Haemophilus influenzae type b. Vaccine. 2003;21:716-20.
4. Termorshuizen F, Dorigo-Zetsma JW, de Melker HE, van den Hof S, Conyn-van Spaendonck MA. The prevalence of antibodies to hepatitis A virus and its determinants in The Netherlands: a population-based survey. Epidemiol Infect. 2000;124:459-66.
5. Kortbeek LM, de Melker HE, Veldhuijzen IK, Conyn-van Spaendonck MA. Population-based Toxoplasma seroprevalence study in The Netherlands. Epidemiol Infect. 2004;132:839-45.
6. Veldhuijzen IK, Conyn-van Spaendonck MA, Dorigo-Zetsma JW. Seroprevalentie van hepatitis B en C in de Nederlandse bevolking. Infectieziekten Bull. 1999;10:182-4.
7. Osborne K, Weinberg J, Miller E. The European Sero-Epidemiology Network. Euro Surveill. 1997;2:29-31.
8. Edmunds WJ, Pebody RG, Aggerback H, et al. The sero-epidemiology of diphtheria in Western Europe. ESEN Project. European Sero-Epidemiology Network. Epidemiol Infect. 2000;125:113-25.
9. De Ory F, Echevarria JM, Kafatos G, et al. European seroepidemiology network 2: Standardisation of assays for seroepidemiology of varicella zoster virus. J Clin Virol. 2006;36:111-8.
10. Pebody RG, Andrews N, Brown D, et al. The seroepidemiology of herpes simplex virus type 1 and 2 in Europe. Sex Transm Infect. 2004;80:185-91.
11. Pebody RG, Gay NJ, Giammanco A, et al. The seroepidemiology of Bordetella pertussis infection in Western Europe. Epidemiol Infect. 2005;133:159-71.
12. De Melker HE, van Lier EH. The National Immunisation Programme in the Netherlands; developments in 2008. 2009; 210021009/2009.
13. Oostvogel PM, van Wijngaarden JK, van der Avoort HG, et al. Poliomyelitis outbreak in an unvaccinated community in The Netherlands, 1992-93. Lancet. 1994;344:665-70.
14. Hahne S, Ward M, Abbink F, et al. Large ongoing rubella outbreak in religious community in the Netherlands since September 2004. Euro Surveill. 2005;10:E050303
15. Karagiannis I, van Lier A, van Binnendijk R, et al. Mumps in a community with low vaccination coverage in the Netherlands. Euro Surveill. 2008;13:pii=18901.
16. Van Binnendijk RS, Hahne S, Timen A, et al. Air travel as a risk factor for introduction of measles in a highly vaccinated population. Vaccine. 2008;26:5775-7.

17. Van den Hof S, Meffre CM, Conyn-van Spaendonck MA, Woonink F, de Melker HE, van Binnendijk RS. Measles outbreak in a community with very low vaccine coverage, the Netherlands. Emerg Infect Dis. 2001;7(3 Suppl):593-7.

18. Van der Wal MF, Diepenmaat AC, Pel JM, Hirasing RA. Vaccination rates in a multicultural population. Arch Dis Child. 2005;90:36-40.

19. Pauw-Plomp H, van Wieringen JCM. Vaccination status of Amsterdam Children. Amsterdam: GG&GD. 1985;

20. Mossong J, Hens N, Jit M, et al. Social contacts and mixing patterns relevant to the spread of infectious diseases. PloS Med. 2008;5:e74

21. McAdam AJ, Greenwald RJ, Levin MA, et al. ICOS is critical for CD40-mediated antibody class switching. Nature. 2001;409:102-5.

22. Dhiman N, Ovsyannikova IG, Vierkant RA, et al. Associations between SNPs in toll-like receptors and related intracellular signaling molecules and immune responses to measles vaccine: preliminary results. Vaccine. 2008;26:1731-6.

23. Kemp T, Pearce N, Fitzharris P, et al. Is infant immunization a risk factor for childhood asthma or allergy? Epidemiology. 1997;8:678-80.

24. Van Gageldonk PG, van Schaijk FG, van der Klis FR, Berbers GA. Development and validation of a multiplex immunoassay for the simultaneous determination of serum antibodies to Bordetella pertussis, diphtheria and tetanus. J Immunol Methods. 2008;335:79-89.

25. De Voer RM, Schepp RM, Versteegh FG, van der Klis FR, Berbers GA. Simultaneous detection of Haemophilus influenzae type b polysaccharide-specific antibodies and Neisseria meningitidis serogroup A, C, Y, and W-135 polysaccharide-specific antibodies in a fluorescent-bead-based multiplex immunoassay. Clin Vaccine Immunol. 2009;16:433-6.

26. Lal G, Balmer P, Joseph H, Dawson M, Borrow R. Development and evaluation of a tetraplex flow cytometric assay for quantitation of serum antibodies to Neisseria meningitidis serogroups A, C, Y, and W-135. Clin Diagn Lab Immunol. 2004;11:272-9.

27. Dias D, Van Doren J, Schlottmann S, et al. Optimization and validation of a multiplexed luminex assay to quantify antibodies to neutralizing epitopes on human papillomaviruses 6, 11, 16, and 18. Clin Diagn Lab Immunol. 2005;12:959-69.

28. Waterboer T, Sehr P, Pawlita M. Suppression of non-specific binding in serological Luminex assays. J Immunol Methods. 2006;309:200-4.

29. McDowell A, Engel A, Massey JT, Maurer K. Plan and operation of the Second National Health and Nutrition Examination Survey, 1976-1980. Vital Health Stat 1. 1981;(15):1-144.

30. Birkner R. Plan and initial program of the health examination survey. Vital Health Stat 1. 1965;(125):1-43.

31. Gidding H. Australia's national serosurveillance program. NSW Public Health Bull. 2003;14:90-3.

32. Viet AL, van den Hof S, Elvers LH, Seidell JC, Otten F, Van Veldhuizen H. RIVM Report: Risk factors and health in The Netherlands, a survey by municipal public. 2000; 260854003/2002.

33. Amsterdam health monitor study, Gezond zijn en gezond leven in Amsterdam. 2006.

34. Trotter CL, Borrow R, Findlow J, et al. Seroprevalence of antibodies against serogroup C meningococci in England in the postvaccination era. Clin Vaccine Immunol. 2008;15:1694-8.

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3. Powell LW, Isselbacher KJ. Hemochromatosis. In: Braunwald E, Fauci AS, Kasper DL, et al., editors. Harrison's Principles of Internal Medicine. 15th edition. New York: McGraw-Hill; 2001. p. 2257-61.

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