Heterophilic Antibody Interference in Immunometric Assays

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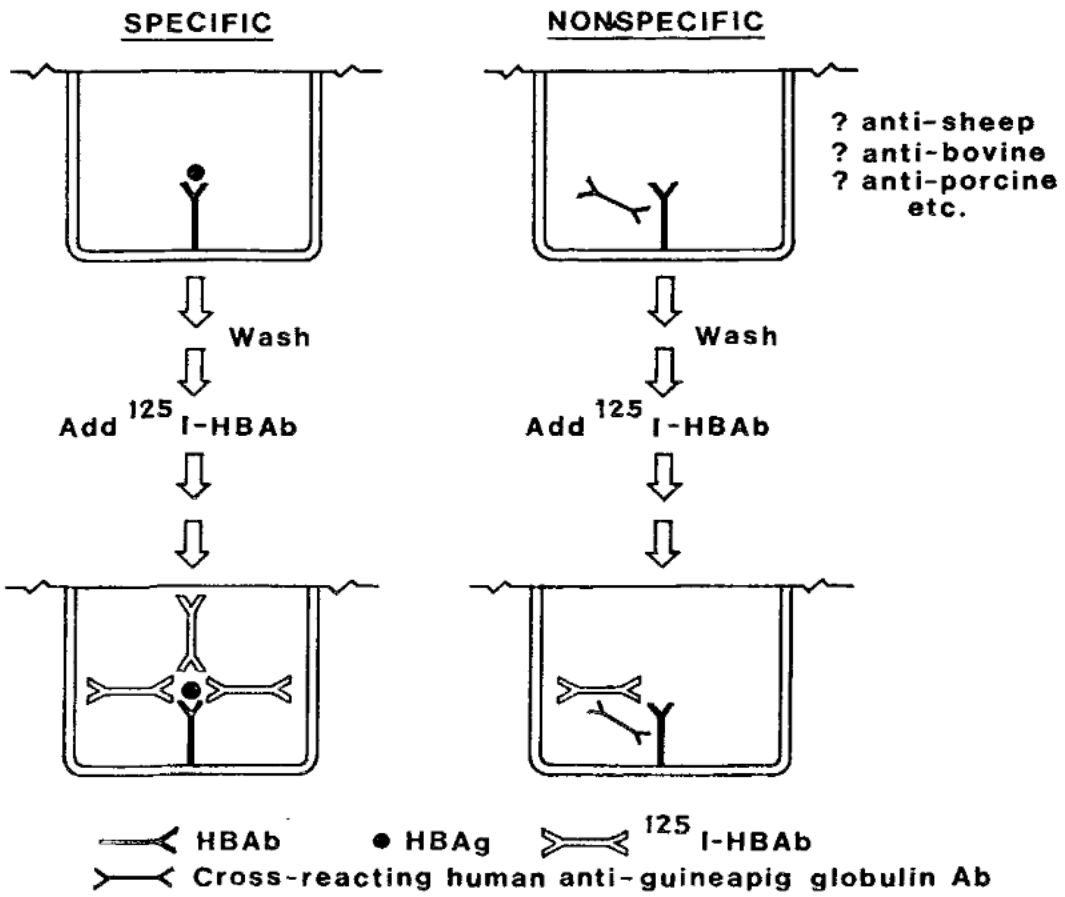

Fig. 2—A model for the mechanism of specific and non-specific direct solid-phase R.I.A. test results.

"Many people are very disturbed when told that they are chronic hepatitis-virus carriers - indeed this label may be both professionally and socially injurious. For this reason the test for HBAg is one clinical laboratory determination which should have, and need have, no false-positives."

Illustration and concluding remark in first publication describing heterophilic antibody interference in immunoassays, Alfred M. Prince et al., The Lancet, 1973 *(1)*. Used with permission obtained Jan. 25th, 2019 (license 4515910411603/4515910540019).

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First and foremost I would like to thank my supervisors Trine Bjøro, David Warren, Johan Bjerner and Kjell Nustad for all their help and generous contributions. Trine, I am extremely grateful that you always consider my opinion and input relevant (even when you don't have to), and that you always support me and my projects. Also, thank you for hiring me and making research and development an integrated part of my job in the department. Dave, thank you for sharing your broad knowledge and brilliant ideas with me. You make work fun, and you always find the best solutions to the problems I have, imagine or create. Johan, thank you for being a kind and supportive friend and colleague, and for helping me use and understand statistics (I can at least pretend). Kjell, thank you for including me in your projects, for your unconditional support and for teaching by example what altruism means. Your boundless energy and unwavering enthusiasm inspire everyone.

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List of contents

Abbreviations

ACTH	Adrenocorticotropic hormone
AFP	α-fetoprotein
ALP	Alkaline phosphatase
AST/ASAT	Aspartate aminotransferase
BNP	Brain natriuretic peptide
CA125	Cancer antigen 125
CEA	Carcinoembryonic antigen
CH1	First constant domain of antibody heavy chains
CV	Coefficient of variation
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamine tetraacetic acid
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
Fab’	Fragment antigen-binding (part of antibody binding to antigen)
$F(ab')_2$	Two Fab’-fragments linked by disulfide bond
FDA	U.S. Food and Drug Administration
Fc	Fragment crystallizable (tail part of antibody)
FSH	Follicle stimulating hormone
FT4	Free thyroxine
HA	Heterophilic antibody
HAMA	Human anti-mouse antibody
HARA	Human anti-rabbit antibody
HbAG	Hepatitis B antigen
hCG	Human choriongonadotropin
HIV	Human immunodeficiency virus
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
Ig-NAR	Immunoglobulin new antigen receptor (shark antibody)

IgY	Immunoglobulin Y (bird, reptile, lungfish antibody)
LH	Luteinizing hormone
MAK33/polyMAK	Mouse antibody to creatinine kinase (used as blocker in assays)
mAb	Monoclonal antibody
MS	Mass spectrometry
NCA-2	Non-specific cross-reacting antigen 2 (truncated CEA-variant)
pAb	Polyclonal antibody
PEG	Polyethylen glycol
PET/CT	Positron emission tomography/computed tomography
RF	Rheumatoid factor
scFv	Single-chain variable fragment
SHBG	Sex-hormone binding globulin
TNF-α	Tumor necrosis factor alpha
TSH	Thyroid stimulating hormone
VHH (nanobodies)	Antigen binding fragment of camel heavy chain only antibodies
V-NAR	Antigen binding single-domain fragment of Ig-NAR

List of papers

Paper I: Bolstad N, Kazaryan AM, Revheim ME, Distante S, Johnsrud K, Warren DJ, Nustad K, Edwin B. A man with abdominal pain: enough evidence for surgery? Clin Chem. 2012 Aug;58(8):1187-90.

Paper II: Bolstad N, Warren DJ, Bjerner J, Kravdal G, Schwettmann L, Olsen KH, Rustad P, Nustad K. Heterophilic antibody interference in commercial immunoassays; a screening study using paired native and pre-blocked sera. Clin Chem Lab Med. 2011 Sep 8;49(12):2001-6.

Paper III: Bjerner J, Bolstad N, Piehler A. Belief is only half the truth--or why screening for heterophilic antibody interference in certain assays makes double sense. Ann Clin Biochem. 2012 Jul;49(Pt 4):381-6.

Paper IV: Bolstad N, Warren DJ, Bjerner J, Aass HCD, Norum LF, Bjøro T, Nustad K. Prevalence and specificities of heterophilic antibodies in a large patient cohort. Manuscript.

Paper V: Bolstad N, Warren DJ, Nustad K. Heterophilic antibody interference in immunometric assays. Best Pract Res Clin Endocrinol Metab. 2013 Oct;27(5):647-61. Review.

Introduction

The work in this thesis was done at the Department of Medical Biochemistry, Oslo University Hospital, Radiumhospitalet. In our department, we have developed and provided in house automated immunometric assays for use in patient care since the 1980s, with particular focus on assays for serum tumor markers. Considerable efforts have been made to identify and prevent interference from heterophilic antibodies and anti-animal antibodies in patient samples, particularly following the hCG-scandal in the United States around the turn of the century, both in research performed at our department but also in our diagnostic routine. The work in this thesis is a result and continuation of this tradition.

Before discussing the details and impact of the projects in this thesis, a brief presentation of the history and recent developments of both immunoassay technology and antibody-mediated interference in immunoassays is given.

Immunoassays

Rosalyn Yalow received the Nobel Prize in Physiology or Medicine in 1977 for her groundbreaking work on radioimmunoassays for peptide hormones, most famously for insulin *(2)*. Yalow worked closely with Solomon A. Berson, who passed away in 1972 and was not eligible for the Nobel Prize in 1977, in developing methods to quantify proteins and peptides present in extremely low concentrations *(3-6)*. Naturally, other prominent reseachers also contributed to the early development of immunoassay technology. Roger Ekins developed some of the methods used by Yalow and Berson in his binding assays for thyroid hormones, where analytes were bound by purified specific binding proteins and not antibodies, and worked closely with Wallac Oy (Turku, Finland) to develop the non-isotopic DELFIA-technology we still use in our laboratory today *(7-9)*.

Exploiting the impressive affinity and specificity of antibodies, immunoassays have since had an enormous impact on modern medicine, both in medical research and clinical management of patients. Where early methods were labor intensive and time-consuming, typically relying on incubations spanning days, modern immunoassays are often fully automated on multi-analyzers and provide results in minutes. Today, both in primary care and specialized hospital departments, physicians rely on results from immunoassays to

diagnose and manage conditions as diverse as infections, pregnancies, malignancies and heart attacks. The ability to deliver reliable and rapid information, at low cost and with little risk of patient harm, ensures that immunoassay technology will continue to aid clinical decisions in the foreseeable future.

From patient antibodies and radioactivity to monoclonal antibodies and non-isotopic labels

Yalow and Berson used anti-insulin antibodies purified from human patient sera in their first assays for bovine insulin. These patients had been treated with bovine insulin, and the antisera did not bind human insulin with sufficient affinity to permit quantification of human insulin in their assays. However, they showed that guinea pigs immunized with bovine insulin produced antibodies that cross-reacted with human insulin with sufficient affinity to permit quantification of human insulin, even in fasting control subjects *(6)*. The insulin assay described by Yalow and Berson was extremely labor intensive and time consuming compared to modern standards. It involved long incubations and electrophoretic separation before counting radioactive emission, typically taking a week to complete. A simplified drawing of the assay principle of the first insulin assay is given in figure 1.

Until the late 1970s, assay antibodies were polyclonal antibodies from animals that had been immunized with the antigen in question. Guinea pigs, rabbits, sheep and goats were the most important antibody sources. A major drawback of polyclonal antibodies is that antibody production relies on individual, living animals, resulting in an unpredictable and inherently limited supply of the reagent an immunoassay is based on. Without a reliable supply of antibodies with predictable and comparable properties, commercial exploitation of immunoassay technology was hampered. This changed with the invention of the monoclonal antibody technology, which has, just as immunoassay technology, been immensely influential on the development of modern medicine *(10-13)*.

The production of monoclonal antibodies was described by Georges J.F. Köhler and César Milstein in 1975 *(14)*. They succeeded in fusing B-cells (producing antibodies) with myeloma cells (immortalized cell line), both from mice, creating hybridoma cell lines. Once the hybridoma producing the desired antibody has been isolated through a screening process,

Figure 1.

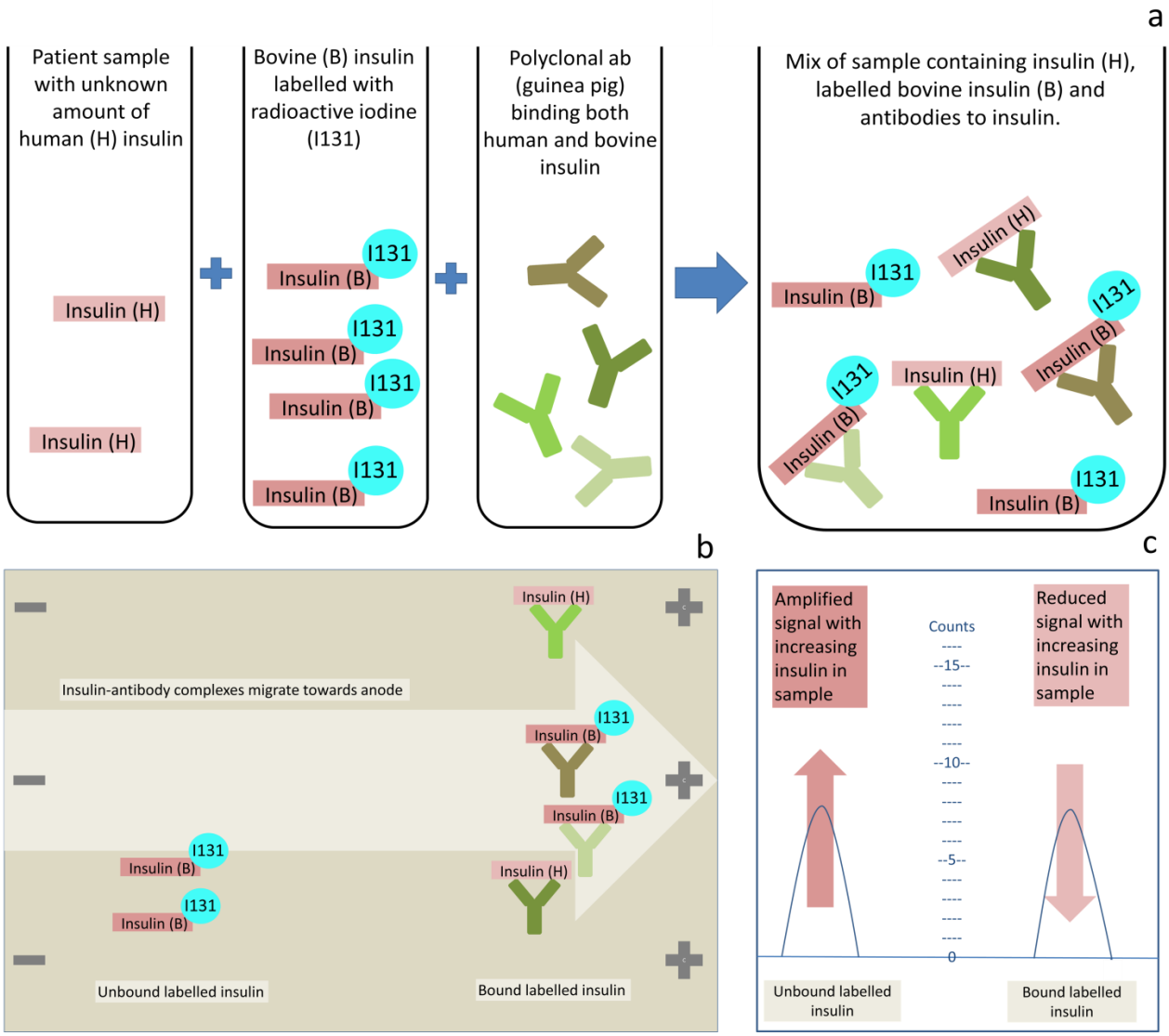

Figure 1. Simplified chart describing the assay for human insulin invented by Yalow and Berson *(5, 6)*. The patient sample, containing an unknown amount of insulin, is mixed with radiolabelled bovine insulin and a polyclonal guinea pig antibody which binds both human insulin and bovine insulin. Since the added antibodies have a limited binding capacity, and the two insulins compete for binding, the amount of unbound labelled bovine insulin is inversely related to the amount of human insulin in the sample (a). After an electrophoretic separation step, where bound insulin migrates away from unbound insulin (b), the amount of radioactivity emitted from unbound and bound insulin can be quantified using a gammacounter (c). More insulin in the sample means less labelled insulin gets bound to the antibodies, giving an increased signal from unbound insulin (cathodal peak) and a decreased signal from bound insulin (anodal peak). The concentration of insulin in the sample is calculated by comparing the signal ratio between bound and unbound labelled insulin in the sample with ratios found in solutions with known and increasing insulin concentrations (a standard curve). (Drawing by candidate.)

this hybridoma can (in theory) produce one identical antibody in cell culture medium indefinitely. The advent of monoclonal antibodies meant that large amounts of identical and specific antibodies could be delivered on demand for commercial immunoassay manufacture. Since the amount of available antibody was no longer a limiting factor, monoclonal antibodies could be combined to create immunometric assays (described below) with superior performance compared to the earlier competitive immunoassays. Another important improvement in immunoassay technology came with the discovery that monodisperse particles could be used as solid phases to reduce non-specific binding and assay durations. This groundbreaking work was performed in our department by my supervisor, Kjell Nustad, and had an enormous impact on clinical laboratory services *(15-18)*. Today, Ugelstad-beads (later Dynabeads) or similar monodisperse beads are used in most automated immunoassays produced by the major immunoassay companies, making it possible to provide clinicians with reliable assessments of e.g. cardiac troponins within minutes.

Current use of monoclonal antibodies in medicine is not limited to immunoassays intended for blood samples. They have largely replaced polyclonal antibodies in immunohistochemistry (pathology) and diagnostic imaging (nuclear medicine). Arguably, the greatest impact of monoclonal antibody technology is currently in therapy, where the use of antibody-based biologic drugs have revolutionised treatment of major disease groups such as inflammatory diseases, neurodegenerative diseases and cancer. The current commercial value of monoclonal antibody technology, and immunoassay technology for that matter, is immense *(19)*. In this regard it should be mentioned that Köhler and Milstein refrained from patenting their invention (as did Yalow and Berson), allegedly believing it belonged to all mankind *(20)*. Köhler and Milstein received the Nobel Prize in Physiology or Medicine in 1984 *(21)*.

Early immunoassays depended on radioactive isotopes as labels. Although radioactive labels may provide acceptable sensitivity, safety concerns and inherent instability complicate and limit their use in immunoassays. From the late 1970s, radioactive labels were increasingly replaced by enzyme labels, fluorophores and chemiluminescent labels *(22)*. In general, these new labels increased sensitivity and assay performance compared to the radioactive isotopes, and the practical advantages meant immunoassays became more accessible to

medical and research laboratories. Together with the availability of monoclonal antibodies, the movement away from radioactive isotopes paved the way for the automation and commercialization of immunoassay technology we know and depend on today *(13, 23, 24)*.

Assay formats

Yalow and Berson used antigen labelled with a radioactive isotope (tracer), hence the name radioimmunoassay, which competed with unlabeled antigen in samples for binding to immobilized antibodies. In such assay formats, the amount of bound labelled antigen is inversely proportional to the amount of antigen in the patient sample. Similar assays, commonly called ***competitive immunoassays***, are still used today to measure small analytes such as peptide hormones or vitamins, and radioactive isotopes have largely been replaced with other tracer molecules *(25)*. Larger analytes are usually measured by using two different antibodies to the antigen, one antibody attached to a solid phase (e.g. well or bead) and one antibody labelled with a tracer molecule. In this assay format, where the two assay antibodies form a sandwich complex with the antigen, the amount of bound labelled antibody is proportional to the analyte concentration. While commonly referred to as "sandwich assays", ***immunometric assays*** is the preferred term. While "ELISA", Enzyme-Linked ImmunoSorbent Assay, is a familiar term often used among medical professionals to refer to any immunoassay, it should be reserved for immunoassays relying on enzymatic processing of substrates to generate signals.

Both competitive and immunometric assays normally rely on one or more wash steps, and assays are often described using the number of wash steps and assay format (e.g. 3-step immunometric assay, 1-step competitive immunoassay etc.). ***Homogenous immunometric assays***, such as assays performed on the Kryptor immunoassay platform, are assays relying on the relative positioning of assay antibodies to create a quantifiable signal. These assays do not require wash steps, as the energy transfer that creates the light signal only occurs when the two assay antibodies (one labelled with donor kryptate, one labelled with acceptor) bind the same antigen *(26)*.

Immunoturbidimetric assays do not use traditional labelling of assay antibodies but rather rely on the formation of aggregates of antigen and assay antibodies to quantify an analyte.

Figure 2.

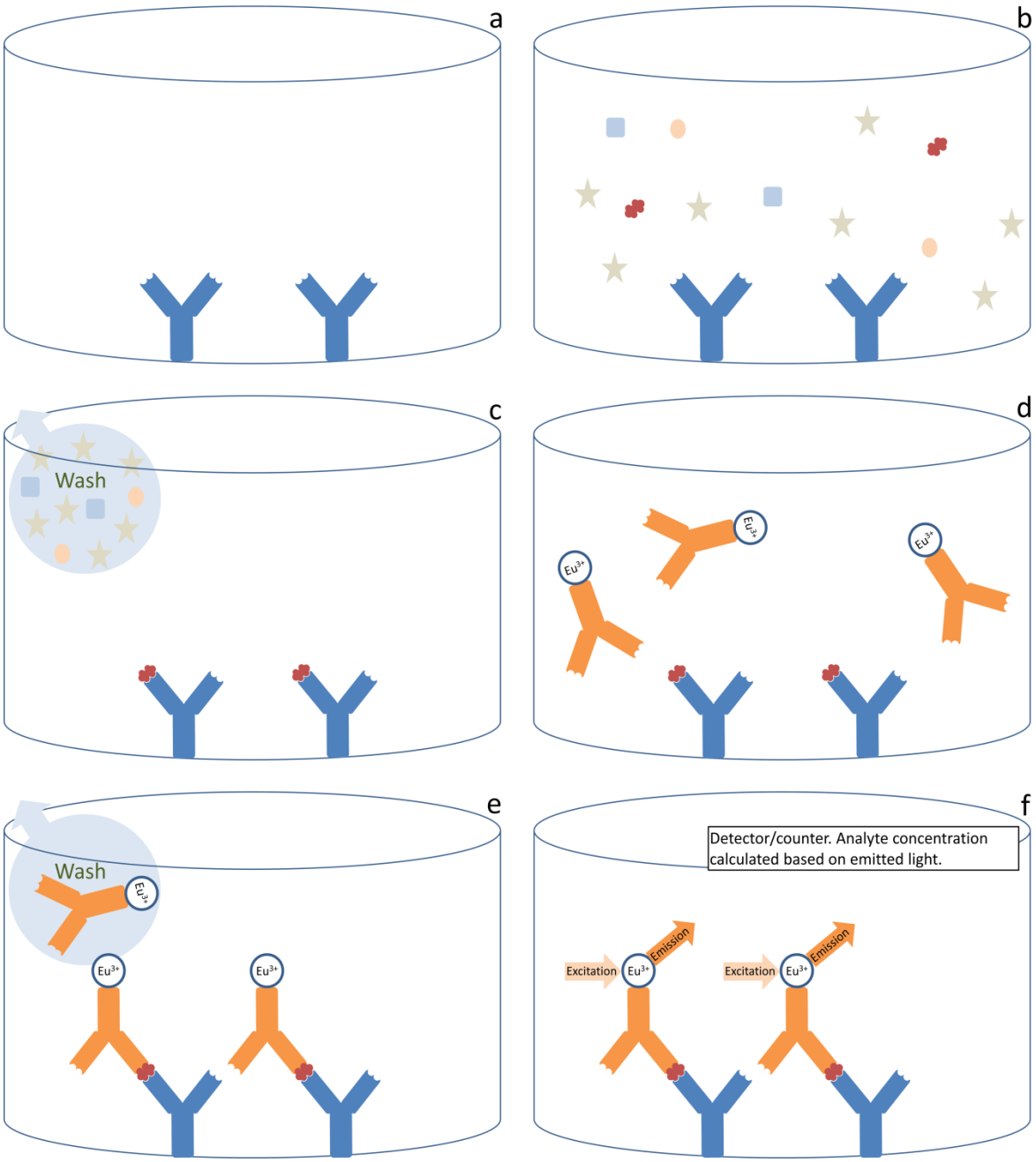

Figure 2. Simplified chart describing a 3-step immunometric assay, resembling our in house assays for tumor markers performed on the AutoDelfia platform, which have been used to generate much of the data in this thesis. Assay wells are coated with a solid phase antibody (a), then incubated with the sample (b) containing both analyte (red) and irrelevant molecules (pale colors). The analyte is immobilized by the solid phase antibody and irrelevant molecules removed in a wash step (c). A second antibody labelled with a tracer molecule is added (d) and binds any analyte immobilized on the solid phase, excess tracer antibody is removed in a wash step (e). Finally, light of a defined wavelength excites the tracer, which then emits light with a different, defined wavelength (f). A detector quantifies the emitted light and analyte concentration is calculated based on, and proportional with, the emitted light.

Increasing aggregation results in decreasing light transmission (increasing turbidity) in the assay well or cuvette. In modern routine diagnostics these assays are usually enhanced by immobilizing the assay antibodies to latex particles or similar entities, and can be performed without wash steps.

Immunoassay technology in future medicine

Alternatives to immunoassay technology exist for measuring most analytes, and in some cases, the alternatives are gradually replacing immunoassays as the preferred technology. The most relevant example is likely the increasing use of mass spectrometry (MS) technology to measure steroid and peptide hormones. In fact, MS could theoretically replace immunoassays for all analytes *(27)*, but the increased cost and reduced sensitivity and throughput of many MS-alternatives (compared to their immunoassay counterparts) limit their application in routine laboratories.

Commercial genetic/DNA plasma markers, often referred to as liquid biopsies, are currently launched with ambition to complement or replace immunoassays. In some cases, large multimarker panels are offered, often at remarkable costs, but with several unresolved challenges related to interpretation and patient consequence *(28)*. Other applications are based on a limited selection, or even single markers. Some of these genetic plasma markers, such as detection of EGFR-mutations which affect prognosis and treatment selection in lung cancer *(29-31)*, will undoubtedly find clinical routine use.

Despite recent advances in alternative technologies, the low cost, speed and high throughput associated with immunoassay measurements will likely ensure the technology will be in use for decades to come, despite the inherent limitations.

Heterophilic antibody interference in immunoassays

The defining virtue of immunoassay technology, the ability to selectively quantify a molecule present in a very low concentration in a medium containing large amounts of similar molecules, is granted by the affinity and specificity of antibodies. As the use of immunoassays, particularly immunometric assays, gained popularity, it became evident that

the reliance on antibodies may in fact be the Achilles heel of the technology. Some patients were shown to have antibodies in their blood that bind the animal antibodies used in immunoassays and produce false results. In rare cases, such antibodies can be expected, i.e. if patients receive animal antibodies as part of treatment or diagnostic procedures. In most cases, there is no certain exposure in the patient's history to explain such anti-animal antibodies. As suggested by Kaplan and Levinson *(32)*, patient antibodies to animal antibodies are usually called heterophilic antibodies when the antigen is not known. In the rare cases where exposure to a defined animal immunoglobulin can be documented, the patient antibody is usually named after the animal antibody, e.g. a human anti-mouse antibody (HAMA).

First reports

In a short communication published in The New England Journal of Medicine in early 1973, James T. Sgouris suggested that false positives in the assay for hepatitis B antigen from Abbott Laboratories were caused by patient antibodies reacting with the guinea pig assay antibodies *(33)*. Sgouris showed that false results were normalized by the addition of normal guinea pig serum. Later in 1973, in a more elaborate study on the properties of the Abbott assay for hepatitis B antigen published in The Lancet *(1)*, Alfred M. Prince et al. gave the first accurate description of heterophilic antibody interference (illustration used with permission on page 3 of this thesis). Again, false positive results in the assay for hepatitis B antigen from Abbott were attributed to patient antibodies reacting with the guinea pig assay antibodies and were normalized with the addition of normal guinea pig serum.

Growing knowledge on incidence and prevention of antibody-mediated interference

In the following years, several publications increased focus on interference from patient antibodies in immunoassays *(34-43)*, albeit not limited to heterophilic type interferences *(44-49)*, illustrating increasing awareness of the inherent limitations of immunoassay technology. A growing understanding of the mechanisms of antibody-mediated interference was evident, and Meurman and Ziola demonstrated that **heat-aggregated IgG** neutralized interference from rheumatoid factor in 1978 *(45)*. In addition, as early as 1979, both

Figure 3.

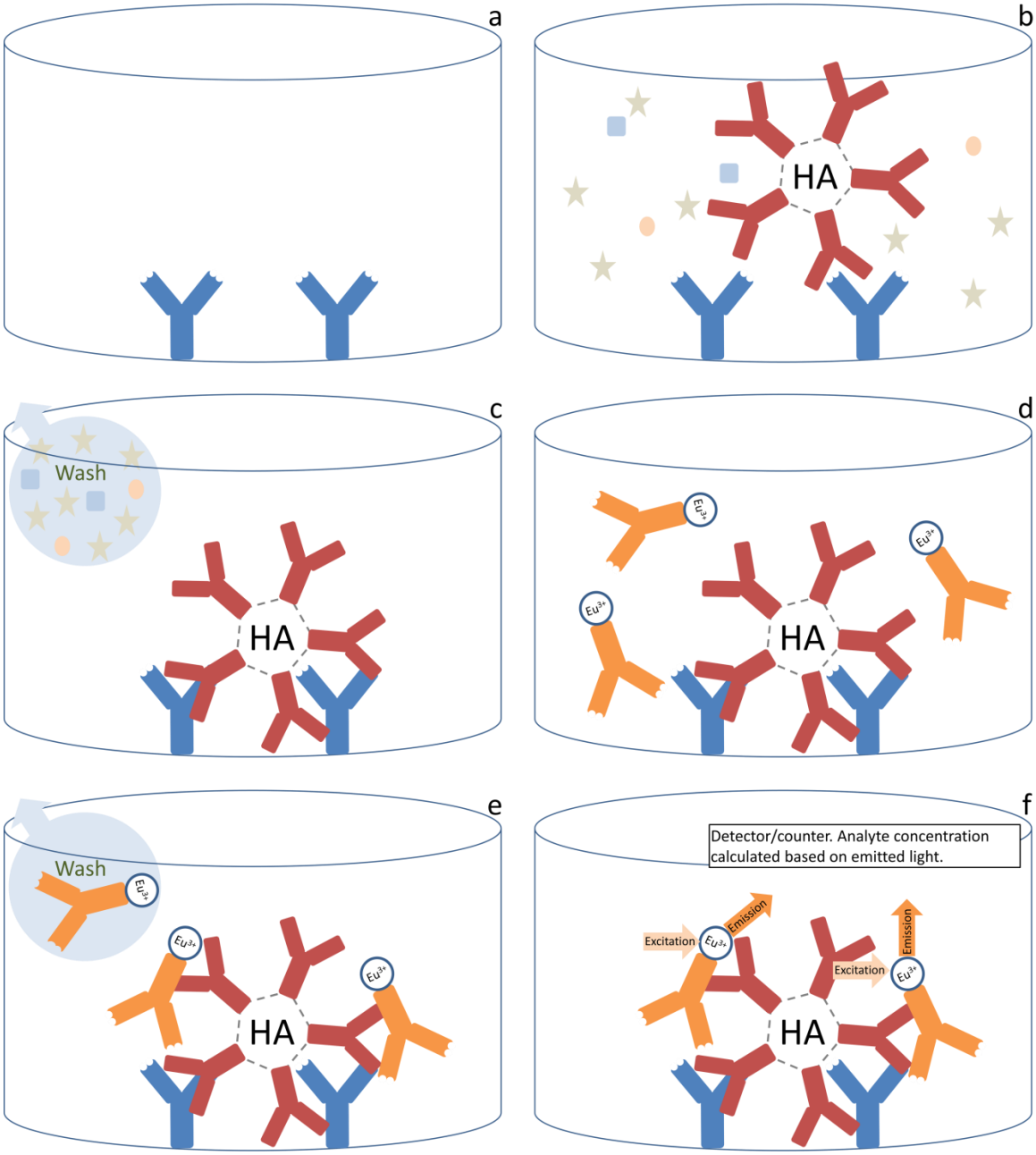

Figure 3. Simplified chart describing interference from heterophilic antibodies in a 3-step immunometric assay. Assay wells are coated with a solid phase antibody (a), then incubated with a sample (b) containing heterophilic antibodies (HA, red) and irrelevant molecules (pale colors), but no analyte. The heterophilic antibody binds the solid phase antibody and is not removed in the next wash (c). A second antibody labelled with a tracer molecule is added (d), is also bound by the heterophilic antibody, and is not removed in the final wash (e). The instrument calculates analyte concentration based on emitted light (f). In this case, the signal is generated by tracer antibody immobilized by the heterophilic antibody, not the analyte, and the result is false.

Duermeyer et al. and Kato et al. demonstrated that use of **Fab' or F(ab')$_2$-fragments in place of intact IgG** in immunoassays dramatically reduced interference from rheumatoid factors *(36, 50)*. These important discoveries unfortunately did not immediately receive the attention they deserved, but were both thoroughly evaluated later both by Vaidya and Beatty *(51)* and by our group *(52)*, and are arguably still the two most important specific protective measures against heterophilic antibody interference.

Anti-bovine antibodies and high-risk populations

During the 1980s and 1990s, several publications documented how commonly occuring patient anti-bovine antibodies may cross-react with assay antibodies from other species and create false results in immunoassays *(53, 54)*. Addition of high concentrations of blocking bovine immunoglobulin to assay buffers became obligatory in assay design. The properties and potential detrimental effects of other cross-reactive or polyreactive endogenous antibodies were explored and described in several landmark publications *(55-62)*. Increasing focus was also put on high-risk populations, particularly patients with seropositive rheumatoid arthritis *(36, 40, 45, 63, 64)*, and both iatrogenic and non-iatrogenic exposure to animal antibodies were shown to cause, or increase risk of, assay interference *(41, 65-70)*. It also became clear that antibody-mediated interference did not disappear with the increasing use of monoclonal antibodies in immunoassays, as some researchers had hoped *(71-73)*.

The hCG scandal

In 2001, a young woman was awarded 16 million USD for damages after false positive hCG-results in the assay from Abbott (case discussed in more detail in paper V) *(74)*. The case increased focus on heterophilic antibody interference, both among immunoassay developers, laboratorians and clinicians. In addition, increased attention from the U.S. Food and Drug Administration (FDA) specifically on this type of interference ensured the immunoassay industry invested more resources than before in protecting their assays from heterophilic antibody interference. Sadly, as documented and discussed also in several of the papers in this thesis, some assays are still vulnerable because the companies have not invested sufficiently in adequate protection.

Recombinant proteins, non-mammalian and non-immunoglobulin alternatives

The revolution in molecular biology towards the end of the 20th century, particularly the availability of recombinant DNA technology, gave new and promising alternatives to antibodies in immunoassay design *(75, 76)*. Use of non-immunoglobulin proteins or engineered antibodies as capture or detection proteins, such as affibodies/aptamers, fusion proteins or single-chain fragments (scFv), effectively limit interference from heterophilic antibodies *(77-82)*. Commercial use of these molecules in immunoassays is still rare, but it is likely such use will increase in the future. Technological advances make targeted design and large-scale production of these molecules (at acceptable cost) increasingly accessible to researchers and industry, not only for use in immunoassays, but also in therapy and imaging *(83, 84)*. As of yet, the affinity, specificity and stability of antibodies means they are still chosen over non-immunoglobulin alternatives in most immunoassays.

In order to limit interference from patient antibodies, particularly rheumatoid factor type antibodies, avian antibodies (IgY) provide an interesting alternative to mammalian antibodies in immunoassays *(85-87)*. Although this has been known for three decades, commercial use of avian antibodies in immunoassays is still limited. Camel antibodies also have unique properties that could make them interesting alternatives to other antibodies, also in immunoassays. Camelids are the only non-extinct members of the suborder Tylopoda, phylogenetically relatively far removed from other mammals such as cattle, mice and humans *(88, 89)*. So far, widespread use has been limited by broad patents, and camel antibodies have received more attention as therapeutic agents than immunoassay reagents. This is particularly true for the naturally occurring heavy-chain camel immunoglobulin lacking light chains and CH1 domains *(90)*, or the small (15 kDa) VHH-fragments derived from heavy chain antibodies called nanobodies *(91, 92)*. Curiously, such small antibodies have also been discovered in sharks, called Ig-NAR (with variable domain V-NAR), which are currently being explored as diagnostic or therapeutic agents *(93, 94)*.

The use of lectins, a group of plant proteins with specific affinity to defined carbohydrate structures, are still being explored in immunoassays, and show particular promise in the detection of malignancy-associated glycoprotein variants *(95, 96)*. Systematic data on resistance to interfering patient antibodies is lacking, but it is highly likely that assays based

on cross-linking of an antibody and a lectin are less vulnerable to heterophilic antibody interference than assays designed using two antibodies. In general, assays designed using non-immunoglobulin capture proteins rather than immunoglobulins largely avoid heterophilic type interference, as antibody-mediated crosslinking of solid phase (non-immunoglobulin) protein and tracer antibody is highly unlikely due to structural differences. When designing assays for certain analytes, assay designers can choose between non-immunoglobulin capture proteins and immunoglobulins as solid phase reagents. Recently, we have established automated assays for several antibody-based biologic drugs in our laboratory, mostly using the drug targets as capture proteins *(97-101)*. In addition, the streptococcal antibody-binding protein called protein A is chosen as tracer protein where possible, meaning that some of the assays do not utilize any antibodies as assay reagents. Avoiding antibody pairs or antibodies in general in these assays is particularly beneficial as patients with rheumatoid arthritis (and potentially interfering rheumatoid factor) constitute one of the largest patient groups in our diagnostic routine.

Future perspectives

Immunoassay designers today have the necessary tools and knowledge to make robust assays. Newer assays from established assay producers are generally well protected against heterophilic antibody interference, but some older assays still have inadequate protection. Use of antibody-fragments and sufficient blocking with irrelevant immunoglobulin has become, and will probably continue to be, common and near obligatory defenses against antibody-mediated interferences in commercial assays. Some of the alternatives discussed in the previous section will find increasing use in commercial assays compared to current use, but it is difficult to envision any of the alternatives to mammalian antibodies gaining dominant positions in the immunoassay market.

The fight to reduce patient harm from heterophilic antibody interference in immunoassays in the coming years should, in my opinion, focus on removing vulnerable assays from use in our laboratories. Ideally, they should be removed from the market. To achieve this, we (the researchers) first have to identify vulnerable assays and document patient harm. Next, regulatory agencies (by limiting market access for poorly protected assays) and clinical laboratories (by not purchasing poorly protected assays) have to force the manufacturers to

improve their (remaining) poorly protected assays. While manufacturers have the tools to properly protect assays, for many older assays they lack the incentive to invest time and resources to do so.

Aims

In general, the research on heterophilic antibody interference in our laboratory has focused on generating systematic data (as opposed to anecdotal reports, which are plentiful) on incidence and prevention of this type of interference in immunometric assays *(52, 53, 79, 102, 103)*. Using methods relevant to our diagnostic routine, large sample cohorts have been examined to document incidence of heterophilic antibody interference, assess assay vulnerability or demonstrate efficacy of protective measures. Since our in house assays almost exclusively have been based on murine monoclonals, focus has naturally been on patient antibodies with affinity to murine antibodies. The main aims in this thesis were:

1. Focus on heterophilic antibody interference in commercial assays. Even though previous research on interference performed in our laboratory generated systematic, high-quality data on interference in our in house assays, most modern commercial assays are quite different from our in house assays, e.g. typically having only one wash step as opposed to the three wash steps often used in our in house assays.
   The argument that methodological differences make previous findings less relevant to modern assays can be used as an excuse for not protecting commercial assays properly. We were interested in evaluating if we could apply the knowledge accumulated in our laboratory in a systematic test of the resistance to interference from heterophilic antibodies in current commercial assays. The ultimate goal was to reveal which assays had inappropriate protection against heterophilic antibody interference, expose these assays and encourage manufacturers to take the necessary steps to improve their assays. In addition, exposing vulnerable commercial assays in use in routine laboratories would increase awareness among laboratorians and make them better equipped to identify samples with potential interference.
2. Improve our interference diagnostics. This means that when interference is suspected, we perform and interpret the adequate interference tests correctly. The reality is that interference testing is more complicated and riddled with pitfalls than what is often

communicated. In our laboratory, this work is not only done in a research setting, but also when patient samples are sent to us for evaluation. Our conclusion on whether an abnormal result most likely is true or false can have an enormous impact on patient care. In order to minimize patient harm from poorly protected assays, an aim of the project was to improve our handling of samples with suspected interference and communicate our experience to the laboratory community.

3. Characterize patient antibodies to murine and non-murine assay antibodies. Where previous focus had been on patient antibodies to murine monoclonal antibodies, we wanted to extend focus to patient antibodies with reactivity to antibodies from other species as well, since these are still used in commercial assays. With the growing availability of non-murine monoclonals, such antibodies might become more common in immunoassays in the future. Our attempt to map all relevant anti-animal reactivities in patient samples was undoubtedly the most ambitious aim of this project. The rationale behind this goal is that we are better equipped to design robust assays and perform interference diagnostics if we know which anti-animal reactivities exist in our patients, how common they are and how they are interrelated.
4. Assess the relationship between the commonly occurring anti-bovine antibodies and other anti-animal reactivities existing in patient samples.

Results, summary of papers

Paper I:

A man with abdominal pain: enough evidence for surgery?

This case report presents the resolution of a complex clinical challenge facing the surgeons at Oslo University Hospital, Rikshospitalet. A patient with a grossly elevated plasma ACTH in the Siemens Immulite assay was thought to have an ACTH-producing pancreatic tumor, which had subsequently been identified using a modern PET/CT-protocol employing the radiotracer ^{68}Ga-DOTA(0)-Phe(1)-Tyr(3)-octreotide (^{68}Ga-DOTATOC). The patient was scheduled for surgery, but a pre-operative conventional CT-scan could not identify the

tumor. The PET/CT-scan and the ACTH-results were thoroughly scrutinized, both in the end determined to be false positive, and the patient was discharged without further treatment.

Previously, when samples were referred to our laboratory because heterophilic antibody interference was suspected, we blocked with aggregated murine IgG1 and tested for heterophilic antibodies in interference assays using combinations of murine IgG1 antibodies. The Immulite assay for ACTH combines a murine monoclonal antibody with a non-complementary polyclonal rabbit antibody, and using our normal approach of blocking with aggregated murine IgG did not reduce interference, even though we observed very high signals in our interference assay designed using two murine IgG1 antibodies. To solve the case we tested the sample in an interference assay closely mimicking the Immulite ACTH-assay, combining a murine monoclonal IgG1 with a polyclonal rabbit antibody. When increased signals were observed also in this assay, we added aggregated rabbit immunoglobulin to the sample and re-assayed in the Immulite ACTH assay. Results were normalized with addition of increasing amounts of aggregated rabbit immunoglobulin, proving that the original result was falsely elevated because the patient's antibodies with reactivity to rabbit immunoglobulin bound the rabbit tracer antibody in the Immulite assay.

Blocking with aggregated murine immunoglobulin did not affect the result in the Immulite ACTH-assay, suggesting that tracer complexes were not bound to the solid phase particles through antibody-mediated cross-linking of murine solid phase antibodies and rabbit tracer antibodies. We believe that cross-linking of solid phase and tracer assay antibodies was caused by the actual ACTH in the sample, but the gross false elevation was due to aggregation of rabbit tracer antibodies by patient antibodies with anti-rabbit reactivity. This suggested mechanism is obviously important to consider when planning and interpreting interference tests when the assays in question use combinations of assay antibodies from different species or different subclasses.

Our experience with the sample in paper I changed our approach when testing for interference in cases where the assays in question are constructed using non-murine antibodies or antibody combinations from different species. After this case, in interference cases involving assays using antibodies other than murine IgG1 or combinations of assay antibodies from different species or different subclasses, blocking experiments are no longer limited to the addition of murine IgG1 antibodies. When facing similar cases, we attempt to

mirror the assay antibodies as closely as possible, both in interference (non-sense) assays and in blocking attempts. We have since solved several cases of suspected interference, particularly involving serology assays, where blocking with polyclonal rabbit, goat or sheep antibodies (mirroring assay tracer antibodies) was necessary to normalize results. Curiously, one such example is the Abbott assay for hepatitis B antigen, the modern version of the assay where heterophilic antibody interference was first described *(1, 33)*. In addition, our experience with this case made us interested in mapping patient antibodies to all animal antibodies used in immunoassays, not only murine antibodies, which ultimately led to paper IV.

Paper II:

Heterophilic antibody interference in commercial immunoassays; a screening study using paired native and pre-blocked sera.

In this paper we show that despite current knowledge and availability of effective protective measures, several widely used immunoassays from leading immunoassay companies are still susceptible to interference from patient antibodies with Fc-reactivity. Most laboratorians probably expected that leading immunoassay producers made sure their assays were properly protected against this type of interference. Rightfully so, considering how the U.S. hCG-scandal had admonished the industry more than a decade earlier.

Two patient samples containing Fc-reactive heterophilic antibodies, originally referred to our laboratory for interference testing, were distributed to 20 laboratories in the Nordic countries. Both native and pre-blocked aliquots of the sera were distributed, and a difference between native and pre-blocked aliquots exceeding 50 % was deemed indicative of interference. In total, 170 different assays were tested, of which 21 were susceptible to interference from heterophilic antibodies in one or both patient sera. Among the vulnerable assays was the hCG assay from Abbott, the purportedly improved and fixed version of the assay involved in the hCG-scandal a decade earlier.

We firmly believe that modern immunoassays should not be vulnerable to interference from Fc-reactive heterophilic antibodies. This type of interference is completely avoided if Fab' or $F(ab')_2$-fragments replace intact antibodies in assays, and greatly reduced if aggregated

immunoglobulin is added to assay buffers, both measures with documented efficacy since the late 1970s. For this reason, we were surprised and disappointed when 21 commercial assays were exposed as vulnerable to Fc-reactive patient antibodies, which are the most common and (importantly) predictable interfering antibodies. Thankfully, many colleagues shared our disappointment, and our results received a fair amount of attention following publication. In addition to the paper, the project was presented at the 2012 Nordic Congress in Clinical Chemistry in Iceland (award-winning poster, oral presentation at Roche symposium) and at the 2012 meeting in the Norwegian Quality Control organization NKK (now Noklus). This additional exposure increased awareness in laboratories using the vulnerable assays, and some laboratories established new routines for scrutinizing and retesting unexpected results in assays exposed as vulnerable to interference from Fc-reactive patient antibodies. We noticed increasing interest in the problem of interference from the 20 Nordic laboratories that participated in the study, when immediately following publication of paper II, each participating laboratory received a report with an interpretation of their results. One of the largest Norwegian hospital laboratories decided to retest every elevated result for D-dimer with an alternative method available at a nearby laboratory until the immunoassay company launched an improved version of the assay with specific protection against heterophilic antibody interference. Several samples with falsely elevated results were discovered, and most were confirmed to contain Fc-reactive heterophilic antibodies when examined in interference assays in our laboratory.

Even though modern assay package inserts always inform of the possibility of heterophilic antibody interference, in effect making sure that the companies avoid future lawsuits, the refusal to invest in available protective measures reveals a conspicuous disregard for patient safety. As discussed in the section on ethics in this thesis, the same criticism applies to laboratories that knowingly buy and offer poorly protected assays because they are subjecting patients to unnecessary risk. As long as companies avoid legal sanctions and customers continue to buy their products, there is little incentive for companies to invest resources to improve fallible products, even though patients are harmed. The type of exposure that some immunoassay producers received in paper II provided some incentive to improving their products. When their assays were exposed, several manufacturers communicated they had decided to improve protection against heterophilic antibody

interference in their vulnerable assays. An improved version of the D-dimer assay from Stago was relaunched as a new product in 2013 called D-dimer Plus *(104)*. We cannot know for certain that the new version of the assay was developed as a result of exposure in our publication, but it seems likely considering the assay was launched a little over a year following publication. In addition, we know that many Nordic laboratories using their D-dimer assay exerted pressure on Stago (or their national/regional distribibutors) to solve the problem of interference. Other assays, such as CA125 from Abbott, soluble transferrin receptor from Roche, and cytokine assays from BioRAD, were reported to have improved protection through personal communication with company representatives.

Paper III:

Belief is only half the truth--or why screening for heterophilic antibody interference in certain assays makes double sense.

In this paper we use mathematics, probabilistic Bayesian reasoning and information theory to discuss and evaluate different strategies to limit damage from heterophilic antibody interference in immunoassays. We also discuss why interference is most often reported in diagnostic tests with a high medical impact, such as tests used in patients suspected of having life-threatening infections or malignancies, which in fact are tests where we would expect the diagnostic industry to invest additional resources in preventing interference. A key hypothesis is that there must be some bias, leading us to notice and report interference for some tests, but not for others. The main question we try to answer is whether we best fight interference before (by making better assays) or after (by reanalyzing elevated or dubious results) interference may appear. Even though, in reality, we must do both to successfully minimize patient harm, we wanted to show how different premises (such as incidence and consequence of false results) favor different strategies.

We conclude that the most cost-effective strategy is to focus on increasing resistance to interference when immunoassays are designed, i.e. by adding sufficient blockers to assay reagents or using antibody fragments in place of intact immunoglobulin, and not by focusing on re-testing of positive or dubious results. This is particularly true in assays where we expect a substantial proportion of true positive results, such as assays for cardiac troponins

used in patients with acute chest pain. Part of the explanation for this is that when the number of true positives increases, according to Bayesian reasoning, the likelihood that an elevated result is false decreases. Simplified, this relationship is expressed as:

$$\text{Likelihood of interference as cause of positive test} = \frac{\text{Frequency of false positives}}{(\text{Frequency of false positives} + \text{Frequency of true positives})}$$

Where we have to re-test a high number of samples to identify the false positives, the added cost per identified false positive sample becomes high, making re-testing a less cost-effective strategy when the frequency of true positives is high.

Conversely, in assays with a low frequency of true positives, but unchanged frequency of false positives, re-testing all positive samples to identify false positive samples adds lower cost per identified false positive sample. In addition, we claim that positive results in assays where we expect the frequency of true positives to be low (i.e. HIV-tests) usually have a greater impact on patient care than positive results in assays where we expect the frequency of true positives to be high (i.e. tests for mononucleosis). Using information theory, we show that the information value of a true positive result is greater when it is unexpected (frequency of true positives is low). On top of the increased likelihood of a positive result being false (as shown using Bayesian reasoning), the added information value (as shown by information theory) further increases cost-effectiveness of a re-testing strategy in assays where we expect a low number of true positives.

As mentioned above, relying entirely on either a priori (making or choosing robust assays) or a posteriori (re-testing) measures to fight interference is a bad idea, we must do both. Most laboratories today do not develop their own immunoassays, but rather purchase assay kits provided by diagnostic companies. Our conclusions in the paper are just as valid for laboratories buying kits as they are for laboratories setting up in house assays. Investing in robust assays, even though these assays may cost more than vulnerable alternatives, is in most cases a more cost-effective strategy than re-testing every elevated result. Sadly, we have not succeeded in communicating this clearly enough to other Norwegian laboratories.

Some laboratories still choose the latter strategy (re-testing) for certain analytes, mostly because they are committed to assays from one diagnostic company, and the additional cost of purchasing and maintaining another instrument to run alternative assays is deemed unacceptable. As an example, at least four laboratories in Norway still use the hCG-assay from Abbott, but warn their users it cannot be used as a tumor marker, i.e. to evaluate patients with known or suspected malignant disease. Every time hCG is ordered as a tumor marker, at least if it is positive, it has to be sent to another laboratory for re-testing. This creates additional costs and potential delays in diagnosis and treatment. In addition, it is a vulnerable strategy increasing the risk of mistakes, and will (sooner or later) lead to mistreatment of patients, which ultimately confers significant cost to patients, the health system and society as a whole. As shown in paper III, knowingly choosing assays vulnerable to heterophilic antibody interference is not only ethically questionable, it is also a flawed strategy in terms of cost-effectiveness.

It is difficult to assess the impact of paper III, but it certainly provided us with new arguments when discussing laboratory investments with colleagues more accustomed to economic than academic reasoning. In addition, our arguments were embraced in a recent paper *(105)* by Adel A.A. Ismail, one of the leading and most prolific theoreticists in this research field *(106-113)*.

Paper IV:

Prevalence and specificities of heterophilic antibodies in a large patient cohort.

Paper IV describes the systematic characterization of heterophilic antibodies in more than 5000 samples from 3072 female patients. One of the methods used to characterize the heterophilic antibodies is what we refer to as an interference assay, or non-sense assay. These assays are designed using combinations of antibodies that do not measure analytes by binding to different epitopes (regions) on the same protein, but rather detect interference by being cross-linked by antibody-binding antibodies (heterophilic antibodies) in the sample. The project has been a massive undertaking for our research group, including more than 50000 immunoassay results in total, and still continues today.

The main findings were that antibodies to rabbit IgG and murine IgG1 were by far the most common in this large female patient population, while antibodies to murine antibodies of IgG2a and IgG2b subclasses were rare. Antibodies to sheep and goat antibodies were also rare, but cross-reactivity from anti-bovine antibodies becomes a significant problem if blocking is not sufficient. We showed that very few samples in our cohort had concomitant reactivity to murine IgG2a antibodies and the other antibodies we tested, prompting us to suggest that combining murine IgG2a assay antibodies with another antibody isotype could contribute to increasing assay robustness. Interestingly, and reassuringly, this finding supports the results in a previous study from our group, where a different analytical approach was used *(103)*.

We also describe how the commonly occurring anti-bovine antibodies affect reactivity to the non-bovine assay antibodies if the anti-bovine antibodies are not effectively blocked. Cross-reactivity from anti-bovine antibodies was significant to all non-bovine antibodies except for rabbit antibodies. Likely related to the close phylogenetic relationship among the Bovidea (including cattle, sheep, goats) we saw vast cross-reactivity from anti-bovine antibodies to sheep and goat antibodies in our cohort when blocking with bovine IgG was removed.

Because we had results for all samples from four different versions of our routine assay for the tumor marker CEA (with varying protection against heterophilic antibody interference) we could compare our findings in the interference assays with the results from our CEA-assays. In this way, we could evaluate our strategy of 1) screening all samples with an interference assay constructed using pools of 6 different antibodies both as solid phase and tracer antibody, and 2) characterizing the samples identified by the screening assay in assays using individual antibodies as solid phase and tracer antibodies. Even though our strategy missed some samples with interference in our CEA-assay, we were satisfied that most (65/91) samples with interference were in fact identified and characterized, and that those we missed were samples were the effect of interference was generally less dramatic.

Paper IV was originally submitted to Clinical Chemistry in December 2018, but was rejected in February 2019. It has not yet published, but the findings described in the paper have sparked new projects in our group. Among them are systematic characterizations of heterophilic or anti-animal antibodies in selected patient groups and in domesticated mammals.

Paper V:

Heterophilic antibody interference in immunometric assays (review).

Paper V is a comprehensive review written for a double-volume special issue of *Best Practice & Research: Clinical Endocrinology & Metabolism* called Endocrine Assays and Pitfalls. In addition to presenting an overview of the literature and knowledge base on the subject, the aim of the review was also to provide practical tools to limit harm from heterophilic antibody interference relevant to laboratorians, clinicians and immunoassay producers.

This is by no means the first review article written about antibody-mediated interference in immunoassays. In fact, considering the modest number of research articles presenting original systematic data in this field, the number of review articles is impressive. Our review article was intended as a practical guide to limit harm from interfering patient antibodies, and we hope it is as relevant to clinicians as it is to laboratorians. It is rewarding to see that this review is referenced comparatively frequently by authors with a clinical rather than immunoassay background.

Methodological considerations

Most hospital laboratories today rely entirely on commercial immunoassay kits and fully automated and closed “black box” analytical instruments. Our laboratory is rather unique in this regard, as we still offer a range of in house immunoassays to tumor markers, biologic drugs and patient anti-drug antibodies. Most in house assays are automated on the AutoDELFIA immunoassay robot, where assay protocols can be modified to create custom test systems for particular questions or problems. This gives us access to an extensive tool kit of reagents and possible assay modifications, and makes us well equipped to investigate occurrence and nature of antibody-mediated interferences. It also allows us to establish custom high-quality immunoassays for clinical trials or other external research projects, which constitutes a rapidly growing activity in our laboratory.

Interference assays and modified routine assays

We are in a beneficial position to establish methods for interference testing in our laboratory, but both interference assays and common test strategies have some inherent limitations worth discussing. When setting up interference assays or test systems, we have to choose among several possible reagents (including assay antibodies/proteins and blockers) and assay formats. Every choice we make in this process usually comes at some cost, either affecting sensitivity (the ability to detect the phenomenon we are interested in) or specificity (the ability to avoid positive signals from other entities) of the assays or test strategy.

One such example is our decision to screen our patient cohort of 3072 patients in paper IV using a screening assay with large amounts of blocking bovine immunoglobulin in the assay buffer. By blocking any anti-bovine antibodies in the samples, we increase the chance of identifying samples containing specific antibodies to the six antibody subclasses we included in the screening assay, but we obviously lose the ability to detect anti-bovine antibodies cross reacting to the six antibody subclasses, which in some immunoassays could be an important source of interference. We chose this approach because most commercial immunoassays used in clinical care of patients are blocked with large amounts of bovine immunoglobulin, and we wanted our results to be relevant for patient care.

Another example is our decision to use only one monoclonal antibody from each murine isotype in our interference assays. We test for antibodies to murine IgG1-antibodies using monoclonal K57, an IgG1-kappa antibody. We believe this is acceptable as most heterophilic antibodies bind the Fc-fragment of the antibodies, which is identical among antibodies of the same isotype. Theoretically, some patient sera might contain antibodies that bind other murine IgG1-monoclonals, but not K57. In fact, some patient antibodies will bind the variable portion of antibodies, and not the Fc-fragment, meaning they will only bind one or a very limited number of murine monoclonals. Despite these concerns, our previous experience using two different antibody combinations to detect antibodies to murine IgG antibodies, showed very little difference between the two assays. All things considered, it would likely be prohibitively demanding to produce and process a number of monoclonal antibodies in order to perform one assay, particularly considering the number of assays we use and the very limited additional information we expect to get from the effort.

The in house routine assays for CEA we have used to detect heterophilic antibodies in this project are 3-step assays constructed using murine monoclonal IgG1-antibodies exclusively. This means that we are only able to detect heterophilic antibodies with affinity or cross-reactivity to murine IgG1. While murine IgG1 is the most important isotype in modern commercial immunoassays, this limits the general applicability of our findings somewhat. However, using established routine assays to test the effect of heterophilic antibodies confers significant advantages over establishing novel "research only" assays using other antibody isotypes, both in terms of resources and interpretation.

Assay formats

All interference assays in this project are 2-step immunometric assays, with wash steps after addition of solid phase antibody and after co-incubation of sample and tracer antibody. If specific protection against heterophilic antibody interference were unchanged, we might have found fewer antibodies had we used 3-step formats, with an additional wash step between addition of patient sample and tracer antibody, as the patient sample with potentially interfering antibodies would not incubate together with assay tracer antibodies in solution. Conversely, we believe we would have detected more heterophilic antibodies had we chosen 1-step assays, where the patient sample and both assay antibodies are present in the assay well simultaneously, expectedly increasing the risk of analyte-independent cross-linkage of assay antibodies.

Most modern automated immunometric assays are run as 1-step assays, at least assays from the large assay manufacturers, as it reduces assay time. Our experience with both interference assays and routine assays on the AutoDELFIA platform comes from 2- or 3-step formats, and we were worried about performance of 1-step interference assays with very little protection against interference from heterophilic antibodies and anti-bovine antibodies. In this format we risk near total aggregation of assay antibodies, a *de facto* consumption, which could at least theoretically result in no signal because the aggregates are not being immobilized to assay wells. As a result, we could increase the risk of not detecting the most potent heterophilic antibodies, which would seriously harm the project. We also wanted to compare results with data from different versions of our CEA-assay, which were run as 3-step assays. We chose to run our interference assays in a 2-step format

as a compromise, and we believe this is probably closer in performance to 3-step assays than 1-step assays. By making this choice, we were able to relate our findings to our data from 3-step routine (particularly CEA) assays, and we used assay formats where we had experience both running assays and interpreting results.

One of the likely consequences of chosing a 2-step format is that we are less likely to detect negative interference. Put simply, heterophilic antibodies may cause negative interference by blocking the antigen binding ability of assay antibodies directly (by binding near the antigen-binding site of the assay antibodies) or by consuming assay antibodies through aggregation. While negative interference in general is more difficult to detect than positive interference, and systematic data are largely lacking, the latter mechanism (aggregation) is probably the most likely to cause negative interference in modern 1-step assays. Our choice of assay format means we are less vulnerable to signal loss due to aggregation, and may miss possible negative interference, but we could still lose signal due to blocking of antigen binding sites. In other assay formats, both competitive assays and 1-step immunometric assays, negative interference could be more likely. This is supported by the results in the cytokine multiplex assays (1-step) in paper IV, where negative interference apparently was quite common. In truth, I believe we still do not have convincing and systematic data to document the extent of negative interference in immunoassays.

Blocking with MAK33

One of the most important tools we use in interference studies, both in testing of individual patient samples where interference in specific assays is suspected and in research projects, is reanalysis after blocking immunoglobulin is added to samples. The tradition in our laboratory has been to block with aggregated murine IgG1 MAK33 (Roche), which is the same murine immunoglobulin we add to our assay buffers. As discussed previously in the presentation of paper I, we now use a repertoire of blocking immunoglobulins, which allows adaptation of our blocking strategy depending on the antibodies used in the assay where interference is suspected. Still, most assays we test are designed using murine IgG1 exclusively, where MAK33 is the blocking reagent we use.

MAK33 is a murine monoclonal to the cardiac marker CK-MB, and should obviously not be used to perform blocking experiments in assays for CK-MB. It is sold in large quantities by Roche and mostly used as irrelevant blocking immunoglobulin in immunoassays. Roche is one of the largest manufacturers of immunoassays, and often use MAK33 as blocking immunoglobulin in their own assays. This means that we risk biased results (favoring Roche assays) when we use MAK33 to block heterophilic antibodies in interference testing, simply because the blocking reagent is already added to assay reagents in Roche assays, greatly reducing the likelihood of a positive interference test. Addition of MAK33 is more likely to affect the result in assays where it is not already added to assay reagents, i.e. assays from other manufacturers. We always try to supplement blocking experiments when we perform interference studies, both with testing in other assays for the same analyte and testing in our interference assays. In addition, we have actually found interference in Roche assays by blocking with MAK33. As an example, and surprisingly considering we bought the blocking reagent from Roche, three of the 21 assays identified as vulnerable using this blocking strategy in paper II were manufactured by Roche.

Previously, we bought non-aggregated MAK33. The freeze-dried immunoglobulin was rehydrated and aggregated by heat-treatment in our laboratory. Different batches of aggregated MAK33 had slightly different properties and ability to block heterophilic antibodies. This was mostly related to the temperature and duration of the heat-treatment, as degree of aggregation certainly affects blocking properties. This means that over time, we used batches of aggregated MAK33 with slight variations in blocking potency. Because all aggregated MAK33 used in paper II was from the same batch, results in this study is unlikely to be affected such variation. However, since analysis was performed over an extended time period, we cannot exclude that results were affected by batch differences in paper IV. In reality, any such effect is likely very limited, as aggregation in every batch was controlled by absorbance testing prior to use. Today, partly to avoid batch differences of heat-aggregated MAK33, we purchase chemically aggregated MAK33 from Roche, polyMAK.

Lack of calibration of interference assays

We have made several unsuccessful attempts to calibrate and standardize our interference assays through the years using a plethora of commercial, in house or patient antibodies

reactive to animal antibodies. Some promising alternatives (e.g. sheep anti-mouse antibodies) proved outright harmful, where presumed calibrator (anti-animal antibody) residues in our immunoassay platforms interfered in other immunoassays performed on the instruments. Although it is far from optimal, we chose to run the interference assays without calibration, but included a diluted sample known to give response in each assay as a positive control to ensure the assay works as intended. We used interference in a working immunometric assay (CEA-interference) as a reference when deciding the signal cut-off to define a positive sample in the anti-murine IgG1 assay and (to some degree) in the interference screening assay in paper IV, which we believe makes sense in a study aiming to identify heterophilic antibodies able to interfere in immunoassays. Lacking references for the other (than murine IgG1) individual interference assays, we chose to use the same cut-off in all assays. While signal blanks (mean buffer blanks ranging 300-1000 cps) and signal ranges (highest responses > 250 000 cps for all but murine IgG2b, where highest response in patient samples was 80 000 cps) were fairly comparable for the six antibody products we used in individual interference assays, we cannot exclude systematic test bias caused by differences in antibody purity, integrity or labelling efficiency between antibody products. Lack of comparable calibrators between interference assays is a limitation of all our interference studies. Because signals (interference) in these assays depend not only on concentration of antibodies, but also on affinity and valency, it will likely remain a challenge in any future attempts to compare reactivities of patient anti-animal antibodies. In light of this, I believe the best strategy is to run a working assay, preferably in two versions with varying protection against interference, as a reference to see when signals in the interference assay gives interference in the working assay. In paper IV, we had such a reference for murine IgG1-reactivity, but did not have existing, or capacity to establish, working assays using antibodies of the other isotypes in the study. This is a weakness of the study that was obvious from the start of the project, clearly limiting the impact of our results, but one we were unable to circumvent.

Choosing interference cut-off, use of mathematical models and statistics

We had to decide on a cut-off for positive interference when we tested the vulnerability of commercial assays in paper II, and decided to define interference as a difference of at least

50 % between blocked and native sample. We have used this definition since when performing blocking experiments. We chose a pragmatic approach, with one cut-off for all assays, because it would be demanding to calculate individual cut-offs for 170 different assays based on their reported or actual analytical variations at the analyte levels of the two patient samples used. By setting such a high cut-off, laboratories in fact rarely accept analytical variation exceeding 20 % in commercial assays, we hoped the positive status we assigned to vulnerable assays would be indisputable. The likelihood of an observed difference of 50 % in an assay with an analytical CV of 21.4 % is p=0.05, meaning that all positive outcomes in our study would have $p<0.05$, which is the threshold often chosen to indicate statistical significance in medical research, as long as assays have analytical CVs below 21.4 %. Most positive results in paper II were so clearly deviant that they were immediately accepted as indicative of interference. In three assays (BNP and SHBG from Abbott, TSH from PerkinElmer) the differences between blocked and native samples were modest enough (60-107 %) that we were asked to calculate p-values based on reported analytical variation. In all three cases the probabilities of the observed differences were $p<0.001$. Even if we opted for a pragmatic approach to define interference, it was satisfying to show that our cut-off was valid also using a more traditional method based on assay properties to define interference. In retrospect, we probably should have documented statistical significance based on the reported assay performance for all individual results, at least for all results defined as positive using our pragmatic approach.

I have been fortunate to collaborate closely with Johan Bjerner, MD, PhD, both in three projects included in this thesis and in several projects on tumor marker assays. He is also one of my supervisors. Dr. Bjerner is a co-author on paper II, and performed the calculations referenced in the paragraph above. Paper III uses Bayesian reasoning and mathematical models based on information theory to discuss validity of different strategies to combat interference. All calculations in this paper were performed by Dr. Bjerner, and while I do have a basic understanding of Bayes' theorem, most of the models and arguments presented in the paper are beyond my knowledge of mathematics.

The 50 % cut-off created some challenges in paper IV, when we observed differences (between blocked and unblocked sample) exceeding 50 % in many results for cytokines, particularly in lower assay ranges. This was not entirely unexpected, as the multiplex assays

for cytokines have a somewhat blemished reputation *(114-117)*, both regarding interference and general assay performance. To increase the likelihood that the positive results were truly positive, i.e. caused by antibody-mediated interference, we decided that we only considered results as positive if one of the results (either blocked or native) were above the fourth calibrator counting from the blank and up. In addition, some results indicated negative interference, i.e. the result in the blocked sample was higher than the result in the native sample. Negative antibody-mediated interference in multiplex cytokine assays has previously been reported *(118)*, it is likely more common in such 1-step formats than in 2- or 3-step formats, and we chose to evaluate these cases as well. In an attempt to allow equal detection of positive and negative interference, interference was determined using the difference between the results as numerator and the lowest result as denominator. We conclude that negative interference was observed in the cytokine multiplex assays in paper IV, but I am not absolutely convinced the observed difference was caused by antibody-mediated interference, at least not in every case we define as interference. Unfortunately, I decided we should run singlicate measurements of blocked samples and singlicate measurements of native samples, allowing analysis of more samples with heterophilic antibodies. Since the kits are very expensive, we could not purchase two kits for this project, which would have allowed duplicate measurements of both blocked and native aliquots. This was a mistake that complicated interpretation of results, but a mistake we will not repeat should we ever use multiplex assays for cytokines in the future.

Ethics

The work described in the articles of this thesis does not fall under the current definition of medical research according to the interpretation of Norwegian law by the regional ethics committee. These projects are defined as quality projects, as they do not involve intervention or change in patient treatment. As such, the projects have not been evaluated by the regional ethics committee. All the individual projects described in papers I, II and IV, and the use of patient samples and patient data, were approved by the Oslo University Hospital Privacy Office. In addition, the patients described in papers I and II gave their written, informed consent to the use of samples and data.

In paper IV, we used surplus sample material from the routine laboratory in our hospital collected in a diagnostic biobank. Considering the nature of this diagnostic biobank, collected from more than 3000 patients with breast tumors more than 10 years ago, it was not feasible to contact the patients still alive, or the relatives of deceased patients, to ask for permission to use samples and data. The privacy office of our institution granted approval to use the samples for the specific project in paper IV with the proviso that all personal data linked to the samples, including patient age and diagnosis, were deleted.

The work in this thesis describes patients that were subjected to unnecessary diagnostic or therapeutic interventions because medical laboratories provided false results from blood tests. One of the serum donors in paper II endured chemotherapy and surgical interventions for a presumed malignant disease she did not have. The patient in paper I spent significant time and money in several European hospitals and underwent a host of radiological and invasive diagnostic procedures because he was told he had a malignant tumor. In view of the potential consequences to patients, I believe certain general ethical aspects should also be discussed in addition to the formal ethical aspects discussed above.

As laboratory physicians, we are in a fortunate position where we rarely risk harming patients directly. Still, when we provide clinicians with confusing or misleading laboratory results, and these results contribute to harmful interventions to patients, we risk accountability. This was demonstrated in the court case following the hCG-scandal mentioned in the introduction of this thesis, where the clinical laboratory received stark criticism for failing to resolve the case thorough adequate control measures before the patient was mistreated. I believe it can be considered unethical to offer immunoassays we know have an increased risk (compared to alternative methods) of giving false results, if these results can contribute to patient mismanagement. If more reliable methods exist, we should choose them, even if they are more expensive or necessitate additional analytical instruments or training of laboratory staff. An obvious example is that medical laboratories still offer the hCG-assay from Abbott, even though false results from this assay repeatedly has contributed to patient harm *(74, 116, 119)*, and in my opinion likely continues to do so today. Another example, increasingly relevant today because of increasing use of biotin supplements, is the challenge of biotin interference in troponin-assays. Unless new and robust assay versions are provided by the manufacturer within a reasonable time frame, I

believe it is unethical for laboratories servicing emergency departments to continue relying on vulnerable troponin assays. As discussed later in this thesis, at least one patient death has been attributed to dietary biotin causing a false negative troponin in a patient with heart attack.

Discussion

Vulnerable immunoassays are still on the market, and removing or improving them is primarily a question of what resources the producers are willing to allocate to improve their assays. It is unavoidable that properly protecting modern 1-step immunoassays against interference from heterophilic antibodies and similar entities increases production costs. Using antibody fragments in place of intact antibodies means that more antibody will have to be purchased or produced, as a loss of 40-60 % of antibody can be expected through the fragmentation and purification process. In addition, the cost of blocking immunoglobulin may be significant, particularly if it is a monoclonal immunoglobulin. When laboratories weight price rather than quality when choosing assays, we risk increasing costs to our hospitals and patients. One false result can spark extremely costly interventions. Examples include direct costs, such as radiological examinations, surgical interventions and hospital stays, and indirect costs, such as patient anxiety, treatment associated adverse events and distrust towards health care providers *(74, 116, 119, 120)*. Such costs are rarely considered in tenders competing for contracts with laboratories. Importantly, Roche Diagnostics has shown that is it possible to provide state-of-the –art protection against heterophilic antibody interference at an acceptable cost in their Elecsys series of immunoassays for tumor markers. As far as I know, all these assays are designed with aggregated immunoglobulin in buffers and antibody fragments as solid phase and tracer antibodies. We have never encountered heterophilic antibody interference in these assays, neither in our routine laboratory, where we use tumor marker assays from Roche, or in our *de facto* function as national reference laboratory for heterophilic antibody interference.

Below, I will discuss some aspects related to, and consequences of, a possible shift away from murine monoclonals to sheep and goat assay monoclonals in immunoassay design. I will briefly discuss the term (and phenomenom) heterophilic antibodies, and present

ongoing and possible future projects related to heterophilic antibodies. Finally, in an attempt to present a more complete and practically useful overview of immunoassay interference, I discuss the most important sources of confusion we face in addition to heterophilic antibodies.

Animal antibodies used in immunoassays, current developments

Today, murine monoclonals of IgG1 isotype dominate commercial immunoassays, and have largely replaced polyclonal antibodies from larger mammals such as rabbits, sheep and goats in immunoassay design. Polyclonal antibodies have advantages over monoclonals in several assays, particularly for analytes that exist in several variants, such as certain hormones or complex tumor markers (e.g. hCG *(121, 122)*). However, polyclonals are not optimal for commercial immunoassay manufacturers, primarily because it is difficult to ensure reagent stability over time when antibody supply is limited and depends on living individual animals *(123)*.

As the methodology to produce them is getting simpler and more accessible, non-murine monoclonal antibodies are getting more common and are likely to be used in commercial assays to a greater extent. Increasingly, sheep and goat monoclonals are promoted and used as alternatives to murine monoclonals *(124-126)*. As yet, we have little experience with assays using sheep or goat monoclonals in our laboratory, but these relatively new antibody options could have several advantages over murine monoclonals both to improve resistance to heterophilic antibody interference but also to reduce costs. I believe this could be the case both when sheep or goat monoclonals are combined with murine monoclonals, and when they are used without murine monoclonals.

As shown in paper IV of this thesis, patient antibodies to sheep and goat antibodies are relatively rare. (Alternatively, they might be quite common but are effectively blocked by bovine immunoglobulin.) Very few samples showed concomitant reactivity to murine and sheep/goat IgG in our study, in fact only one sample showed reactivity to murine IgG1 and goat IgG and three to murine IgG1 and sheep IgG. No samples showed concomitant reactivity to either murine IgG2a or IgG2b and sheep/goat IgG, and only one sample showed concomitant reactivity to rabbit IgG and sheep/goat IgG. These were found after testing

more than 3000 samples, without specific blockers in assay reagents, indicating that such antibodies are indeed very rare. Combinations of murine monoclonals and e.g. sheep monoclonals could be particularly resistant to interference if one or both antibodies were fragmented. As an example, the new Tropinin-assay from Siemens (ADVIA Centaur High-Sensitivity Troponin I) is designed using a sheep Fab as tracer antibody *(127)*, albeit combined with both a murine monoclonal and a sheep monoclonal as solid-phase antibodies.

If assays are constructed using non-murine antibodies, e.g. by combining two sheep monoclonals, manufacturers can also reduce costs by omitting blocking murine immunoglobulin from assay reagents. Sheep, goat and bovine immunoglobulin are all comparatively cheap compared to immunoglobulin from small mammals, particularly rodents. In this setting, blocking with polyclonal bovine (always) and sheep or goat immunoglobulin (depending on assay antibodies) could become a cheap and ready-made formula providing relevant protection. Blocking experiments when facing questionable results could also be simpler and more available as a mixture of polyclonal bovine, sheep and goat immunoglobulin is likely to be sufficient in most cases.

A possible challenge related to use of goat and sheep antibodies is their likeness to bovine antibodies. Bovine (cattle) and Caprinae (sheep and goats) are both subfamilies belonging to the Bovidea family. Since anti-bovine antibodies are extremely common in our population, most likely caused by our exposure to beef and dairy products, anti-bovine crossreactivity to sheep and goat antibodies is probably a greater challenge than crossreactivity to murine and particularly rabbit antibodies. We found clear indications of this in paper IV, where the effect of anti-bovine reactivity was very different on the reactivities to the six antibody products we tested. There was no correlation between anti-bovine reactivity and anti-rabbit reactivity, even without bovine immunoglobulin in assay buffers, indicating that anti-bovine crossreactivity to rabbit IgG is virtually non-existent. In contrast, the correlation between anti-bovine and anti-sheep/goat reactivity was clear and greatly increased when blocking bovine immunoglobulin was removed from assay buffers. Thus, it is possible that a switch to sheep and goat monoclonals could affect interference patterns. Cross-reactivity from rheumatoid factor type antibodies to murine and rabbit antibodies, or rare specific anti-murine or anti-rabbit antibodies, could become rarer sources of interference, while cross-

reactivity from common anti-bovine antibodies could become an even bigger problem than today. We could also risk that samples with high concentrations of anti-bovine antibodies exhaust the capacity of the blocking bovine immunoglobulin creating broad interference in multiple assays and increasing confusion. (We have already encountered such broad interference in one patient, also involving assays only using murine IgG, unpublished.) As a consequence, blocking with sufficient bovine immunoglobulin likely becomes even more essential if sheep and goat monoclonals become more common in immunoassays.

The systematic data on the prevalence and relationship of patient antibodies to different animal antibodies presented in paper IV could become more relevant as the repertoire of monoclonals available to assay developers increases. Hopefully, future assay developers will choose antibody isotypes or combinations of antibodies with these data in mind. Targeted selection and combinations of antibody isotypes could add another layer of protection against heterophilic antibody interference, particularly relevant if whole assay antibodies (as opposed to fragments) are preferred.

Effect of hybridoma medium

Another interesting finding in paper IV is the effect the choice of hybridoma medium has on assay resistance to crossreacting anti-bovine antibodies. It is well-known that monoclonal antibodies produced in medium containing fetal calf serum may be contaminated with bovine immunoglobulin even after stringent purification protocols *(128, 129)*. In such cases, the bovine immunoglobulin is labelled and incorporated in the assay along with the (usually murine) assay antibodies, and anti-bovine antibodies in the sample may bind both assay antibodies and labelled bovine immunoglobulin. To my knowledge, paper IV is the first to document how this may affect interference in immunoassays, as reactivity to murine IgG1 was assessed in parallel using both murine IgG1 without contaminating bovine immunoglobulin and murine IgG1 with contaminating bovine immunoglobulin.

Without contaminating bovine immunoglobulin: When anti-murine IgG1 reactivity was assessed with blocking bovine immunoglobulin in assay buffers, the correlation with anti-bovine reactivity was 0.06 and the number of samples with anti-murine IgG1 reactivity was 148. When assessed without blocking bovine immunoglobulin in assay buffers, the

correlation with anti-bovine reactivity increased to 0.22 and the number of samples with anti-murine IgG1 reactivity increased to 183. Since anti-murine reactivity was assessed using murine IgG1 produced in chemically defined hybridoma medium free of bovine immunoglobulin, the increase in correlation when bovine blocking is removed represents the true cross-reactivity from anti-bovine antibodies to murine IgG1.

With contaminating bovine immunoglobulin: When anti-murine IgG1 reactivity was assessed with blocking bovine immunoglobulin in assay buffers, the correlation with anti-bovine reactivity was 0.09 and the number of samples with anti-murine IgG1 reactivity was 158. When assessed without blocking bovine immunoglobulin in assay buffers, the correlation with anti-bovine reactivity increased to 0.47 (!) and the number of samples with anti-murine IgG1 reactivity increased to 210. Since anti-murine reactivity was assessed using murine IgG1 produced hybridoma medium containing bovine immunoglobulin, the increase in correlation when bovine blocking is removed represents both cross-reactivity from anti-bovine antibodies to murine IgG1 and binding of anti-bovine antibodies to bovine immunoglobulin contaminating the murine IgG1 solid phase and tracer antibody.

Thankfully, there is a clear movement away from serum-containing hybridoma media to chemically defined media, particularly in the monoclonal antibody and diagnostics industry *(130, 131)*. Consequently, most antibodies used in commercial immunoassays are produced in serum-free hybridoma media, and patient anti-bovine antibodies rarely encounter contaminating bovine immunoglobulin in the clinical laboratory routine. Also, as shown by our results, the potential interference caused by contaminating bovine immunoglobulin is effectively neutralized by the ever-present blocking bovine immunoglobulin in assay buffers. As discussed previously, it is imperative that buffers contain sufficient blocking bovine immunoglobulin to neutralize even high concentrations of patient anti-bovine antibodies. If not, contaminating bovine immunoglobulin could exacerbate the broad interference caused by insufficient blocking of anti-bovine reactivity.

Rheumatoid factor, heterophilic, anti-animal and polyreactive antibodies.

We generally do not know why we have antibodies that bind animal antibodies, and several possible explanations exist. Antibodies to bovine antibodies are different in this regard, as

our extensive exposure to bovine immunoglobulin from early age is usually considered the likely cause that anti-bovine antibodies are so prevalent in our population. In some cases, prior exposure can explain antibodies to other animal antibodies as well, but certain prior exposure is rare in our experience from investigating assay interference from anti-animal or heterophilic antibodies in patient samples referred to our laboratory for evaluation. The work of Abner et al. allows for an intriguing understanding of the mechanism behind heterophilic antibodies and similar molecules *(58, 59)*. They conclude that individual antibodies can be polyspecific, by demonstrating that individual monoclonal antibodies can bind several different molecules such as human Fc, insulin etc. Similar results have been replicated in our laboratory *(132)*, and in a comprehensive overview published by Rubin and Theofilopoulos *(133)*.

The distinction between heterophilic antibodies, human anti-animal antibodies and rheumatoid factor is not absolute, and attempts have been made to clarify the relationships between the entities and terms used to describe them. It is well established that when patients are exposed to animal immunoglobulin in a medical setting (iatrogenic), such as murine antibodies used in diagnosis or therapy, the patient's antibody response to the animal antibody should be named after the animal antibody *(32)*. If patients are treated with unmodified murine monoclonal antibodies, which may still occur in early phase clinical trials, the patient antibody (often) produced in response to the murine monoclonal should be called a human anti-mouse antibody, a HAMA. If patients were treated with unmodified rabbit antibodies (to my knowledge, this is not done in modern medicine), the patient antibody produced in response to the rabbit antibodies should be called a human anti-rabbit antibody, a HARA.

The relationship between heterophilic and human anti-animal antibodies, and I believe we should include rheumatoid factor in this fold, is more complicated in cases without iatrogenic exposure to animal immunoglobulin. Classification of detected antibodies in these cases often depends as much on perspective and expectations as on the property or origin (which is unknown, after all) of the antibodies. Antibodies to rabbit immunoglobulin were the most prevalent antibodies in our study population in paper IV, present in 168 out of 3072 (5.5 %) samples using our criteria. Unlike the other reactivities we tested, anti-rabbit reactivity was not correlated to anti-bovine reactivity in our study, and the correlation was

not affected by removal of bovine immunoglobulin from assay buffers (Table 3, paper IV). This observation may indicate that the anti-animal reactivities we found can be classified into two main groups: one where anti-animal antibodies are likely related to exposure through diet or handling of animals, animal products or droppings (anti-murine, anti-sheep, anti-goat and anti-bovine) and another where anti-animal reactivities are likely related to auto-immunity (anti-rabbit). The high prevalence of antibodies to rabbit immunoglobulin found in our female cohort is comparable to, and could be related to, the prevalence of rheumatoid factor. Rheumatoid Factor (RF) is a term used to describe patient antibodies with reactivity to human antibodies, which are present in 5-10 % of the Norwegian population depending on the method used to measure them, and tests for rheumatoid factor are often designed using rabbit immunoglobulin. While some patients with RF may have antibodies with high affinity e.g. anti-mouse reactivity, most RF bind assay antibodies with low affinity, and likely rely more on high concentrations to cause interference. For this reason, assay interference may be more likely in patients with seropositive rheumatoid arthritis with high disease activity, where the highest concentrations of RF are normally seen *(114)*. Most package inserts contain data on assay resilience to RF up to a certain concentration, but this should not lead to exclusion of RF-interference, as RF may be present in much higher concentrations in patients and have different properties than the RF used to validate the assays.

The rarity of antibodies to sheep immunoglobulin compared to antibodies to rabbit immunoglobulin seen in our study is perhaps in conflict with an understanding that exposure is an important cause of anti-animal antibodies in humans, and may support a relationship between anti-rabbit antibodies and autoimmunity. Sheep meat is an important part of our diet, and most of the patients in our cohort would have handled and consumed sheep meat (and blood remnants) repeatedly. This is probably not the case with rabbit meat. Admittedly, our screening strategy might underestimate the number of samples with anti-sheep antibodies, as the screening was done using a buffer with blocking bovine immunoglobulin. Bovine and sheep antibodies have enough structural similarities that the bovine immunoglobulin might mask anti-sheep antibodies. This could be the case with anti-goat antibodies as well, also seen rarely in our cohort.

Kaplan and Levinson suggested that the term heterophilic antibodies should only be used when a sample contains antibodies reactive to more than one animal antibody, although they do not specify how many reactivities should be assessed before concluding that a sample only reacts to one animal antibody *(32)*. While attractive in theoretical discussions, in my opinion this definition remains entirely theoretical and is of little practical use or relevance to laboratorians working with assay interference. In interference testing, or any form of detection or characterization of antibodies to animal antibodies, results are highly influenced by choice of detection methods and strategy. As detection assays vary widely, and are usually in house assays, there is no consensus on how to define a positive or negative anti-antibody test. Most patients have some antibodies to bovine immunoglobulin, which usually cross react with sheep and goat antibodies, and sometimes with murine monoclonals, unless blocking bovine immunoglobulin is added to assay buffers. This means that if you have a test system including a range of animal antibodies, but are not careful to block against anti-bovine reactivity, you find a lot of heterophilic antibodies with reactivity to several animal antibodies.

In practice, the terms we use to describe anti-animal reactivities are determined by our own position and perspective, the information we possess at the time and the person we communicate with. The exact property of the anti-animal antibody, if known, is less important. If we discover antibodies to murine antibodies in a sample with suspected assay interference in our laboratory, we would usually call it a heterophilic antibody, even if the patient reported occasional exposure to mice or mouse droppings. If this sample was collected from a patient with seropositive (positive RF) rheumatoid arthritis, and we discussed our findings with a rheumatologist, we would call it a rheumatoid factor with reactivity to murine antibodies. If the same patient worked in an animal research facility and routinely performed necropsies of laboratory mice, we would probably call it a human anti-mouse antibody, even though the patient had RF and was not given mouse antibodies as part of diagnostics or therapy.

Current focus: Heterophilic antibodies and HAMAs in the age of biologics

When we started the work in this thesis we were highly interested in how the increasing use of antibody-based biologics would affect antibody-mediated interference in our laboratory

(7, 134, 135). Biologic drugs are diverse entities, but have in common that they require production in living cells, as opposed to most drugs that are produced synthetically (chemically). Biologic drugs are used extensively in modern medicine, and the use and associated costs are increasing rapidly. This is particularly true for inhibitors of tumor necrosis factor alpha (TNF-inhibitors) used in treatment of inflammatory diseases, leukocyte inhibitors to treat multiple sclerosis and checkpoint inhibitors used in treatment of an increasing range of malignancies. In fact, in the last decade or so, the three individual drugs with the highest direct drug costs to Norwegian healthcare are all TNF-inhibitors.

The reason for our interest is obvious, as many important biologic drugs are antibodies, antibody fragments or fusion proteins with antibody components. Patients receiving these treatments occasionally form antibodies to the drugs, increasing the risk of treatment failure and adverse infusion- or injection-related events *(136-140)*. We expected increased interference in assays using murine monoclonals (which is the case for most immunoassays in our laboratory) since one of the biologic drugs used most extensively (infliximab) is a chimeric antibody with murine and human components. In our initial experiments, even after testing a large number of patients treated with infliximab, we have not found a correlation between anti-drug antibodies and anti-murine reactivity using our interference assays (unpublished). This is most likely because the anti-drug antibodies formed are usually anti-idiotypic antibodies, and do not cross-react with other biologic drugs or the murine antibodies we use in our assays. In fact, it is quite possible that anti-animal reactivities are reduced in patients treated with antibody-based immunosuppressive drugs, since this treatment reduces disease activity and presumably the concentration of interfering antibodies. (Theoretically, and somewhat worrying from an interference perspective, the effect could be opposite in patients receiving checkpoint inhibitors in oncology, where the immune system is stimulated.)

To clarify the relationship between biologic drugs and antibody-mediated assay interference further, we are planning larger studies using clinical biobanks with samples collected before and after treatment with several biologic drugs. This is made possible through close collaboration between our laboratory and leading researchers in gastroenterology and rheumatology established in recent years. Particular focus will be on patients with seropositive rheumatoid arthritis, where we want to study the effect of biologic treatment

on interference from rheumatoid factor. In addition, in collaboration with the Norwegian veterinary school, we hope to characterize anti-animal reactivities in a selection of domesticated mammals.

Additional sources of confusing results in immunoassays

Antibody-mediated crosslinking, aggregation or blocking of assay antibodies are not the only sources of false and confusing results in immunoassays. Our laboratory is a reference laboratory for immunoassay interference. We often get samples referred from other Norwegian laboratories and occasionally from laboratories abroad. In most cases when heterophilic antibody interference is suspected we conclude that other mechanisms are more likely explanations. Extensive testing is often needed to identify the true causes of unexpected results, and we believe this complexity is important to recognize. Some causes of false or confusing immunoassay results may be related to unknown assay vulnerabilities or unrecognized pathological processes or substance use. In addition, some sources of interference, both patient-related (i.e. biotin) and assay/instrument-related (i.e. carryover), are general sources of false results that may produce confusion in other assays or patients where consequences may be serious if left undetected. The complexity and potential gravity of immunoassay interference is, in our opinion, an argument for centralized interference testing. Interpretation of interference tests is, as discussed both in the articles and elsewhere in this thesis, not a simple matter. In a setting where a laboratory is only equipped and experienced to elucidate some sources of interferences, there is an increased chance that interference may go undetected or falsely attributed to the limited range of interferences the laboratory focuses on. I believe false attribution to heterophilic antibody interference is not uncommon when unexpected laboratory results are discussed, be it in the clinical routine or in the literature on the subject. In a clinical setting, such false attribution could obviously be harmful to patient care, as potentially serious conditions could go undiagnosed and untreated. This is illustrated in a publication from the USA hCG Reference Service *(141)*, where 114 cases of elevated hCG in patients without clinical signs of disease (or pregnancy) were evaluated. Out of the 114, 51 had false positive hCG due to heterophilic antibody interference. The remaining 63 patients had real elevated hCG, according to the authors, attributed to quiescent (assumed premalignant/preinvasive)

gestational trophoblastic disease. Some of the patients later developed clinically detectable malignant disease.

Some alternative sources of confusion are described below, where the sources and mechanisms are discussed in varying detail depending on assumed clinical impact and relevance to heterophilic type interference.

Interference related to tracer molecules

In immunoassays using alkaline phosphatase (ALP) to generate signals, and this includes automated immunoassays from major immunoassay producers, endogenous ALP may interfere with analyte quantification *(142-144)*. As assays based on quantification using ALP will always include a wash step before addition of substrate, (barring instrument malfunction) only samples with extreme concentrations of ALP can be expected to give false results. Thus, such interference is likely rare.

Macro-analytes

Analyte plasma values associated with both health and disease are essentially products of analyte production and clearance, and plasma half-lives and clearance varies widely between analytes. In some individuals, endogenous antibodies bind the analyte, altering the plasma clearance and half-life of the analyte to effectively mirror that of the antibody subclass(es) in question. Since antibodies generally speaking have very long half-lives, this usually leads to an increased plasma concentration of the analyte. In these cases, plasma concentration of the analyte may increase severalfold without increased production or actual pathology.

Macro-prolactin and macro-amylase are familiar entities to all laboratorians and most clinicians working with these analytes *(145, 146)*, but macro-variants of troponins, TSH and aspartate aminotransferase (AST, in Scandinavia: ASAT) have all been reported in the literature *(147-149)* and encountered in our laboratory. Typical tests for macro-analytes include gel electrophoresis with immunofixation/western blot (to determine size of immunoreactive analyte) or reassay after PEG-precipitation, protein A/G affinity

chromatography or size-exclusion chromatography (to separate analyte and immunoglobulin/immune complexes).

Particular focus might be warranted on troponins in this regard, as the consequences to patient care are often dramatic and immediate. We have seen several cases of macrotroponins referred to our laboratory, all associated with significant impact on follow-up of the patients, ranging from additional invasive diagnostic procedures to significant limitations on personal and professional activities. We lack systematic data on risk and incidence of macrotroponins, but our impression is that some patient groups may have an increased risk of developing this phenomenon. These include patients who have endured invasive cardiac procedures, ischemic cardiac events and (particularly) myocarditis. A temporary release of cardiac troponins into plasma is expected in all these situations, where the cardiac troponins presumably elicit immune responses in some patients *(150)*. In two patients referred to our laboratorium, both with serious ischemic cardiac events in their medical history but without signs of ongoing ischemia, we did not see obvious changes in the troponin result over time. In these patients, all samples were collected several months after the initial event and within a limited time period, which could contribute to this lack of dynamic. One patient, with very low risk of ischemic heart disease and suspected recent myocarditis, was followed with frequent troponin measurements. Shortly after the suspected bout of myocarditis, when he was free from any symptoms or other signs of cardiac disease, his troponin levels started to increase. Samples were sent to our laboratory, where we found no evidence of heterophilic antibodies in interference assays or blocking experiments. However, protein G affinity chromatography removed all troponin in the samples, indicating the troponin was bound to immunoglobulins. During the next weeks his troponin levels continued to increase before they gradually decreased over a few months to undetectable levels, very much mirroring the expected dynamics of an immune response to a foreign antigen.

Extensive focus is put on macro-analytes in this section because they create particular analytical challenges also in testing for heterophilic antibody interference and interpretation of results. In a laboratory where interference test repertoire is limited to reassaying after immunoglobulin removal through PEG-precipitation, protein A/G affinity chromatography or size-exclusion chromatography (which is not uncommon), it is not possible to distinguish

between heterophilic antibody interference and macro-analyte. Such equivocality probably has little consequence for patient care, but it limits our understanding of both the biology and assay vulnerabilities creating confusion in these cases. It is also a potential (likely quite common) source of false attribution, particularly to heterophilic antibody interference, when cases of incongruent immunoassay results are published.

It should be mentioned that autoantibodies to troponins can also give false low results in cases where the antibodies block the epitopes of assay antibodies *(151, 152)*, but we have not identified any such cases in our laboratory. Admittedly, we are probably not equipped to identify such cases, but have sent samples to collaborators who are.

Cross reacting substances

Pharmaceutical. Several pharmaceutical drugs are known to give false result in immunoassays. Examples include prednisolone which cross reacts in most assays for cortisol, which is not surprising considering structure homology, and furosemide which interferes in some assays for thyroid hormones through an unknown mechanism. These interferences are usually well documented in assay package inserts, but still create problems because many clinicians are not aware of the possibility.

Hormones: Among related endogenous hormones, such as the plethora of steroid hormones, but also among the larger and more complex pituitary hormones, structural similarities create analytical challenges. This used to be a significant problem when steroid hormone analysis was limited to immunoassay technology, and the lack of specificity of certain antibodies used to measure pituitary hormones was less understood. Today, these problems are reduced as steroid hormones are increasingly measured using mass spectrometry, if not as the primary measurement then at least in confirmatory analysis. Also, specific antibodies to what we now recognize as unique β-subunit epitopes of individual pituitary hormones are usually preferred in immunoassay design, replacing antibodies to common α- subunit epitopes or non-unique β- subunit epitopes. Previously, cross-reactivity in assays for hCG, TSH, LH and FSH created ample confusion in the follow-up of pregnancies and hCG-producing tumors such as testicular cancer and gestational trophoblastic disease, to name a few examples.

Metabolites and protein/peptide variants: Immunoassays often have varying abilities to detect metabolites of endogenous (i.e. hormones, precursor peptides) or pharmaceutical compounds (i.e. methotrexate *(153)*, cyclosporine A *(154)*) depending on the properties of assay antibodies. In this regard, assay properties are usually known and documented in package inserts, but clinicians using these assays may need reminders of this interaction.

In addition, and more relevant to the subject in question, certain protein variants (i.e. truncated forms) may be detected in some assays but not in others. Important examples of the latter are unequal detection of the six major hCG-variants, the varying ability of immunoassays for carcinoembryonal antigen (CEA) to detect the common truncated variant non-specific cross reacting antigen 2 (NCA-2), and the unpredictable detection of different ACTH-related peptides produced by malignant tumors. In oncology, this may create significant confusion if patients are followed in different medical centres using assays with different properties, which is common since care for cancer patients is often shared between highly specialized central hospitals and general local hospitals or primary physicians. It is also a source of confusion in interference testing, as reassay with a different method is one of the most accessible and (generally speaking) reliable interference tests when heterophilic antibody interference is suspected. When two commercial immunoassays give very different results, it is generally interpreted as a positive interference test, and the elevated result is attributed to interference and not a pathological process. This illustrates that we need to know the properties of the immunoassays we use in our laboratories, particularly when evaluating interference as possible cause of assay results that may also indicate serious disease. It is also highly relevant when establishing reference intervals, or updating these when changing analytical methods, but this discussion goes beyond the scope of this thesis.

Clearance

Most analytical compounds are removed from the circulation in the liver or kidneys. Large and complex molecules, but also some smaller pharmaceutical compounds, are primarily removed by the liver, while most small compounds including peptides are primarily removed by the kidneys. Hepatic clearance is impaired in patients with hepatic failure, and increased concentration of analytes normally removed by the liver can be found in the circulation,

without increased production due to other pathological conditions. Correspondingly, renal clearance is impaired in patients with impaired renal function, and increased concentration of analytes normally removed by the kidneys can be found in the circulation without increased production. This is a general challenge in clinical chemistry and not directly relevant to heterophilic antibody interference.

In continuation of the discussion on renal failure, it is worth mentioning a mechanism relevant to antibody-mediated interference whereby dialysis may create confusion in immunoassays. Membranes used in hemodialysis have varying properties and may treat biomarkers, and particularly variants of biomarkers, differently. In such cases, certain molecular variants may be effectively cleared in dialysis while others are retained. If the retained variants, e.g. of a tumor marker like hCG, are measured to give an elevated result, it may create additional challenges in the already complicated care of these patients. We were involved in the work up of one patient where a scheduled kidney transplantation was delayed due to suspicion of malignant disease. The combination of an uncertain radiological finding and an increasing hCG-level (to pathological levels > 6 times the upper reference limit) led to several weeks of additional work up and a host of diagnostic procedures. After we concluded that the hCG-result was most likely related to selective retainment of an unknown hCG-variant by the dialysis membrane in question, the patient received a new kidney and the hCG-level returned to undetectable levels. Additional confusion may be created if immunoassays see the retained variants differently, where different results in different assays could lead to a false attribution to heterophilic antibody interference *(155)*.

Rare variants, genetic causes

Occasionally we observe abherrant immunoassay results we cannot explain, even after extensive experiments and testing, or unexpected results caused by extremely rare conditions. Three cases in our laboratory were all related to tumor marker assays, had grossly elevated results, and had obvious medical and emotional consequences to the patients.

One was a case of clearly elevated hCG that led to invasive diagnostics procedures and treatment with chemotherapy, that had no effect on the hCG-result. Extensive interference

testing did not indicate the result was false, and it was only when we tested family members of the patient we were able to understand the mechanism. Some members of the family had hCG-levels comparable to the level in the index patient, the rest had undetectable (normal) levels. This was perceived as examples of what is known as familial hCG, a genetically determined hCG-production thought to be extremely rare. Since hCG is actually present in plasma, these patients have increased risk of false diagnosis caused by positive pregnancy tests in both plasma and urine, elevated hCG levels when measured as tumor marker and even possible positive outcome in sports doping testing.

Two other patients had grossly elevated levels of CEA and alfa-fetoprotein (AFP), respectively. To our knowledge, both tests were originally ordered in primary care, as part of health controls without strong suspicion of malignant disease. In both cases, interference and malignant disease were considered, but neither extensive interference testing nor prolonged diagnostic work up could provide explanations for the results. Both patients expressed significant distress during and following investigations, which illustrates the potential emotional impact of abnormal laboratory results, and is highly relevant in a discussion on heterophilic antibody interference. It also serves as a reminder to use tumor marker assays conservatively.

When assays are designed using monoclonal antibodies, substitution of one amino acid in the analyte may be sufficient to change the ability of the assay to measure the analyte. Many examples likely exist, but few have been documented in the literature. A notable exception is the description of a rare TSH-mutation (a point mutation in the β-chain of TSH) that gave falsely undetectable TSH in 4 commercial TSH-assays *(156)*. The mutation was found in 20 of approximately 2 million individuals, all of South Asian ethnicity, and is not expected to affect biological activity of TSH.

Samples

Hemolysis, lipemia, icterus or clots in samples may affect assay results, but this is familiar to most laboratorians and will not be discussed further. Monoclonal components, as found in patients with multiple myeloma or Waldenström's macroglobulinemia, may form aggregates or precipitates in certain assays. To my knowledge, this is most common in assays involving

an acidic dissociation step to separate analytes from binding proteins, such as assays for vitamin D. Aggregation or precipitation of monoclonal components in these assays may interfere either by inhibiting binding of analytes to reagents, or by increasing opacity in assay wells and disturbing the quantification step. Analyzing the analyte in alternative assays, preferably using other methodologies such as mass spectrometry, usually reveal interference. Serial dilutions may also help, but interpretation may be challenging, both because individual components and samples behave differently when diluted, but also because the assays themselves do not always give linear responses upon dilution.

In addition, particularly when sample material is transferred to secondary tubes before transportation or analysis, there is a risk that analysis may be performed in the wrong medium (e.g. EDTA-plasma and not serum) or the wrong sample (due to mistakes in sample transferal or labelling). In such cases, it may be very difficult to identify false results, at least if comparison with previous results is not possible.

Biotin, anti-streptavidin antibodies and anti-tracer antibodies

Interference from biotin in patient samples is a potentially serious problem in immunoassays. The impressive binding affinity between biotin (vitamin B7) and streptavidin (a protein isolated from a streptococcus genus) is used by many immunoassay producers to immobilize assay antibodies on solid phases (beads or wells). Patients taking high doses of biotin supplements may have sufficient biotin in samples to interfere with the interaction between biotinylated antibodies and streptavidin solid phases necessary for assay performance. Biotin interference is particularly ominous because it often leads to false results in several assays, e.g. a falsely elevated thyroxine (FT4)-level and a false low result for thyroid-stimulating hormone (TSH). If only one of these results were abnormal, we would perhaps expect interference, but seen together, these results validate each other and increase the risk of false diagnosis, in this case hyperthyroidism.

The problem has been actualized recently both because biotin is marketed as a “beauty vitamin”, with claims of beneficial effects on hair and skin, and because extremely high biotin doses has shown promise in the treatment of progressive multiple sclerosis. A recent warning from the U.S. Food and Drug Administration (FDA), following the death of a patient

related to false negative Troponin measurements after a heart attack, puts increased focus on this potential interferent. Our department is located at a hospital that cares for children with inborn errors of metabolism, including children with biotinidase/carboxylase deficiencies that depend on high doses of biotin to reduce the risk of devastating disease manifestations. We also buy the majority of our immunoassay kits from a producer (Roche Diagnostics) that uses the biotin-streptavidin interaction in most immunoassays they offer. As such, we have particular focus on interference from biotin in our department. It is difficult to find satisfactory solutions to this problem in a modern hospital laboratory such as ours. Because of costs, we cannot have confirmatory assays available in our department that don't rely on biotin-streptavidin interaction, at least not for all these markers. For certain markers such as troponin, either we or the assay manufacturer will have to find solutions to control the potentially detrimental effect of biotin interference before patients are hurt. Thankfully, an improved version of the Roche assay for troponin T is currently being launched.

Similar effects may be seen if patients have antibodies to streptavidin or anti-tracer antibodies that bind tracer-protein complexes such as antibodies labelled with ruthenium (acting as a hapten), but both these mechanisms are likely very rare. (While patient anti-hapten antibodies may lead to aggregation of assay antibodies, they have little in common with heterophilic antibodies and are discussed separately.) The past 10 years we have only identified one patient with anti-streptavidin antibodies, none with anti-tracer antibodies, but I expect they are both more common than we presume. These mechanisms are important to be aware of since they are antibody-mediated interferences that may be confused with heterophilic antibody interference in some interference tests involving antibody removal.

Instruments

Carryover from samples with extremely high concentrations of analyte is a particular concern in our laboratory, since the tumor marker assays we offer can have significant impact on patient care, and because results in many of these assays vary by a factor of millions. Most modern analytical instruments have mechanisms to detect carryover, but these are not guarantees that erroneous results escape detection. Technical issues including carryover, but also mishaps like bubbles, clots or clogged pipettes, can usually be resolved by

reassay on the same instrument that gave the suspect result in the first place, and this should always be the first interference test undertaken.

High-dose hook effect, antigen excess

False results generated by antigen excess, in immunoassays often referred to as the high-dose hook effect, may cause confusion if elevated biomarker levels are expected, but can easily go undetected if elevated biomarker levels are not expected. The mechanism behind the high-dose hook effect is that the analyte is present in such high concentrations that the two assay antibodies are fully saturated with analyte, but are bound to separate analyte molecules and are unable to form the sandwich complex on which detection is based.

Binding proteins

Some analytes, particularly vitamins and hormones, are normally bound to binding or carrier proteins in plasma. In some cases, high levels of the binding protein may give falsely elevated results in competitive immunoassays through the binding and consumption of tracer, which is usually labelled analyte. In metric assays, high levels of binding proteins should in theory give false low results. Another potential source of false results is incomplete separation of analyte from binding protein, causing the analyte to remain undetectable by the assay. This problem should be rare in modern commercial assays, but could confuse in older reports or in house research assays.

Conclusion

As mentioned previously in this thesis, immunoassay developers today have the knowledge and tools to design assays with very high resistance to heterophilic antibody interference. New immunoassay applications, along with new reagents, are continuously being introduced into the field. In fact, with increasing access to non-murine monoclonals, recombinant antibodies and non-immunoglobulin alternatives, assay developers will likely have even more tools to improve assay resistance in the future. As laboratory physicians, we should encourage the diagnostics industry to use the available tools to limit interference, both in

scientific research and communication, but also when purchasing immunoassay kits and instruments. In addition to providing new possibilities to limit interference, however, every new application or reagent likely also comes with new possibilities for interference. In this regard, it is important to remember and use the knowledge accumulated over the past decades to reduce damage from interference in the future.

The most important consequence of the work in this thesis, at least on a national level, is probably that our laboratory continues as a reference laboratory for immunoassay interference. While this status is not formalized in any way, the work of my supervisors in recent decades has established our position, and we regularly receive samples for evaluation or are consulted when interference is suspected in other laboratories. In many of these cases, we help clinicians avoid or cancel unnecessary diagnostic procedures and treatments. In addition, we have helped several European laboratories, a few biotech companies and the Danish quality control organization DEKS with advice and sample evaluation when needed. When heterophilic antibody interference (or interference of any kind) is suspected in immunoassays, experience with execution and interpretation of interference tests is vital. This is discussed in detail when listing other sources of false results previously in this thesis, and also in paper V. As the consequences of false attribution to (but also missed identification of) heterophilic antibody interference can have serious consequences to patients, it is beneficial to have a centralized service for consultation, testing and interpretation. It also gives us the experience and confidence to continue with systematic research on assay interference when new immunoassay applications, sources of interference or patient groups emerge.

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Paper II

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Heterophilic antibody interference in commercial immunoassays; a screening study using paired native and pre-blocked sera

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Abstract

Background: Heterophilic antibodies are still an important source of interference in immunoassays. We have conducted a screening study for interference in a panel of commercially available assays using two sera known to contain high titer Fc-reactive heterophilic antibodies.
Methods: The sera were distributed to laboratories participating in the Nordic External Quality Assessment cooperation (EQANord). Duplicate samples pre-blocked with aggregated murine monoclonal MAK33 were also supplied. Discrepancies (>50%) between the results for native and blocked samples were used to classify the tested assays as susceptible to interference. A total of 170 different assay kits covering 91 analytes were tested.
Results: We found that 21 assays, covering 19 different analytes, were susceptible to interference from the heterophilic antibodies in the two sera. Many of these are clinically and commercially important assays. Some of the false results were grossly elevated and could have been detrimental to patient care in a clinical setting.
Conclusions: Heterophilic antibodies with Fc-reactivity remain a threat. A more widespread use of antibody fragments and aggregated immunoglobulin could potentially improve the heterophilic antibody resistance of assays intended for clinical use.

Keywords: HAMAs; heterophile antibody; heterophilic; interference; immunoassay.

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Introduction

Immunoassay techniques have revolutionized the determination of clinically relevant protein and peptide analytes. However, these methods do not always give the ''correct'' result (1–4), and extreme caution is needed when clinical findings and assay results are discordant. The unnecessary diagnostic and therapeutic interventions that often follow such discordance can be costly to both patients and hospitals (5–11). In these situations troubleshooting is complicated by the proprietary nature of most information relating to the assay kits.

Immunometric assays are particularly sensitive to interference by multivalent antibody-binding moieties that can bridge the reagent antibodies. Such cross-linking results in the generation of positive assay signals in the absence of analyte. Heterophilic or human anti-mouse antibodies (HAMAs) present in patient sera are the usual culprits (1, 3, 4). Several approaches can be effective in limiting heterophilic antibody interference including sample pretreatment with heterophilic blocking tubes (HBT) (12), polyethylene glycol (PEG) precipitation (13), affinity chromatography on protein A (14) or size exclusion chromatography (15). These methods are however not well suited to the high-throughput assays used in clinical laboratories. Indeed, optimal reductions in assay interference can most probably only be achieved by focusing on this problem during the assay design phase (16).

In a previous study, we found that the frequency of interference in our in-house immunometric assay for carcinoembryonic antigen (CEA) was 4.0% (1). The addition of a heat-aggregated irrelevant murine monoclonal antibody, MAK33, to the assay buffer reduced the frequency to 0.86%. Significantly, the use of $F(ab')_2$ fragments as assay solid phases was found to reduce the frequency of interference to 0.1% even in the absence of irrelevant mouse immunoglobulin. This is in agreement with previous studies which indicated that most interfering immunoglobulin target the Fc portion of assay antibodies (17, 18).

Most commercial immunoassays have irrelevant animal immunoglobulin added to the assay reagents in order to limit interference. Some manufacturers aggregate the immunoglobulin by heat or chemicals, but this is rarely detailed in package inserts. To our knowledge, only a limited number of commercial assays are constructed using $F(ab')_2$ or Fab' fragments.

Herein we describe the results from a screening study of heterophilic antibody interference in commercially available immunoassays using two high titer sera originally referred to

our laboratory for testing. Both sera, shown to contain Fc-reactive heterophilic antibodies, were distributed to 18 clinical laboratories through the Nordic External Quality Assessment cooperation (EQAnord). The sera were supplied in both native form and pre-blocked by the addition of heat-aggregated MAK33 non-specific immunoglobulin.

Our aims were firstly to investigate if selected sera, supplied as paired native and pre-blocked specimens, can be used as screening tools for assay interference and secondly, how well a panel of commercial assays were protected against Fc-reactive heterophilic antibodies.

Materials and methods

Human test sera used for assay screening

Serum 1 was from a man in his fifties. Interference was suspected when analysis of soluble transferrin receptor (Tina-quant® sTfR on the Cobas Integra 800; Roche Diagnostics, Mannheim, Germany) gave an elevated result without corresponding laboratory results or clinical symptoms. The result was normalized after addition of aggregated MAK33 to the sample. Approximately 6 months after he donated serum to our study, he was diagnosed with rheumatoid arthritis.

Serum 2 was from a woman in her thirties. Interference was suspected when analysis of β-hCG (total β-hCG on the Architect i2000 SR; Abbott Diagnostics, Abbott Park, IL, USA) gave an elevated result without concomitant pregnancy or malignancy. The result was normalized after addition of MAK33. Re-analysis of β-hCG with a different method (hCG+β on the Cobas e601; Roche) confirmed the normal result. Tragically, prior to our identification of heterophilic antibody interference, this donor endured unnecessary chemotherapy and three inappropriate surgical procedures, including the laparoscopic removal of a fallopian tube.

Both sera were obtained with informed consent following national and institutional guidelines. The study has been approved by the Oslo university hospital privacy office.

Characterization of heterophilic antibodies

All assays used to characterize the sera were manual 3-step methods performed using streptavidin-coated DELFIA® microtitration strips. The wells were coated with biotinylated antibodies, washed, and then incubated with the serum samples. Following additional washing, the assays were developed using europium-labeled tracer antibodies. Methodological details are given in a supplemental file (web only) and in Bjerner et al. (19).

To detect heterophilic antibodies, and characterize their reactivity to murine immunoglobulin, assays were established using intact IgG, F(ab')$_2$, and Fc fragments of the IgG1 monoclonal antibodies K57 (anti-α-fetoprotein) and T84.66 (anti-carcinoembryonic antigen) as solid phase reagents and K57-IgG as tracer. These are non-sense immunometric assays since they use non-complementary assay pairs. A positive signal indicates the cross-linking of the solid phase and tracer antibodies in the absence of analyte.

Species specificity of the heterophilic antibodies was determined using polyclonal murine, rabbit, ovine, equine, bovine, and human IgG as solid phase antibodies and K57-IgG as tracer antibody.

The size of the interfering antibodies was estimated by gel-permeation chromatography on a pre-calibrated Superdex S200 column. Column fractions were assayed using a non-sense method (solid phase K57-IgG, tracer K57). Isotyping was performed using K57-IgG as solid phase antibody and commercially available rabbit F(ab')$_2$ antibodies to human heavy and light chains as tracers.

Selection of tested assays and participating laboratories

EQAnord provided invaluable assistance in selecting representative laboratories and methods. Particular focus was directed at including the assays most widely used in the Nordic countries. Where possible, assays performed on different instrument models from the same manufacturer were tested. Of the 19 laboratories invited to participate in our study, only one laboratory declined, citing reorganization of laboratory services. The participating laboratories were invited to include immunoassays at will if they had surplus test sera. For this reason, some in-house assays and non-immunometric (competitive) assays were included in the study. A total of 170 commercial immunoassay kits were tested.

Heat treatment of MAK33

Murine monoclonal IgG1κ antibody MAK33 (Roche Molecular Biochemicals, Mannheim, Germany) was stored at a concentration of 2 g/L in 0.15 mol/L NaCl, 0.01 mol/L Na_2HPO_4, pH 7.4, at –30°C. Aliquots were heat-treated by incubation in a 60°C water bath for 10 min (1). The change in absorption at 595 nm from approximately 0.02 to approximately 1.0 was used to monitor aggregation.

Screening of commercial immunometric assays

Aliquots of the two patient sera with known interference from heterophilic antibodies were distributed to participating laboratories. Duplicate samples pre-blocked with 180 μg/mL aggregated murine monoclonal MAK33 were also supplied. The participating laboratories were informed about the purpose and design of the study, but were not informed about which aliquots were blocked with MAK33. Laboratories were instructed to perform analyses and report results as for routine samples.

Prior to the study, we set a cut-off limit of 50% for the difference between the results from native and pre-blocked sera to indicate if the method tested is vulnerable to heterophilic antibody interference. As assays are subject to analytical variation, such differences may occur by chance. The probabilities for an observed 50% difference between native and pre-blocked sera by chance are $p=0.001$ at an analytical CV of 11.4%, $p=0.01$ at a CV of 15.1% and $p=0.05$ at a CV of 21.4%. The three smallest differences considered to be significant in the study were in the SHBG and BNP assays from Abbott laboratories, with observed differences of 60% and 107%, and analytical CV of 10% and 12% as stated by the manufacturer, and the AutoDELFIA TSH assay from Perkin Elmer Life Sciences, with an observed difference of 60%, where analytical CV has been reported as 2.8% (20). This corresponds to $p<0.001$ for all the three assays. Thus, all differences reported in the study correspond to $p<0.001$. Our screening method might not be suitable for some of the included assays, e.g., competitive assays or assays using non-murine antibodies. As we have little experience with interference in competitive assays, we have not classified these assays based on our test. However, results from all tested assays are presented in Table 2 in the electronic supplement that accompanies this paper.

Results

Characterization of sera

Both sera gave grossly elevated responses in non-sense assays indicating the presence of heterophilic antibodies with

affinity for whole IgG and the Fc fragments of IgG1 antibodies. They also displayed high titers and could be diluted 1:300 (serum 2) and 1:3,000 (serum 1) before a positive assay signal was lost. Responses were normalized after the addition of heat-aggregated MAK33 immunoglobulin to the sera. Very little binding to $F(ab')_2$ fragments was observed (Figure 1).

In addition to their strong reactivity to murine IgG1-antibodies, the heterophilic antibodies in serum 1 showed some cross-reactivity to rabbit IgG, but minimal cross-reactivity to human, bovine, and equine IgG, while the heterophilic antibodies in serum 2 showed some cross-reactivity to bovine IgG (Figure 2).

Gel-permeation chromatography indicated that the size of the heterophilic antibodies was >650 kDa suggesting that they are most likely IgMs (data not shown). Isotyping using a modified non-sense assay gave strong signals for μ and κ but comparatively low signals for λ light chain. However, we were unable to detect monoclonal components using the routine methods available at our hospital: capillary zone electrophoresis with immunotyping and immunofixation electrophoresis.

Screening of commercial immunoassays

Analysis of one or both sera showed interference in 21 assay kits covering 19 different analytes (Table 1). As expected, interference was not limited to assays from one or a few manufacturers. False results were seen for both test sera in 13 assays, while 8 assays gave false results for either serum 1 (6 assays) or serum 2 (2 assays). The degree of false elevation varied between assays. In 15 assays, results for one or both native samples were increased at least five-fold compared to corresponding blocked samples. In 11 assays, the difference between native and blocked sera was >10-fold. No assays displayed negative interference in this study. The

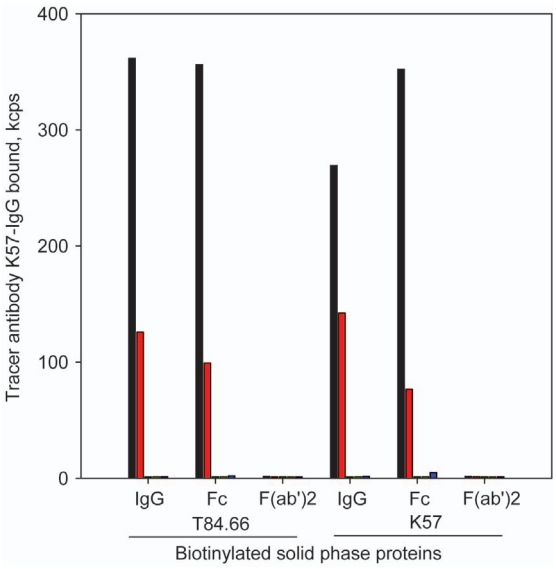

Figure 1 Reactivity of heterophilic antibodies to murine IgG1 fragments.

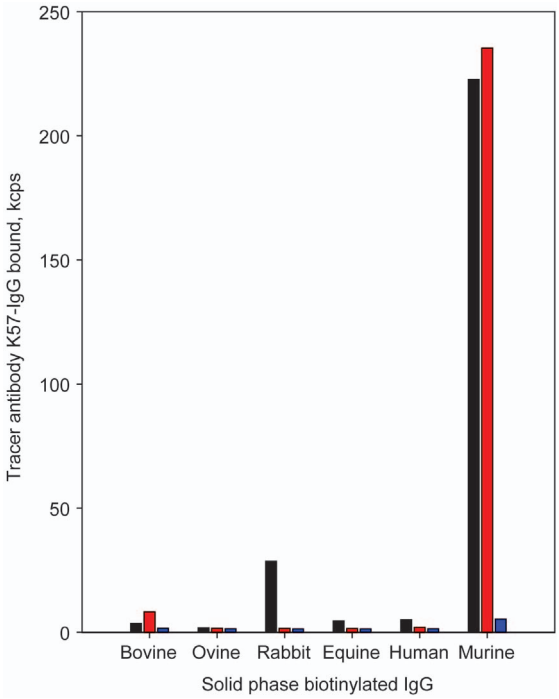

Figure 2 Reactivity of heterophilic antibodies to IgG from different animal species.

results for the pre-blocked samples in vulnerable assays were comparable to corresponding results for the native samples in resistant assays (Table 2, electronic supplement).

For one assay, CA125 (CA125II, Abbott) on the Architect platform, our results could indicate a significant variation between lots with respect to vulnerability from interference (lot numbers are reported in the electronic supplement). For the other interference-positive assays where different lots were tested, such as β-hCG (total β-hCG, Abbott) and soluble transferrin receptor (Tina-quant® sTfR, Roche), inter-batch variation was not observed in this study.

CA125 on Abbott Architect models i2000 and ci8200, and β-hCG on Abbott Architect models i2000SR, ci8200 and ci16200 showed falsely elevated results for both sera tested, with little or no difference between instruments. All results were normalized when adding heat-treated MAK33. The results obtained for CA125 and β-hCG on the Architect instruments using pre-blocked sera are comparable to results from assays negative for interference.

D-dimer (STA®-LIATEST® D-DI) on STA-R Evolution and STA Compact; (Diagnostica Stago, Gennevilliers, France, www.stago.com) showed grossly elevated results for both sera tested. Although significantly lower than in the native sample, results after addition of heat treated MAK33 in serum 1 differed from results obtained with assays negative for interference. This is most likely due to the addition of inadequate amounts of aggregated immunoglobulin to completely block interference in this sample in this particular assay. Results for serum 2 with MAK33 are comparable to those from assays negative for interference. The values obtained in serum samples are higher than in a plasma sam-

Table 1 Assays vulnerable to inference in the present study[a].

Analyte, unit	Manufacturer	Instrument	Serum 1 blocked	Serum 1	Serum 2 blocked	Serum 2
AFP, μg/L	Diasorin	Liaison	2.6	**4.2**	0.6	**2.8**
BNP, pmol/L	Abbott	Architect i2000SR	<2.9	**6.0**	<2.9	<2.9
		Architect ci16200	<2.9	3.4	<2.9	<2.9
CA125, kU/L	Abbott	Architect i2000	8	**256**	6	**96**
		Architect ci8200	7	**278**	6	**84**
		Architect i2000	7	**20**	6.7	**20**
	Diasorin	Liaison	5.5	7	4.2	**74**
CA19-9, kU/L	Diasorin	Liaison	17	18	7.3	**89**
CEA, μg/L	Diasorin	Liaison	<1	**1.9**	<1	**3.6**
Crosslaps, ng/L	Roche	Modular E 170	75.6	**1838**	11.5	**32.8**
CRP[b], mg/L	Roche	Cobas Integra 800	1.1	**3.4**	<0.6	0.7
		Modular P	<0.6	**4.3**	<0.6	<0.6
D-dimer[b], mg/L	Diagnostica Stago	STA-R Evolution	1.8	**>4.0**	0.6	**>4.0**
		STA Compact	2.5	**>20**	0.5	**2.3**
		STA Compact	2.1	**>20**	0.6	**2.2**
GH, μg/L	PerkinElmer	AutoDELFIA	0.1	**1.5**	0.1	**0.5**
hCG, U/L	Abbott	Architect ci16200	<1.0	**42**	<1.0	**113**
(Total β)		Architect i2000 SR	<1.2	**59**	<1.2	**147**
		Architect ci8200	<2	**36**	<2	**117**
Insulin, pmol/L	Siemens	Immulite 2000	351	**1191**	22	**131**
Interleukin 1β, ng/L	Bio-Rad	Bio-Plex 200	<0.13	**134**	<0.13	**2.8**
Interleukin 2, ng/L	Bio-Rad	Bio-Plex 200	<0.13	**685**	<0.13	**4.8**
Interleukin 6, ng/L	Bio-Rad	Bio-Plex 200	18.8	**232**	1.6	**11**
PAPP-A, U/L	PerkinElmer	AutoDELFIA	0.003	**0.16**	0.002	**0.21**
SHBG, nmol/L	Abbott	Architect i2000	32.8	**52.6**	97.3	98
	Beckman Coulter	UniCel DxI 800	29	**99.5**	73.7	85
sTfR[b], mg/L	Roche	Modular P	3.5	**26.5**	1.7	1.8
		Cobas Integra 800	4.4	**19.2**	1.9	**3.1**
		Hitachi 917	2.7	**25.7**	1.7	1.8
TNF-α, ng/L	Bio-Rad	Bio-Plex 200	1.2	**95.5**	4.8	5.3
TSH, mU/L	PerkinElmer	AutoDELFIA	1.5	**2.3**	1.6	2.1

[a]Results indicating interference (difference >50% between native and blocked sample) in bold. [b]Immunoturbidimetric assays. Assays resistant to interference in our study (listed by manufacturer): Abbott: AFP, anti-CCP, anti-CMV IgG, anti-HAV IgG, anti-HAV IgM, anti-HBcII, anti-HBs, anti-HCV, anti-HTLV I/II, anti-rubella IgG, anti-toxo IgG, anti-toxo IgM, anti-TPO, B12, CA15-3, CA19-9, CEA, CRP, ferritin, FSH, FT3, FT4, HBsAG, HIV Ag/Ab combo, LH, prolactin, PSA, PSA(free), PTH, transferrin, tTroponin I, TSH. Beckman Coulter: anti-TPO, BNP, CA125, CEA, estradiol, ferritin, FSH, hCG (total β), LH, prolactin, PSA, PTH, testosterone, troponin I, TSH. Biomerieux: anti-CMV IgM. Bio-RAD: anti-toxo IgG, anti-toxo IgM, anti-mycoplasma IgM. Biotest: anti-EBNA IgG. Biotrin: anti-parvo B19 IgG, anti-parvo B19 IgM. Brahms: AFP, CA19-9, procalcitonin, TgAB**.** Dade Behring (Siemens): anti-borrelia IgG, anti-borrelia IgM, anti-HSV IgG, anti-syphilis, anti-varicella IgG. Diasorin: CA15-3, NSE. Instrumentation Lab: d-dimer. Novitec (Orion): anti-EBV VCA IgG, anti-EBV VCA IgM. Ortho: BNP, CRP, troponin I. PerkinElmer: α-1-antitrypsin, LH, FSH, β-hCG (free), PSA, PSA (free). Roche: AFP, ApoA1, ApoB, CA15-3, CA19-9, CA72-4, CA125, CEA, cortisol, C-peptide, cyfra 21-1, D-dimer, DHEAS, estradiol, ferritin, FSH, FT3, FT4, haptoglobin, hCG (hCG+β), IgA, IgG, IgM, insulin, LH, myoglobin, NSE, NT-proBNP, progesterone, prolactin, PSA, PTH, S-100, SHBG, testosterone, transferrin, troponin T, troponin T hs, TSH, TSH-receptor-Ab. Siemens (Advia-platforms): anti-TPO, B12, CRP, ferritin, FSH, hCG (ThCG), LH, prolactin, PSA, PTH, TSH. Siemens (Immulite-platforms): ACTH, calcitonin, CDT (%), C-peptide, GH, hCG, IGF1, interleukin 1β, interleukin 6, interleukin 8, interleukin 10, NT-proBNP, sIL-2R, TgAB, Tg (thyroglobulin), TNF-α.

ple due to the expected formation of some d-dimer fragments during the coagulation phase prior to sample centrifugation.

Insulin on Immulite2000 (Siemens Healthcare Diagnostics, Erlangen, Germany, www.medical.siemens.com) showed falsely elevated results for both sera tested. Results after addition of heat-treated MAK33 are very similar to the results from another insulin-assay (Roche).

Interleukins 1β, 2, and 6 analyzed on Bio-Plex 200 (Bio-Rad Laboratories, Hercules, CA, USA, www.bio-rad.com) all gave falsely elevated results for both sera tested. TNF-α gave a falsely elevated result for the native serum from donor 1. For all four samples, duplicate results were reported for each analyte. All results are given in the electronic supplement, while only the first result for each analyte is given in the printed table. It should be noted that for serum 1 (both native and blocked), the Bio-Plex instrument gave a warning signal with the result, possibly due to bead aggregation.

Discussion

Characterization of the sera used in this study indicated that they contained heterophilic antibodies belonging to the IgM class with principle reactivity to the Fc domain of murine

IgG1. In our non-sense assays this reactivity was effectively blocked by the addition of heat-aggregated MAK33 immunoglobulin. These observations permitted the use of the sera, as paired native and pre-blocked samples, to test if commercial immunoassays are sufficiently protected against interference from Fc-reactive heterophilic antibodies.

The fact that such a large number of the tested assays are vulnerable to heterophilic antibodies with classic Fc-reactivity is a cause for concern. We have previously shown that this interference could probably have been avoided by removing the Fc fragment from the solid phase assay antibodies (2). It is therefore surprising that this approach is not used more frequently.

In the rare event that heterophilic antibodies bind the $F(ab')_2$-region of the assay antibodies, the inclusion of blocking immunoglobulin in the assay buffers provides a final, but important, line of defense. As demonstrated herein, and in a number of previous studies, aggregated antibodies are potent blockers of interference (1, 21, 22). This efficacy is most probably related to the stable binding of low-affinity interfering IgMs (23) to the reiterative epitopes displayed on aggregated immunoglobulin. MAK33 is a good choice for blocking reagent since IgG1 monoclonal antibodies are commonly chosen as capture antibodies to prevent consumption of the solid phase through complement activation (24).

As long as immunoassays are vulnerable, it is important that clinical laboratories implement strategies for identifying samples with a high probability of interference. To identify samples, Ismail et al. (25) suggest a probabilistic approach, i.e., elevated results in assays known to have a low rate of true positive results should be retested for interference. We agree with this probabilistic approach, but we also think that the impact of the assay result should guide which samples to retest for interference. A false-positive HIV-1, hCG or troponin I result probably has more impact than a falsely elevated interleukin 6, although interference may be equally probable in all these assays. An optimal strategy would be based on detailed knowledge of (and experience with) the assay, analyte, and interference tests in question (26, 27). We stress this because interpretation of interference tests is rarely as simple and straightforward as we would like. An extensive discussion on this subject has been published previously (28). A general rule is that a negative interference test does not exclude heterophilic antibody interference. A positive test, given appropriate controls and correct interpretation, can normally be trusted as a proof of interference.

In this study, we relied on the ability of the commercially available immunoglobulin MAK33 to block heterophilic antibodies when added to sera prior to assay. This approach was chosen because in a previous study, using a panel of 11,261 sera, we demonstrated that this reagent was able to reduce the level of interference to $<1\%$ (1). We believe that re-assay after blocking with aggregated MAK33, or other commercially available heterophilic blocking reagents (HBRs) (29) for that matter, may prove a good testing alternative when interference is suspected in the routine diagnostic laboratory. Not only is aggregated MAK33 commercially available in a form that has undergone stringent quality control, but also it is easy to use and interpretation is relatively simple. It should, however, only be used with assays containing murine antibodies and, since MAK33 is an antibody to CK-MB, its use is inappropriate with assays for this particular analyte.

Herein we show that a surprising number of immunoassay kits (21 out of 170 tested) are vulnerable to Fc-reactive heterophilic antibodies. Had more sera with heterophilic antibodies been included, or more assays been tested, it is likely that additional vulnerable assays would have been identified. The fact that 149 assays proved resistant to Fc-reactive heterophilic antibodies in our study should not lead to a false sense of security when using these particular assays. As with other interference tests, negative results do not exclude the possibility of interference.

Based on this study and our previous findings (1, 30), we argue that some immunoassay kits need to be better protected against Fc-reactive heterophilic antibodies. This could be accomplished using either $F(ab')_2$, Fab' or scFv assay antibodies and adequate concentrations of aggregated irrelevant IgG in the assay reagents. The added blocking immunoglobulin should be similar to the assay antibodies with respect to species and subclass.

A potential limitation of this study, given the marked heterogeneity of heterophilic antibodies, is the small number of sera used. However, in a prior investigation, using 198 interference-positive sera (selected from 11,261 tested specimens) we observed that 194 demonstrated Fc-reactivity (2). Thus, the two sera we used in this study contained heterophilic antibodies with the reactivity most commonly associated with antibody interference.

In conclusion, this article describes a simple way of screening immunoassays for interference using small panels of native and pre-blocked sera. Using this method we demonstrate that some commercial assays are poorly protected against heterophilic antibodies with Fc-reactivity.

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Erratum

Heterophilic antibody interference in commercial immunoassays; a screening study using paired native and pre-blocked sera

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Due to a technical error, Figures 1 and 2 have been published only with the titles but without the legends. The correct Figures in the article published in Clin Chem Lab Med 2011;49(12):2011–6 are complete as below.

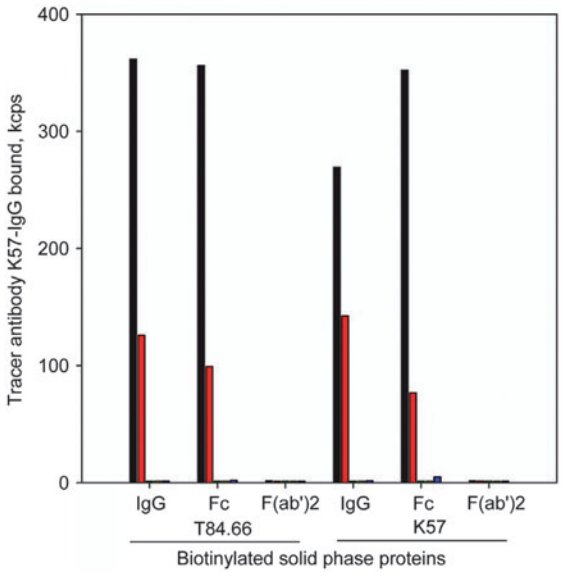

Figure 1 Reactivity of heterophilic antibodies to murine IgG1 fragments.
Data are from non-sense assays using monoclonal antibodies T84.66 or K57 as solid phases and a europium-labeled K57-IgG tracer. The solid phase reagents were either whole IgG1, F(ab')2 fragments or Fc made by bromelain cleavage. Serum 1 (black), serum 2 (red), serum 1 pre-blocked with MAK33 (green), serum 2 pre-blocked with MAK33 (yellow). Control serum (blue).

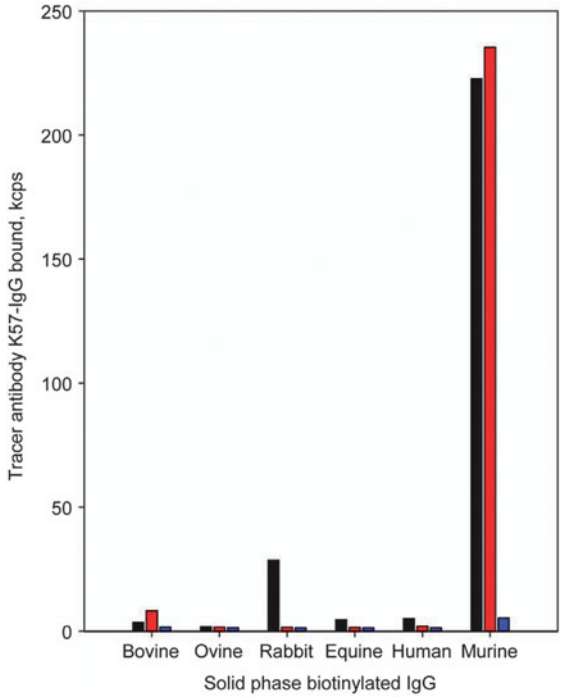

Figure 2 Reactivity of the heterophilic antibodies to IgG from different animal species.
Assays utilized polyclonal non-immune IgG from the different species as solid phases, and europium-labeled mouse monoclonal antibody K57 as tracer. Serum 1 (black), serum 2 (red). Control serum (blue).

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Supplemental data

Interference assays

All assays described below are 3-step interference assays (1, 2) using biotinylated solid phase antibodies and Europium-labeled tracer antibodies. Streptavidin-coated Delfia® microtitration strips, a Wallac Plate Shaker 1296–001, a Wallac Plate Washer 1296-026, and a Victor 1420 multilabel counter (all from PerkinElmer, Waltham, MA) were used for all interference assays.

Solid phase antibodies

The monoclonal antibody to carcinoembryonic antigen T84.66 was purified from hybridoma culture supernatant (ATCC; HB-8747). Antibody purification, fragmentation and biotinylation have previously been described in detail (2). Antibody K57 is a mouse monoclonal antibody of IgG1 subclass to α-fetoprotein (AFP) previously characterized and described (3). Polyclonal human, rabbit, bovine, and equine IgG were purified from sera and biotinylated as described above.

Tracer antibodies

All tracer antibodies were labeled with an Europium chelate (PerkinElmer) using a 12.5-fold molar excess giving about 4 Eu-molecules to each antibody (2).

Antibody K57 IgG was used as tracer antibody both in the screening assay and in the specificity assays described below.

Rabbit polyclonal antibodies to human IgM, IgG, kappa, and lambda (Dako, Glostrup, Denmark) were used as tracer antibodies in the isotype assay. Those not sold as $F(ab')_2$ fragments were prepared ''in-house'' by papain cleavage as described by Bjerner et al. 2005 (4), and used in concentrations of 1 μg/mL.

Buffers

The assay buffer used in all interference assays consisted of 0.05 mol/L Tris (Sigma), 0.15 mol/L NaCl (Merck), 0.2 mmol/L diethylene-triamine pentaacetic acid (Sigma), 0.05 g/L tartrazine (Aldrich), 1 g/L Germall II, 0.01% Tween20 (Serva), 5 g/L BSA (A4503, Sigma), and 0.5 g/L bovine IgG (G7516, Sigma) at a final pH of 7.8.

Plate washing buffer was made using the DELFIA® wash concentrate (PerkinElmer).

Gel filtration of sera

The two sera with heterophilic antibodies were gel-filtered using a Superdex 200 column (1×30 cm) on the Äkta Prime chromatographic system (both from GE Healthcare, Buckinghamshire, England). Fractions of 0.5 mL were analyzed for heterophilic antibodies (ability to cross-link murine IgG1) in the non-sense screening assay described below. For each serum, a fraction containing heterophilic antibodies was analyzed in the subclass assay.

General assay procedure

Biotinylated solid phase antibodies were added to streptavidin-coated microwells and incubated with shaking for 30 min, then washed x3. Serum samples (1:6 dilution in assay buffer, total volume 150 μL/well) were added in duplicates, incubated with shaking for 60 min and washed×6. Tracer antibodies (150 μL/well of 0.5 μg/mL in assay buffer) were added, incubated with shaking for 30 min and washed×6. Enhancement solution (200 μL/well, PerkinElmer) was added, incubated for 2 min with shaking prior to counting. A serum sample without heterophilic antibodies was always included as a negative control. A sample of affinity purified sheep antibody to mouse IgG (0.16 μg/mL in assay buffer) served as a positive control.

Heterophilic antibody screening and specificity assays

To detect heterophilic antibodies with affinity to murine IgG1, and determine the specificity of the antibodies, biotinylated IgG (1.6 μg/mL), $F(ab')_2$ (1.0 μg/mL) and Fc (1.0 μg/mL) of both K57 and T84.66 were used as solid phase antibodies. K57 IgG was used as tracer antibody. (In the relatively rare event that heterophilic antibodies do not display Fc-reactivity, but bind the $F(ab')_2$ fragment, we also test for reactivity to Fab', single-chain (scFv), humanized $F(ab')_2$, and humanized Fab' variants of T84.66.) This is a non-sense assay as suggested by Boscato and Stuart (1), since AFP has only one binding site for K57(3). Combining monoclonal antibodies for AFP and CEA also creates (for obvious reasons) a non-sense assay.

An additional assay was run to determine the species specificity of the heterophilic antibodies. Polyclonal human (gammanorm, Pharmacia/GE Healthcare), Bovine (Sigma), Equine (Sigma), Ovine and Rabbit (in-house) IgG were purified on protein A columns, biotinylated as described above, and used as solid phase antibodies. K57 IgG was used as tracer antibody.

Heterophilic antibody subclass assay

This assay was designed using biotinylated K57 IgG as the solid phase antibody, while tracer antibodies were $F(ab')_2$ variants of rabbit polyclonal antibodies to human IgM, IgG, kappa, and lambda chain described above. Heterophilic antibodies with affinity to murine IgG will bind to the solid phase antibody, while the tracer antibodies will bind their epitopes on the heterophilic antibodies.

Table 2 Results for all tested assays.

Analyte, unit	Manufacturer	Instrument	Lot number	Serum 1 blocked	Serum 1	Serum 2 blocked	Serum 2	Hospital/Laboratory
ACTH, pmol/L	Siemens	Immulite 2000		1.43	<1.11	1.55	1.9	Rikshospitalet, Norway (SAFE)
AFP, μg/L	Abbott	Architect i2000	82598LF00	2.88	3.11	1.1	1.19	Aleris Medilab, Täby, Sweden
AFP, kU/L	Brahms	Kryptor		4.606	4.412	1.531	1.686	Radiumhospitalet, Norway
AFP, μg/L	Diasorin	Liaison		2.6	**4.2**	0.6	**2.8**	Karolinska, Solna, Sweden
AFP, kU/L	Roche	Modular E170	156,607	1.7	2.0	0.5	0.5	St. Olavs Hospital, Norway
α-1-antitrypsin, g/L	Roche	Modular P	618096	1.15	1.26	1.35	1.45	Rikshospitalet, Norway (clin.chem.)
Anti EBNA IgG, % CO	Biotest	BEP 2000	3929081	21.5	23.6	1027.2	1093.20	Rikshospitalet, Norway (microbiology)
Anti HAV-IgG, S/CO	Abbott	Architect i2000	83203HN00	4.54	4.07	0.18	0.17	Rikshospitalet, Norway (microbiology)
Anti HAV-IgM, S/CO	Abbott	Architect i2000	82015HN00	0.34	0.35	0.30	0.30	Rikshospitalet, Norway (microbiology)
Anti Hbs, U/L	Abbott	Architect i2000	83149LF00	0.71	0.43	0.13	0.78	Rikshospitalet, Norway (microbiology)
Anti-varicella IgG, OD	Dade Behring	BEP 2000	38818	1.068	1.004	0.883	0.949	Rikshospitalet, Norway (microbiology)
Anti-CCP, kU/L	Abbott	Architect i2000 SR		22.7	20.8	0.7	0.5	Ålesund, Norway
Anti-HBcII, S/CO	Abbott	Architect i2000	82304HN00	0.07	0.07	0.08	0.06	Rikshospitalet, Norway (microbiology)
Anti-HCV, S/CO	Abbott	Architect i2000	82595HN00	0.17	0.15	0.04	0.05	Rikshospitalet, Norway (microbiology)
Anti-TPO, kU/L	Abbott	Architect ci16200	34747UN09	0.61	0.26	0.18	0.22	Bærum, Norway
Anti-TPO, kU/L	Abbott	Architect i2000 SR		1.0	0.2	0.3	0.2	Ålesund, Norway
Anti-TPO, kU/L	Beckman Coulter	UniCel DxI 800	912975	0.6	0.6	1.1	0.9	Akershus, Norway
Anti-TPO, kU/L	Siemens	Advia Centaur XP	46446178	19	14	30	33	Fürst, Norway
ApoA1, g/L	Roche	Modular P	618835	1.37	1.5	1.08	1.2	Rikshospitalet, Norway (clin.chem.)
ApoB, g/L	Roche	Modular P	618841	0.8	0.86	0.67	0.74	Rikshospitalet, Norway (clin.chem.)
B12, pmol/L	Abbott	Architect ci16200	83900JN00	328	303	169	131	Bærum, Norway
B12, pmol/L	Abbott	Architect ci8200	83900JN00	342	361	139	161	Haugesund, Norway
B12, pmol/L	Ortho	Vitros ECI	1420	301	279	186	Insuff. mat.	Akranes, Iceland
B12, pmol/L	Siemens	Advia Centaur XP	44698201	256	262	155	134	Fürst, Norway
BNP, pmol/L	Abbott	Architect ci16200	79346M100	<2.9	3.4	<2.9	<2.9	Bærum, Norway
BNP, pmol/L	Abbott	Architect i2000 SR		<2.9	**6.0**	<2.9	<2.9	Ålesund, Norway
BNP, ng/L	Beckman Coulter	UniCel DxI 800	912630	2	2	3	3	Akershus, Norway
BNP, ng/L	Ortho	Vitros ECI	550	46.3	46	45.1	44.8	Akranes, Iceland
Borrelia IgM, OD	Dade Behring	BEP 2000	39345	0.073	0.072	0.179	0.170	Rikshospitalet, Norway (microbiology)

(Table 2 continued)

Analyte, unit	Manufacturer	Instrument	Lot number	Serum 1 blocked	Serum 1	Serum 2 blocked	Serum 2	Hospital/Laboratory
Borrelia IgG, OD	Dade Behring	BEP 2000	39105	0.017	0.016	0.023	0.028	Rikshospitalet, Norway (microbiology)
CA125, kU/L	Abbott	Architect ci8200	74255M100	7	**278**	6	**84**	Haugesund, Norway
CA125, kU/L	Abbott	Architect i2000	75248M100	8.4	**256.0**	6.4	**96.4**	Ullevål, Norway
CA125, kU/L	Abbott	Architect i2000	80155M100	7.4	**20.1**	6.7	**20.1**	Aleris Medilab, Täby, Sweden
CA125, kU/L	Beckman Coulter	UniCel DxI 800	910079	4.9	4.4	5.1	5.0	Akershus, Norway
CA125, kU/L	Diasorin	Liaison		5.5	7.0	4.2	**74**	Karolinska, Solna, Sweden
CA125, kU/L	In house	AutoDELFIA		6.7	8.1	7.8	9.3	Radiumhospitalet, Norway
CA125, kU/L	Roche	Modular E	155734	8.06	7.97	5.68	5.68	Bærum, Norway
CA15-3, kU/L	Abbott	Architect i2000	79335MI00	9.9	11.6	10.1	11.2	Aleris Medilab, Täby, Sweden
CA15-3, kU/L	Diasorin	Liaison		12	13	13	13	Karolinska, Solna, Sweden
CA15-3, kU/L	Roche	Modular E170	156.423	12.0	13.3	12.6	13.2	St. Olavs Hospital, Norway
CA19-9, kU/L	Abbott	Architect i2000	80161MI00	10.08	10.56	6.03	6.85	Aleris Medilab, Täby, Sweden
CA19-9, kU/L	Brahms	Kryptor		24.85	24.05	13.55	11.02	Radiumhospitalet, Norway
CA19-9, kU/L	Diasorin	Liaison		17	18	7.3	**89**	Karolinska, Solna, Sweden
CA19-9, kU/L	Roche	Modular E170	156,960	19.8	21.4	7.5	8.3	St. Olavs Hospital, Norway
CA72-4, kU/L	Roche	Modular E170	154,236	0.7	0.7	1.0	1.0	St. Olavs Hospital, Norway
Calcitonin, pmol/L	Siemens	Immulite 2000		<0.59	<0.59	<0.59	<0.59	Rikshospitalet, Norway (SAFE)
CDT%, %	Siemens	BN ProSpec	110217	1.51	1.45	1.97	1.82	St. Olavs Hospital, Norway
CEA, μg/L	Abbott	Architect ci8200	80594LF00	<0.5	<0.5	1.4	1.5	Haugesund, Norway
CEA, μg/L	Abbott	Architect i2000	79351LF00	<0.50	<0.50	0.74	0.99	Ullevål, Norway
CEA, μg/L	Beckman Coulter	UniCel DxI 800	924063	0.51	0.49	1.34	1.30	Akershus, Norway
CEA, μg/L	Diasorin	Liaison		<1	**1.9**	<1	**3.6**	Karolinska, Solna, Sweden
CEA, μg/L	In house	AutoDELFIA		0.9	1.3	1.6	1.7	Radiumhospitalet, Norway
CEA, μg/L	Roche	Modular E	155880	0.855	0.873	1.89	1.89	Bærum, Norway
CMV IgG, kU/L (AU/mL)	Abbott	Architect i2000	82503LF00	>250.0	>250.0	166.70	176.80	Rikshospitalet, Norway (microbiology)
CMV IgM, TV	BioMerieux	MiniVIDAS	100617-0	0.16	0.17	0.27	0.31	Rikshospitalet, Norway (microbiology)
Cortisol, nmol/L	Roche	Modular E		223.1	275.0	175.2	202.6	Karolinska, Huddinge, Sweden
C-peptide, nmol/L	Roche	Modular E		1.97	2.17	0.903	0.659	Karolinska, Huddinge, Sweden
C-peptide, nmol/L	Siemens	Immulite 2000		2.51	2.47	0.609	0.662	Rikshospitalet, Norway (SAFE)
Crosslaps, ng/L	Roche	Modular E		75.57	**1838**	11.45	32.81	Karolinska, Huddinge, Sweden
CRP, mg/L	Abbott	Architect ci16200	90341	0.5	0.5	0.4	0.4	Bærum, Norway
CRP, mg/L	Abbott	Architect ci8200	90380D10	0.3	0.2	0.2	0.2	Haugesund, Norway
CRP, mg/L	Ortho	Vitros 5,1	90253	0.2	0.2	0.3	0.1	Akershus, Norway
CRP, mg/L	Roche	Cobas integra 800	618652	1.06	**3.35**	0.35	0.66	Ullevål, Norway
CRP, mg/L	Roche	Modular P	613638	<0.6	**4.31**	<0.6	<0.6	Rikshospitalet, Norway (clin.chem.)
CRP, mg/L	Siemens	Advia 2400 Chemistry System	163531	0	0	0	0	Fürst, Norway
Cyfra 21-1, μg/L	Roche	Modular E170	156,703	0.8	0.8	0.2	0.4	St. Olavs Hospital, Norway
D-dimer, mg/L	Diag. Stago	STA Compact		2.48	**>20**	0.54	**2.37**	Radiumhospitalet, Norway

(Table 2 continued)

Analyte, unit	Manufacturer	Instrument	Lot number	Serum 1 blocked	Serum 1	Serum 2 blocked	Serum 2	Hospital/Laboratory
D-dimer, mg/L	Diag. Stago	STA Compact	104375	2.1	**>20**	0.6	**2.2**	Haugesund, Norway
D-dimer, mg/L	Diag. Stago	STA-R Evolution	104375	1.84	**>4.0**	0.59	**>4.0**	Ullevål, Norway
D-dimer, mg/L	Instrumentation Lab (IL)	ACL TOP	B91087	0.57	0.59	0.84	0.88	Akershus, Norway
D-dimer, mg/L	Instrumentation Lab (IL)	ACL TOP	B91087	0.332	0.324	0.491	0.495	Bærum, Norway
D-dimer, mg/L	Roche	Modular P	619,442	0.4	0.5	0.1	0.1	St. Olavs Hospital, Norway
DHEAS, μmol/L	Roche	Modular E		0.809	0.878	3.47	3.79	Karolinska, Huddinge, Sweden
EBV VCA IgG, % CO	Novitec	Manual	91646	997	989	1090	1056	Rikshospitalet, Norway (microbiology)
EBV VCA IgM, % CO	Novitec	Manual	90852	13	17	54	58	Rikshospitalet, Norway (microbiology)
Estradiol, pmol/L	Beckman Coulter	UniCel DxI 800		<150	<150	<150	487	Karolinska, Solna, Sweden
Estradiol, nmol/L	Roche	Modular E	157272	0.059	0.064	0.511	0.552	Rikshospitalet, Norway (clin.chem.)
Ferritin, μg/L	Abbott	Architect ci16200	81911JN00	58.38	60.55	19.63	18.17	Bærum, Norway
Ferritin, μg/L	Abbott	Architect ci8200	78902JN00	62	65	21	21	Haugesund, Norway
Ferritin, μg/L	Abbott	Architect i2000 SR		57.5	58.4	19.3	19.3	Ålesund, Norway
Ferritin, μg/L	Beckman Coulter	UniCel DxI 800	970061	47.4	45.8	15.5	14.4	Akershus, Norway
Ferritin, μg/L	Ortho	Vitros ECI	1160	49.5	48.1	16.6	Insuff. mat.	Akranes, Iceland
Ferritin, μg/L	Roche	Cobas e411		80.8	80.1	23.7	22.6	Volda, Norway
Ferritin, μg/L	Roche	Modular E	156085	76.21	75.05	22.47	22.43	Rikshospitalet, Norway (clin.chem.)
Ferritin, μg/L	Siemens	Advia 2400 Chemistry System	175917	95	71	35	17	Fürst, Norway
FSH, U/L	Abbott	Architect ci8200	78909JN00	5	5	4	4	Haugesund, Norway
FSH, U/L	Beckman Coulter	UniCel DxI 800	870068	7.1	6.7	Insuff. mat.	4.78	Akershus, Norway
FSH, U/L	PerkinElmer	AutoDELFIA		4.2	4.6	3.2	3.0	Karolinska, Solna, Sweden
FSH, U/L	Roche	Modular E	154152	5.42	5.31	4.12	4.22	Rikshospitalet, Norway (clin.chem.)
FSH, U/L	Roche	Modular E	156812	4.67	4.6	3.52	3.61	Bærum, Norway
FSH, U/L	Siemens	Advia Centaur XP	44641149	4.5	4.9	3.5	3.7	Fürst, Norway
Ft3, pmol/L	Abbott	Architect i2000	82911JN00	3.74	3.588	3.68	4.46	Aleris Medilab, Täby, Sweden
Ft3, pmol/L	Roche	Modular E	154608	5.35	5.47	4.23	4.13	Rikshospitalet, Norway (clin.chem.)
Ft4, pmol/L	Abbott	Architect i2000	83919JN00	13.53	14.8	13.93	14.25	Aleris Medilab, Täby, Sweden
Ft4, pmol/L	Roche	Modular E	156980	15.7	16.38	15.24	15.3	Rikshospitalet, Norway (clin.chem.)
GH, μg/L	PerkinElmer	AutoDELFIA		0.07	**1.5**	0.1	**0.5**	Karolinska, Solna, Sweden
GH, mU/L	Siemens	Immulite 2000		0.246	0.261	0.489	0.558	Rikshospitalet, Norway (SAFE)

(Table 2 continued)

Analyte, unit	Manufacturer	Instrument	Lot number	Serum 1 blocked	Serum 1	Serum 2 blocked	Serum 2	Hospital/Laboratory
Haptoglobin, g/L	Roche	Modular P	617115	1.93	2.08	1.2	1.28	Rikshospitalet, Norway (clin.chem.)
HBsAg, S/CO	Abbott	Architect i2000	83603LF00	0.33	0.31	0.42	0.34	Akershus, Norway
HBsAg, S/CO	Abbott	Architect i2000 SR	79513LF00	0.55	0.6	0.57	0.67	Fürst, Norway
HBsAg, S/CO	Abbott	Architect i2000	83244LF00	0.53	0.30	0.31	0.21	Rikshospitalet, Norway (microbiology)
hCG (ThCG), U/L	Siemens	Advia Centaur XP	48650227	0	0	0	1	Fürst, Norway
hCG (free β-hCG), U/L	PerkinElmer	AutoDELFIA		<2	<2	<2	<2	Karolinska, Solna, Sweden
hCG (hCG+β), U/L	Roche	Cobas 6000		<1	<1	<1	<1	Radiumhospitalet, Norway
hCG (hCG+β), U/L	Roche	Cobas e601	155484	<0.100	<0.100	<0.100	<0.100	Ullevål, Norway
hCG (hCG+β), U/L	Roche	Cobas e411		<0.1	<0.1	<0.1	<0.1	Volda, Norway
hCG (hCG+β), U/L	Roche	Cobas e411	155484	<0.1	<0.1	<0.1	<0.1	Akershus, Norway
hCG (hCG+β), U/L	Roche	Modular E	156537	<1	<1	<1	<1	Rikshospitalet, Norway (clin.chem.)
hCG (hCG), U/L	Siemens	Immulite 2000		<1	<1	<1	<1	Rikshospitalet, Norway (SAFE)
hCG (total β-hCG), U/L	Abbott	Architect ci8200	81912JN00	<2	**36**	<2	**117**	Haugesund, Norway
hCG (total β-hCG), U/L	Abbott	Architect i2000 SR	81912JN01	<1.2	**59.2**	<1.2	**147.5**	Ålesund, Norway
hCG (total β-hCG), U/L	Abbott	Architect ci16200	81919JN00	<1.0	**42.07**	<1.0	**113.47**	Bærum, Norway
hCG (total β-hCG), U/L	Beckman Coulter	UniCel DxI 800		<2	<2	<2	<2	Karolinska, Solna, Sweden
hCG (total β-hCG), U/L	Ortho	Vitros ECI	390	0	0.01	0	Insuff. mat.	Akranes, Iceland
Herpes IgG, OD	Dade Behring	BEP 2000	38853	0.898	0.864	0.780	0.796	Rikshospitalet, Norway (microbiology)
HIV Ag/Ab combo, S/CO	Abbott	Architect i2000	81890HN00	0.10	0.09	0.10	0.12	Rikshospitalet, Norway (microbiology)
HTLV-I/II, S/CO	Abbott	Architect i2000	79479HN00	0.18	0.20	0.21	0.20	Rikshospitalet, Norway (microbiology)
IgA, g/L	Roche	Modular P	622216	1.56	1.72	1.81	1.98	Rikshospitalet, Norway (clin.chem.)
IGF1, nmol/L	Siemens	Immulite 2000		34.8	31.1	19.8	20	Rikshospitalet, Norway (SAFE)
IgG, g/L	Roche	Modular P	618445	10.42	11.39	6.87	7.47	Rikshospitalet, Norway (clin.chem.)
IgM, g/L	Roche	Modular P	622657	0.57	0.64	1.28	1.39	Rikshospitalet, Norway (clin.chem.)
Insulin, pmol/L	Roche	Modular E		300.9	335.8	19.85	21.37	Karolinska, Huddinge, Sweden
Insulin, pmol/L	Siemens	Immulite 2000		351	**1191**	22	**131**	Rikshospitalet, Norway (SAFE)
Interleukin 10, ng/L	Siemens	Immulite 1000	LKXP1 317	<5	<5	<5	<5	Lund, Sweden
Interleukin 1beta, ng/L	Bio-Rad	Bio-Plex 200	1660907	0.00	**11.39**	0.06	**2.00**	Institutet för Hälsa och Välfärd, Åbo, Finland
Interleukin 1beta, ng/L	Bio-Rad	Bio-Plex 200	1660907	<0.13	**133.6**	<0.13	**2.79**	Institutet för Hälsa och Välfärd, Åbo, Finland

(Table 2 continued)

Analyte, unit	Manufacturer	Instrument	Lot number	Serum 1 blocked	Serum 1	Serum 2 blocked	Serum 2	Hospital/Laboratory
Interleukin 1beta, ng/L	Siemens	Immulite 1000	LKL11 150	<5	<5	<5	<5	Lund, Sweden
Interleukin 2, ng/L	Bio-Rad	Bio-Plex 200	1660907	OOR	**<55.4**	OOR	**<3.53**	Institutet för Hälsa och Välfärd, Åbo, Finland
Interleukin 2, ng/L	Bio-Rad	Bio-Plex 200	1660907	<0.13	**685.01**	<0.13	**4.79**	Institutet för Hälsa och Välfärd, Åbo, Finland
Interleukin 6, ng/L	Bio-Rad	Bio-Plex 200	1660907	15.21	**47.56**	2.71	**8.42**	Institutet för Hälsa och Välfärd, Åbo, Finland
Interleukin 6, ng/L	Bio-Rad	Bio-Plex 200	1660907	18.84	**232.2**	1.6	**10.96**	Institutet för Hälsa och Välfärd, Åbo, Finland
Interleukin 6, ng/L	Siemens	Immulite 1000	LK6P1 217	8	8	<2.8	4	Lund, Sweden
Interleukin 8, ng/L	Siemens	Immulite 1000	LK8P1 165	<5	<5	<5	<5	Lund, Sweden
LH, U/L	Abbott	Architect ci8200	73415M200	4	5	9	10	Haugesund, Norway
LH, U/L	Beckman Coulter	UniCel DxI 800	870086	5.36	4.52	Insuff. mat.	13.35	Akershus, Norway
LH, U/L	Perkin Elmer	AutoDELFIA		5.4	5.5	13	15	Karolinska, Solna, Sweden
LH, U/L	Roche	Modular E	152109	6.52	6.39	13.95	14.17	Rikshospitalet, Norway (clin.chem.)
LH, U/L	Roche	Modular E	154339	6.06	6.36	13.65	13.36	Bærum, Norway
LH, U/L	Siemens	Advia Centaur XP	48826172	5.2	6.3	14.7	13.8	Fürst, Norway
MBL, μg/L	In House	ELISA		81	93	370	600	Lund, Sweden
MUC1 (CA15-3), kU/L	In house	AutoDELFIA		10.9	11.4	11.7	10.2	Radiumhospitalet, Norway
Mycoplasma IgM, % CO	Biorad	Manual (Platelia®)	9E 2027	55	47	85	78	Rikshospitalet, Norway (microbiology)
Myoglobin, μg/L	Roche	Modular E	154602	32.23	34.35	<21	<21	Rikshospitalet, Norway (clin.chem.)
Myoglobin, μg/L	Roche	Modular E		31.46	34.38	21.00	21.00	Karolinska, Huddinge, Sweden
NSE, μg/L	Diasorin	Liaison		4.6	5.2	8.3	8.6	Karolinska, Solna, Sweden
NSE, μg/L	In house	AutoDELFIA		4.2	4.5	9.8	9.3	Radiumhospitalet, Norway
NSE, μg/L	Roche	Modular E170	154,748	4.6	4.2	10.0	10.8	St. Olavs Hospital, Norway
Nt-proBNP, pmol/L	Roche	Cobas e601	155234	4.03	4.04	3.92	4.08	Ullevål, Norway
Nt-proBNP, pmol/L	Roche	Modular E	OD155234	3.83	4.23	3.97	4.23	Fürst, Norway
Nt-proBNP, ng/L	Roche	Cobas e411		34.1	37.9	36.9	34.8	Volda, Norway
Nt-proBNP, pmol/L	Roche	Modular E	155234	4.73	4.85	4.89	5.00	Rikshospitalet, Norway (clin.chem.)
Nt-proBNP, ng/L	Siemens	Immulite 2000	203	41.6	44.8	89	107	Aleris Medilab, Täby, Sweden
PAPP-A, U/L	PerkinElmer	AutoDELFIA		0.003	**0.156**	0.002	**0.21**	Karolinska, Solna, Sweden
Parvo B19 IgG, % CO	Biotrin	Manual	75KT037	18	14	11	9	Rikshospitalet, Norway (microbiology)
Parvo B19 IgM, % CO	Biotrin	Manual	76KT029J	16	16	28	29	Rikshospitalet, Norway (microbiology)
Procalcitonin, μg/L	Brahms	Modular E	154910	<0.1	<0.1	<0.1	<0.1	Rikshospitalet, Norway (clin.chem.)
Procalcitonin, μg/L	Brahms	Cobas e411		<0.1	<0.1	Insuff. mat.	Insuff. mat.	Volda, Norway
Progesterone, nmol/L	Roche	Modular E170	152,855	0.5	0.5	26.4	29.2	St. Olavs Hospital, Norway

(Table 2 continued)

Analyte, unit	Manufacturer	Instrument	Lot number	Serum 1 blocked	Serum 1	Serum 2 blocked	Serum 2	Hospital/Laboratory
ProGRP, ng/L	In house	AutoDELFIA		46	40	44	42	Radiumhospitalet, Norway
Prolactin, mU/L	Abbott	Architect ci8200	78905JN00	186	192	135	134	Haugesund, Norway
Prolactin, mU/L	Beckman Coulter	UniCel DxI 800	970047	134.3	135.1	93.5	80.9	Akershus, Norway
Prolactin, mU/L	Roche	Modular E	152936	186.4	189.8	116.9	116.50	Rikshospitalet, Norway (clin.chem.)
Prolactin, mU/L	Roche	Modular E	156154	184.2	185.7	113.8	114.4	Bærum, Norway
Prolactin, mU/L	Siemens	Advia Centaur XP	45664125	144	128	91	92	Fürst, Norway
PSA, µg/L	Abbott	Architect ci8200	82445LF00	1	1	<0.1	<0.1	Haugesund, Norway
PSA, µg/L	Abbott	Architect i2000	80200FL00	1.17	1.29	0.00	0.03	Ullevål, Norway
PSA, µg/L	Abbott	Architect i2000 SR		1.2	1.4	<0.01	0.01	Ålesund, Norway
PSA, µg/L	Beckman Coulter	UniCel DxI 800	970068	1.170	1.196	0.002	0.005	Akershus, Norway
PSA, µg/L	Ortho	Vitros ECI	2571	1.29	1.3	0	Insuff. mat.	Akranes, Iceland
PSA, µg/L	PerkinElmer	AutoDELFIA		1.33	1.36	0	0	Radiumhospitalet, Norway
PSA, µg/L	Roche	Cobas e411		1.3	1.3	<0.1	<0.1	Volda, Norway
PSA, µg/L	Roche	Modular E	156171	1.32	1.32	0.0003	0.0003	Bærum, Norway
PSA, µg/L	Siemens	Advia Centaur XP	49528216	1.1	1	0.0	0.0	Fürst, Norway
PSA, free, µg/L	Abbott	Architect i2000	85435LF00	0.162	0.183	<0.1	<0.1	Aleris Medilab, Täby, Sweden
PSA, free, µg/L	PerkinElmer	AutoDELFIA		0.23	0.24	0	0	Radiumhospitalet, Norway
PTH, pmol/L	Abbott	Architect ci16200	1409600	3.76	3.71	2.78	2.71	Bærum, Norway
PTH, pmol/L	Beckman Coulter	UniCel DxI 800	909836	2.16	2.15	1.40	1.38	Akershus, Norway
PTH, pmol/L	Roche	Modular E	156701	3.54	3.65	2.96	3.02	Rikshospitalet, Norway (clin.chem.)
PTH, pmol/L	Siemens	Advia Centaur XP	42407152	2.5	2.5	1.8	1.9	Fürst, Norway
Rubella IgG, kU/L	Abbott	Architect i2000	33963UN09	198.5	197.9	36.40	36.30	Rikshospitalet, Norway (microbiology)
S-100, µg/L	Roche	Modular E		0.021	0.022	0.041	0.045	Karolinska, Huddinge, Sweden
SHBG, nmol/L	Abbott	Architect i2000	42091000	32.8	**52.6**	97.3	98	Aleris Medilab, Täby, Sweden
SHBG, nmol/L	Beckman Coulter	UniCel DxI 800		29.00	**99.48**	73.74	84.99	Karolinska, Huddinge, Sweden
SHBG, nmol/L	Roche	Modular E170	157,271	29.0	32.0	77.0	84.0	St. Olavs Hospital, Norway
sIL-2R, kU/L	Siemens	Immulite 1000	LKIP1229	441	475	490	464	Lund, Sweden
sTfR, Tina-quant®, mg/L	Roche	Cobas Integra 8000	618356	4.43	**19.22**	1.90	3.06	Akershus, Norway
sTfR, Tina-quant®, mg/L	Roche	Modular P	622490	3.35	**26.52**	1.74	1.80	Rikshospitalet, Norway (clin.chem.)
sTfR, Tina-quant®, mg/L	Roche	Hitachi 917	614822	2.71	**25.67**	1.73	1.84	Ullevål, Norway
Syphilis ab, OD	Dade Behring	BEP 2000	39285	1.747	1.713	1.783	1.987	Rikshospitalet, Norway (microbiology)
Testosterone, nmol/L	Beckman Coulter	UniCel DxI 800		6.2	8.8	3.2	2.3	Karolinska, Solna, Sweden
Testosterone, nmol/L	Roche	Modular E	156970	10.07	10.34	0.802	0.793	Rikshospitalet, Norway (clin.chem.)
TgAb, kU/L	Brahms	Kryptor		24.7	24.1	31.2	30.3	Radiumhospitalet, Norway
TgAb, kU/L	Siemens	Immulite 1000	LKTG 229	<20	<20	<20	<20	Lund, Sweden
TgAb, kU/L	Siemens	Immulite 2000		<20	<20	<20	<20	Rikshospitalet, Norway (SAFE)

(Table 2 continued)

Analyte, unit	Manufacturer	Instrument	Lot number	Serum 1 blocked	Serum 1	Serum 2 blocked	Serum 2	Hospital/Laboratory
Thyroglobulin (Tg), μg/L	Inhouse	AutoDELFIA		0.6	0.75	88.2	87.7	Radiumhospitalet, Norway
Thyroglobulin (Tg), μg/L	Siemens	Immulite 2000		0.965	0.896	142	127	Rikshospitalet, Norway (SAFE)
TNF-α, ng/L	Siemens	Immulite 1000′	LKNF1 177	7	10	13	16	Lund, Sweden
TNF-α, ng/L	Bio-Rad	Bio-Plex 200	1660907	0.98	**13.97**	4.45	4.52	Institutet för Hälsa och Välfärd, Åbo, Finland
TNF-α, ng/L	Bio-Rad	Bio-Plex 200	1660907	1.2	**95.48**	4.78	5.3	Institutet för Hälsa och Välfärd, Åbo, Finland
Toxo IgG, kU/L	Abbott	Architect i2000	83289HN00	0.10	0.10	0.00	0.00	Rikshospitalet, Norway (microbiology)
Toxo IgG, kU/L	Biorad	Manual (Platelia®)	9K0115	0	0	0	0	Rikshospitalet, Norway (microbiology)
Toxo IgM, Indeks	Abbott	Architect i2000	83075HN00	0.064	0.057	0.030	0.037	Rikshospitalet, Norway (microbiology)
Toxo IgM, % CO	Biorad	Manual (Platelia®)	9B0014	9	12	9	8	Rikshospitalet, Norway (microbiology)
Transferrin, g/L	Abbott	Architect ci16200	78052HW00	2.08	2.11	2.61	2.62	Bærum, Norway
Transferrin, g/L	Roche	Modular P	622171	2.06	2.23	2.49	2.67	Rikshospitalet, Norway (clin.chem.)
Troponin I, ng/L	Abbott	Architect ci16200	34609UN09	0	0	0	0	Bærum, Norway
Troponin I, ng/L	Abbott	Architect ci8200	34573UN09	<30	<30	<30	<30	Haugesund, Norway
Troponin I, ng/L	Abbott	Architect i2000 SR		<10	<10	<10	<10	Ålesund, Norway
Troponin I, μg/L	Beckman Coulter	UniCel DxI 800		<0.03	<0.03	<0.03	<0.03	Karolinska, Solna, Sweden
Troponin I, μg/L	Ortho	Vitros ECI	500	0.003	0.005	0.001	0.001	Akranes, Iceland
Troponin T, ng/L	Roche	Cobas e411		4.5	4.5	3.0	3.0	Volda, Norway
Troponin T hs, ng/L	Roche	Cobas e601	153401	6.95	7.17	3.00	3.00	Ullevål, Norway
Troponin T hs, ng/L	Roche	Cobas e411	153404	11.77	11.57	4.33	<3.00	Akershus, Norway
Troponin T hs, ng/L	Roche	Modular E	153401	8.52	8.5	<3	<3	Rikshospitalet, Norway (clin.chem.)
TSH, mU/L	Abbott	Architect ci8200	79907JN00	1.5	1.6	1.6	1.7	Haugesund, Norway
TSH, mU/L	Abbott	Architect i2000	84957JN00	1.528	1.5257	1.4785	1.7032	Aleris Medilab, Täby, Sweden
TSH, mU/L	Abbott	Architect i2000 SR		1.52	1.58	1.63	1.54	Ålesund, Norway
TSH, mU/L	Beckman Coulter	UniCel DxI 800		1.9	1.8	1.9	1.7	Karolinska, Solna, Sweden
TSH, mU/L	Beckman Coulter	UniCel DxI 800	970072	1.628	1.623	2.010	1.618	Akershus, Norway
TSH, mU/L	Ortho	Vitros ECI	2720	1.7	1.76	1.51	Insuff. mat.	Akranes, Iceland
TSH, mU/L	PerkinElmer	AutoDELFIA		1.5	**2.3**	1.6	**2.1**	Karolinska, Solna, Sweden
TSH, mU/L	Roche	Cobas e411		1.81	1.80	1.66	1.60	Volda, Norway
TSH, mU/L	Roche	Modular E	156978	1.91	1.89	1.77	1.76	Rikshospitalet, Norway (clin.chem.)
TSH, mU/L	Roche	Modular E	157118	1.79	1.78	1.62	1.71	Bærum, Norway
TSH, mU/L	Siemens	Advia Centaur XP	43391219	1.61	1.54	1.71	1.69	Fürst, Norway
TSH-receptor-Ab, U/L	Roche	Modular E		0.300	0.300	0.300	0.300	Karolinska, Huddinge, Sweden

Complete results from screening of commercial immunoassays

The results for all tested assays are given in Table 2

We have not tried to evaluate the results for competitive assays, as we have little experience with detecting and neutralizing heterophilic antibody interference in these assays. The results for competitive assays (and for in-house assays included) are still presented in the complete result file.

The results for the anti-TPO assay performed on the Advia Centaur platform (Siemens) are very high (20-fold) compared to results from Abbott and Beckman assays. We do not know if this is due to interference or differences between assays.

The results for CA125 reported from one of the participating laboratories warranted confirmation, as they were different from results from other laboratories. In both native samples, the result was 20.1 kU/L, much lower than the results from other laboratories using the same method. Upon request, the print-outs from the instrument were scrutinized, and the reported results were confirmed. We have no explanation for this. It could be caused by differences between lots, but this remains purely speculative.

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Paper III

Original Article

Belief is only half the truth - or why screening for heterophilic antibody interference in certain assays makes double sense

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Abstract

Background: Interference in immunoassays may cause both false-negative and false-positive results. It may be detected using a number of affirmative tests such as reanalysis of certain samples using different assay platforms with known bias, after the addition of blocker antibodies, or assessment of linearity and parallelism following serial doubling dilutions. One should look for interference where it is likely and has high medical impact. Probabilistic Bayesian reasoning is a statistical tool to identify samples where interference is most likely. But when looking for interference where it is likely, do we find it where it has the largest population health consequences?

Methods: We used information theory to quantify the effect of assay interference by calculating the Shannon information content (using logarithms with base 2). We then obtained lower bounds of the population health consequences of a particular test and combined these expressions to get lower bounds of the population health consequences of interference.

Results and conclusion: We suggest that assays having a low frequency of true positives should be the primary target of retesting because: (i) assays with a low frequency of true positives exhibit a high likelihood of interference and (ii) the population health consequences of false-positive results are generally higher for assays with a low frequency of true positives. Finally, we give a worked example having a realistic frequency of interference and test costs. In some immunoassays (e.g., tumour markers), adding a blocker to all tests can be a more cost-efficient mean than retesting positive samples.

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Introduction

In a recent article, Ismail *et al.*[1] used Bayes' theorem to show that the relative number of false positives tends to be higher when using immunoassays with a low rate of true positives to diagnose disorders with low prevalence. The expression obtained by probabilistic Bayesian reasoning for the probability of identifying a false-positive test result by retesting the sample with a different assay is $f/(t+f)$ and thus dependent both on the frequency of false positives (f) due to interference and on the frequency of true positives (t). In non-Bayesian terms, we would refer to this expression as the positive predictive value of interference detection by retesting. Such retesting will, in practice, identify most of the interferences, but not all. For simplicity, our calculations will be based on retesting for interferences as previously described.[1]

Probabilistic Bayesian reasoning provides a statistical basis for identifying the samples where interference is most likely *a posteriori* (post-test, i.e. it may be applied only when a test result, e.g. 'positive'/'negative' is available). However, probabilistic Bayesian reasoning is not an *a priori* measure (pre-test, i.e. a measure that may be applied before a test result is available), so it is not an informative measure when reducing interference in an assay, where an *a priori* measure is needed. Furthermore, it does not estimate the population health consequences of interference. Depending on the assay, the population health benefits of test results vary greatly. The detection of human immunodeficiency virus (HIV) antibodies by an immunoassay has much greater impact on population health (since a positive HIV test may both reduce further spreading of the disease and offer the affected individual an opportunity to receive effective treatment) than an immunoassay test result suggesting slightly elevated testosterone concentrations in a male adult.

This paper introduces information theory both as an *a priori* measure of interferences and as a tool to identify assay interference with the most substantial population health consequences. After a brief introduction into

information theory, we show that retesting positive samples from assays with a low frequency of true positives makes double sense because of both a higher likelihood of interference being present in these tests and generally higher population health consequences of a true-positive test result.

Preliminaries of information theory

Information theory is the aspect of quantitatively treating information content, or knowledge. The theoretical foundations were first laid by Claude Shannon[2] and can be found in different standard text books.[3,4] Like in several previous scientific papers published by others,[5–8] we will concentrate on information theory in the context of diagnostic tests. The framework of information theory is as follows.

When performing a test for a diagnostic purpose, we gain knowledge after having received the test result. This knowledge, in a mathematical aspect, should be additive. If the same information is obtained by either two sequential tests or a single test, the sum of knowledge of the two sequential tests should equal the knowledge obtained by the single test. Furthermore, if we get to know something that seemed unlikely before testing (i.e. had a low probability *a priori*), it will count more compared with a more likely result. The impact of a test must thus be a monotonically decreasing function of the probability of the test result. Mathematically, the only way to combine additivity and a decreasing function of the probability is to apply a logarithmic function. A commonly used base of the logarithm is 2^2, with the resulting unit of measurement being bits. The Shannon information content of the test outcome is thus:

$$h(x) = -\log_2(p(x))$$

Here, $p(x)$ is the probability of the test outcome and $h(x)$ is the information content in bits. It is also referred to as the self-information of the particular outcome.

Information theory and diagnostic tests

A definitive test may be considered as a test with the highest accuracy; thus, it entails no false results (positive or negative) and provides a final answer irrespective of the disease prevalence in the tested population. Therefore, the information content of a definitive test solely depends on the prevalence of the disease in the tested population. If hepatitis C antibodies are to be found in 1.7% of the Norwegian population, a definitive immunoassay test for demonstrating them will have an information content of $h(x) = -\log_2(0.017) = 5.88$ bits. Ruling out hepatitis C antibodies will have an information content of $h(x) = -\log_2(0.983) = 0.025$ bits. An immunoassay test result stating the absence of hepatitis C antibodies thus adds little to our knowledge, since the disease has low prevalence and the negative result was highly likely *a priori*. A result stating the presence of hepatitis C antibodies is less likely *a priori* and would thus add more to our knowledge.

Before taking the test, we do not know the outcome. However, we can ask for how much information we can expect to be returned by the test, i.e. the expectancy of a test. The expectancy of a definitive test P is the entropy H of the disease states D with the prevalence $p(d)$ and expressed by:

$$H(D) = -\sum_{j=1}^{n} p(d_j)\log_2(p(d_j))$$

The expected information H is referred to as the Shannon entropy of the test/disease and can be regarded as an *a priori* measure of a test. For a test with only two outcomes (true positives and true negatives) occurring with probabilities t and $(1 - t)$ we have:

$$H(D) = -t\log_2(t) - (1-t)\log_2(1-t)$$

or

$$H(D) = (t-1)\log_2(1-t) - t\log_2(t)$$

A definitive immunoassay test for hepatitis C antibodies thus has the entropy:

$$H(D) = -0.017\log_2(0.017) - 0.983\log_2(0.983) = 0.124\,\text{bits}$$

The highest entropy is reached when all outcomes are equally probable. With two outcomes, the highest entropy is 1 bit and is reached at $P = 0.5$, and lowest when P approaches 0 or 1.

In tests fraught with false positives, i.e. tests having specificities less than 100%, it seems appropriate to distinguish between two categories. In the first case, the test is optimal, but a fraction of healthy individuals also exhibits the same biochemical markers as the test is intended to identify. An example not related to immunoassays is diagnosing bacterial pharyngotonsillitis by throat culture, where some healthy children and adults are asymptomatic carriers.[9] For such tests, the aetiological predictive value has been discussed as an alternative Bayesian *a posteriori* measure of the test.[10] In the second case, the test is clearly suboptimal, i.e. the test gives a false-positive result in individuals which do not express the biochemical marker that the test is intended to identify. For this latter case, it makes sense to calculate the gains of improving/fixing a test, and the term 'suboptimal' will refer to such a test.

The true prevalence of thyroid disease in a young population is 1%. However, testing for thyroid disease (in the youth) by measuring thyroid-stimulating hormone (TSH) typically yields a rate of true negatives of 98.6%, a rate of true positives of 1.0% and a rate of false positives due to assay interference of 0.4%.[11,12]

If the TSH test was definitive, having no false positives, the entropy would be equal to the information entropy of the disease:

$$H(D) = -0.01\log_2(0.01) - 0.99\log_2(0.99) = 0.0808\,\text{bits}$$

We now consider a suboptimal test R having true positives (the fraction of true positives denoted t) and false positives (denoted f). After having performed this test, we have separated samples into one set of true negatives $(1 - t - f)$

and one set of samples containing both true positives and false positives ($t+f$). We have the entropy:

$$H(R) = -(t+f)\log_2(t+f) - (1-t-f)\log_2(1-t-f)$$

Surely we are not satisfied yet, so we now apply an additional, affirmative test A on all positive samples ($t+f$). This test is to be regarded as a definitive test, and may separate the positives into true positives and false positives.

We now have the complete information on which samples are true negatives, false positives and true positives. This information has the entropy:

$$H(R,A) = -t\log_2 t - f\ \log_2 f - (1-t-f)\log_2(1-t-f)$$

The second test A has then contributed with the entropy:

$$\begin{aligned} H(A) &= H(R,A) - H(R) \\ &= (t+f)\log_2(t+f) - t\log_2 t - f\ \log_2 f \end{aligned}$$

which may also be regarded as the information lost by the test interference.

For completeness, we finally state that the entropy shared between a definitive test and the suboptimal test, their mutual information, $I(D;R)$ is:

$$\begin{aligned} I(D;R) &= H(D) - H(A) \\ &= (t-1)\log_2(1-t) - (t+f)\log_2(t+f) + f\ \log_2 f \end{aligned}$$

For the TSH test, we have:

$$\begin{aligned} H(A) &= 0.014\log_2 0.014 - 0.01\log_2 0.01 - 0.004\log_2 0.004 \\ &= 0.0121 \end{aligned}$$

The mutual information is:

$$I(D;R) = H(D) - H(A) = 0.0808 - 0.0121 = 0.0687$$

As the information content of a definitive TSH test is 0.081, interference thus reduces the information content by approximately 15% in the suboptimal test.

If we do the same exercise for elderly women having a rate of true negatives of 82.6%, a rate of true positives of 17% and a rate of false positives of 0.4%,[11,12] we get:

$$H(D) = -0.17\log_2(0.17) - 0.83\log_2(0.83) = 0.6577$$

$$\begin{aligned} H(A) &= 0.174\log_2 0.174 - 0.170\log_2 0.170 \\ &\quad - 0.004\log_2 0.004 = 0.0275 \end{aligned}$$

$$I(D;R) = 0.6577 - 0.0275 = 0.6302$$

If we consider to eliminate interference by retesting all positive samples with a definitive test, this will increase the average information content by 4% in the elderly population (having a high prevalence of the disease) compared with an increase by 18% in the young population (having a low prevalence of the disease).

Thus, the results so far are close to what may be obtained with Bayes' theorem. It should be noted that we are giving a value, albeit small, not only to 'ruling in' diseases but also to 'ruling out' diseases.

The concept may be further generalized to samples having false negatives as well. Finally, we may be interested in applying the concept on quantitative tests. The Shannon information is not defined for continuous probability densities, but there exists a generalized expression for the asymmetric information gain from R to D, defined on continuous probability densities, denoted the Kullback–Leibler information. To clarify, we must emphasize that we here refer to the asymmetric Kullback–Leibler information in the original sense, and not to the symmetrised version, i.e. the Kullback–Leibler divergence.[13] An overview of the proper use of the Kullback–Leibler divergence for diagnostic tests is given in ref.[8]

An *a priori* measure and population health benefit of a diagnostic test

Information theory shows that our efforts on reducing interference at the sample level will be most efficient when concentrating on retesting a few positive samples in an assay with a low rate of true positives. On the assay level, we have demonstrated how to calculate the gain in information content of applying a definitive test over a suboptimal test. However, in order to calculate the population health benefit of a definitive test over a suboptimal test, we must first know the population health benefit for a unit of information content of a particular assay. We must simply calculate the 'benefit per bit' for our assays!

A brief and simplified model of health economics is provided by Claxton *et al*.[14] in a report commissioned by the Department of Health, UK. Every technology, when adopted, infers an additional cost, Δc, on the health-care sector. However, with the adaption of the technology follows a population health benefit, Δh. Since the health budget is not infinite, there exists a threshold, k, for what a society is willing to pay for health. So only techniques where $\Delta c \leq k\Delta h$ will be adapted.

A diagnostic test result provides a road to a potential health benefit, but a diagnostic test result is by no means a health benefit *per se*. Hence, how can we translate a diagnostic test result into a quantitative health benefit? We see two possibilities. The first one is to assign the value of the upcoming health intervention to the test. The second one is to use the direct value of information content of the diagnostic test. In this paper, we follow the second one. For further discussion on prognostic tests and their cost–benefit, we recommend the paper by Moons *et al*.[15]

For the least cost-effective tests in the laboratory, the ratio between cost and benefit will approximate k. Tests having a higher cost per unit of benefit than this threshold should be abandoned. Highly cost-effective tests may have a cost per unit of benefit considerably lower than the threshold k, which can be reflected in willingness-to-pay, i.e. even if this test were more expensive, it would still be in use.

We now split the population health benefit Δh of a test into the information content gained in bits (ΔH) and the

population health benefit of a bit of information content for this particular test (U_t):

$$\Delta h = \Delta H \cdot U_t$$

We thus have:

$$U_t \geq \frac{\Delta c}{k \Delta H}$$

There must be some sort of trade-off in the laboratory between the population health benefit and the disease prevalence. It is not tested for rare diseases unless they are important! For diagnosing thyroid disease, the cost per TSH test is equal for the young and elderly. If we believe that cost-effectiveness is equal for both young and elderly subjects, then the population health benefit of a bit of information content must be higher for the younger subjects (where the disease prevalence is lower and hence the information entropy ΔH is lower). Knuteson[16,17] discusses a similar trade-off in the context of scientific experiments with unknown outcome, and concludes that under equal cost-effectiveness, the benefit must be proportional to the inverse of the information entropy. We have the following expression for the benefit of a bit of information from a particular test:

$$U_t \geq \frac{\Delta c}{k((t-1)\log_2(1-t) - t\log_2(t))}$$

Quantitatively, the ratio between the benefit of a bit of information in the young and elderly, respectively, is: $U(\text{TSH} - \text{younger})/U(\text{TSH} - \text{elderly})$ $0.6577/0.0808 = 8.14$. Hence in fact, the population health benefit of a bit of information on ruling in or ruling out thyroid diseases is considered far more worth (eight times) in younger than in elderly subjects.

We now have expressions both for the population health benefit of a bit of information content in an assay (U_t) and for the loss of information content by interference measured in bits calculated as the difference of the information content between a definitive and a suboptimal test. Combining these two expressions, we get the following expression for the population health benefit of having a definitive test rather than a suboptimal one (assay level):

$$\begin{aligned}\Delta h &= \Delta H U_t = H(A) U_t \\ &\geq \frac{\Delta c((t+f)\log_2(t+f) - t\log_2 t - f\log_2 f)}{k((t-1)\log_2(1-t) - t\log_2(t))}\end{aligned}$$

For the young population, we have:

$$\Delta h \geq \frac{\Delta c \cdot 0.0121}{k \cdot 0.0808} = 0.1498 \frac{\Delta c}{k}$$

and for the elderly population:

$$\Delta h \geq \frac{\Delta c \cdot 0.0275}{k \cdot 0.6577} = 0.0418 \frac{\Delta c}{k}$$

Given the assumptions above, the population health benefit of a definitive TSH assay is 3.6 times higher in a sample taken from a young individual (having a lower prevalence of the disease) than in a sample taken from an elderly individual.

To illustrate how the benefit depends on the rates of false and true positives, we have plotted false-positive rates (interferences) ranging from 0.001 to 0.020 and true-positive rates ranging from 0.01 to 0.50 and calculated the greatest lower bounds of the population health benefit of having a definitive test rather than a suboptimal one (Figure 1).

We want to underline that our calculated and graphically presented benefits are actually the greatest lower bounds of the benefits. Some tests are more cost-effective than others, i.e. the willingness-to-pay greatly for those tests greatly exceeds the threshold. Cost-effective tests will probably be tests for common diseases having large clinical consequences. Examples are troponins (for myocardial infarction), glycated haemoglobin (for diabetes) and D-dimer (for thromboembolic events), which are all tests for common diseases where the test outcome has immediate influence on the future care of the tested patient.

An *a posteriori* measure of the benefits of eliminating interference from a single sample

As pointed out by Ismail *et al.*,[1] the *a posteriori* (i.e. given that the sample is tested positive) probability of finding interference (the positive predictive value of interference) is:

$$\frac{f}{(t+f)}$$

But when considering where to look for interferences, we not only have to take the probability of finding interference

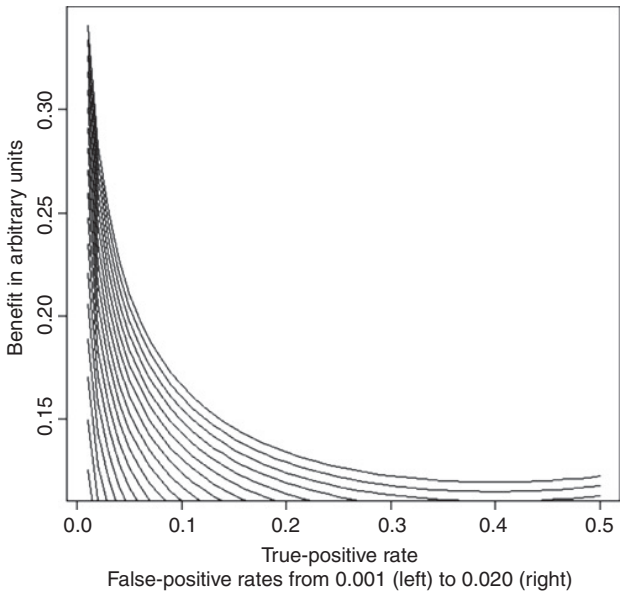

Figure 1 The greatest lower bounds of the gain of eliminating interference from an assay is illustrated as a function of the rate of true positives (p) and false positives (f)

into account, but also the population health benefit, Δh. We thus have an *a posteriori* benefit of retesting a positive sample exceeding:

$$\frac{f}{(t+f)}\Delta h$$

Thus, the population health benefit of retesting a positive sample steeply increases when the prevalence of true positives is low (Figure 2). This is due to both an increasing probability of identifying interference upon retesting and higher population health benefits of identifying interferences in diagnostic tests for diseases having low prevalence.

Adding blockers *a priori* or *a posteriori*?

So far, we have succeeded in finding expressions for the population health benefit of applying definitive tests rather than suboptimal ones. But what constitutes a definitive test? We may eliminate interference *a priori* (by adding blockers to all tests) or *a posteriori* (by adding blockers to all positive tests only and retest). Three sources of cost associated with the definitive test may be: the direct cost of the test Δc, the cost Δc_b of blockers and finally extra costs Δc_e associated with retrieving positive samples and adding blockers.

If we add blockers *a priori*, the cost will be:

$$\Delta c + \Delta c_b$$

If we add blockers *a posteriori*, we only have to add blockers to positive tests. The cost of adding blockers *a posteriori* will be:

$$\Delta c + (f + t)(\Delta c + \Delta c_b + \Delta c_e)$$

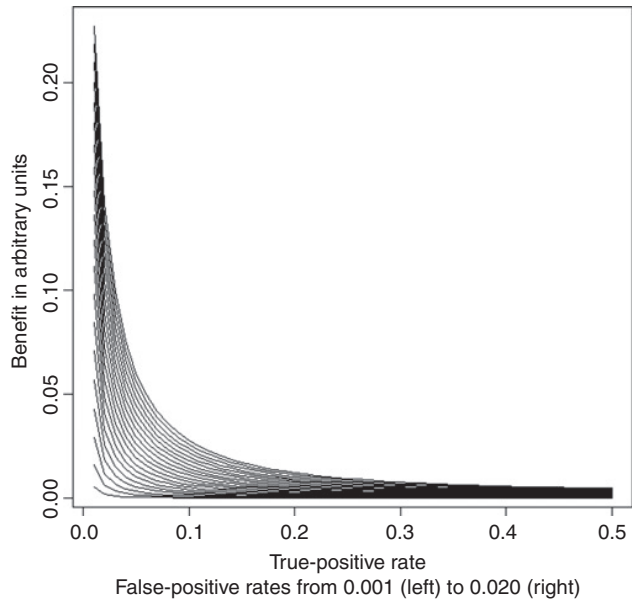

Figure 2 The greatest lower bounds of the gain of eliminating interference from a single sample by retesting it using an assay devoid of interference is illustrated as a function of the rate of true positives (p) and false positives (f)

Thus *a priori* addition of blockers would be preferred if:

$$\Delta c + \Delta c_b < \Delta c + (f + t)(\Delta c + \Delta c_b + \Delta c_e)$$

which can be expressed as:

$$\Delta c_b < \frac{(f + t)(\Delta c + \Delta c_e)}{1 - f - t}$$

We previously found a prevalence of heterophilic antibody interference in an in-house assay for carcinoembryonic antigen (CEA) of 4.0%.[18] We conservatively assume the costs Δc of our CEA test to be £3, the blocker Δc_b 20p and extra costs Δc_e £3 per test. If 10% of samples in our hospital have results over the upper reference limit, adding blockers *a priori* would be an cheaper option (at 20p when costs are shared by all samples) than the *a posteriori* retesting with blockers added (at 87p when costs are shared by all samples).

Although heterophilic antibody interference may be considered a heterogeneous entity, most interference could be eliminated by simple and inexpensive means such as adding immunoglobulins to the buffer,[18] removing the interference-prone Fc-fragment from assay antibodies[18] or deliberately combining antibodies from different subclasses in assays.[19]

Thus, in our view, the high extent of heterophilic antibody interference and the low cost associated with avoiding them strongly favours general *a priori* over *a posteriori* means.

Discussion

The antigen–antibody interaction in an immunoassay takes place in serum samples obtained from different patients who have a huge range of endogenous immunoglobulin antibodies of different classes and subclasses. The vast heterogeneity of these potentially interfering antibodies makes it almost impossible to eliminate all interferences from this unpredictable source. Because of this, the described information theory must be regarded as a relatively blunt but nevertheless useful tool which helps in understanding some of the important features and consequences of heterophilic antibody interference. In a recent paper, we have tried to take a more integral approach on assay interference by looking for interference simultaneously in several immunoassays.[20] Information theory has here helped us to understand some aspects of heterophilic antibody interfence. Some of this knowledge is summarized below.

Most, but not all, interferences may be eliminated by retesting all positives.[1] However, given the observed high prevalence of such false positives, assay design and buffer additives, i.e. *a priori* measures, are the most cost-effective and realistic primary line of defence.

As noted previously, we will probably have some residual interference even if we have optimal assay design and buffer additives. Such interference is most likely when the rate of true positives is low (as shown previously by probabilistic

Bayesian reasoning).[1] In such a test, the population health benefit of identifying interferences also tends to be higher (as shown in the present paper by information theory).

There is thus good reason to believe that assay interference reporting is biased to tests with a low frequency of true positives, where the outcome has large clinical consequences, such as tumour markers and certain hormones. In these cases, interference is easy to identify (by probabilistic Bayesian reasoning) and considered sufficiently important to report (by information theory).

In assays with a high rate of true positives, interference is difficult to identify by probabilistic Bayesian reasoning, and the gains of eliminating interference are smaller as shown by information theory. A more cost-effective approach is to increase test robustness *a priori* as demonstrated by information theory.

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2

Heterophilic antibody interference in immunometric assays

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Immunometric assays are inherently vulnerable to interference from heterophilic antibodies, endogenous antibodies that bind assay antibodies. The consequences of such interference can be devastating. In this review, we discuss strategies that reduce the damage caused by heterophilic antibodies. Clinicians should only order blood tests that are indicated for the patient and clinical setting at hand, and have the confidence to question laboratory results discordant with the clinical picture. Laboratorians should familiarize themselves with the vulnerability of the assays they offer, and be able to perform and interpret adequate confirmatory measures correctly. When designing immunoassays, the immunoassay industry should invest the necessary resources in specific protective measures against heterophilic antibody interference. Examples include using antibody fragments and the addition of effective blockers to assay reagents. The increasing use of modified monoclonal mouse antibodies both in therapy and diagnostics could present a particular challenge in the future.

Introduction

Rosalyn Yalow and Solomon Berson first described their competitive immunoassay for insulin in 1959.[1] The technology revolutionized the measurement of clinically relevant protein and peptide analytes, and enabled the molecular approach to health and disease in modern medicine. For her contribution to medicine, Rosalyn Yalow received the Nobel Prize in physiology or medicine in 1977.

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In the clinical laboratory, we still rely on animal antibodies to quantify important proteins or peptides. The affinity and specificity of vertebrate antibodies permit the accurate measurement of analytes present in very low concentrations (pmol/L) even in complex and protein-rich solutions such as human serum. Although the immunoassay technology is now more than 50 years old, the sensitivity and specificity of well-designed immunoassays are rarely equaled by other analytical techniques.

Our focus in this review will be on interference from human antibodies (with affinity for animal antibodies) in immunometric "sandwich" assays. In this assay format, a solid phase (capture) antibody immobilizes the analyte in the sample, while a second (tracer) antibody coupled to a signal molecule binds to another region (epitope) of the analyte, creating a sandwich (Fig. 1, left). After a thorough wash, although homogeneous (no wash) immunometric assays also exist, the signal is measured and compared to a standard curve to determine the concentration of the analyte. False results occur when heterophilic antibodies in the patient sample cross-link the assay antibodies, even in the complete absence of analyte, thus mimicking the analyte the assay was intended to measure (Fig. 1, right).

Heterophilic antibodies may cause false results even in competitive assays,[2,3] where the analyte in the sample (of unknown concentration) competes with added labeled analyte of known concentration for the limited binding capacity of the assay antibodies. However, heterophilic antibody interference is relatively rare in these assays, unlike the more common problem of interference from cross-reacting endogenous or exogenous molecules.[4,5] In hospital laboratories, competitive assays are still frequently used for small peptides such as steroid and thyroid hormones. The particular challenges associated with the measurement of these hormones, and other sources of confusion such as macro-hormones (prolactin), anti-analyte antibodies (thyroglobulin) or binding proteins (IGF-1 etc.) will be treated in detail in other reviews in this issue, and will not be discussed here. Previous publications provide excellent overviews of general interferences in immunoassays.[4,6,7]

A brief history of interference in immunoassays

Although immunoassay technology has been extensively refined in the 50 years that has passed since its introduction, the use of animal antibodies to bind antigen is the unchanging premise of the methodology. While the sensitivity and specificity provided by the animal antibodies make immunoassays irreplaceable, the reliance on antibodies constitutes the Achilles' heel of the technology; a vulnerability to any antibody-binding entities present in the sample.[8] The first report to describe the detrimental effects of human antibodies with affinity for assay antibodies was published in the early 1970s.[9] The 1970s and 80s saw the development of hybridoma technology,[10] and monoclonal antibodies have since gradually (but not completely) replaced polyclonal antibodies in immunoassays. Concomitantly, the immunometric "sandwich" assay format has largely replaced other formats such as competitive assays.[11] While immunometric assays using monoclonal antibodies are generally robust and rapid assays (important reasons why this format is generally preferred when developing assays for clinically important analytes), they are particularly prone to interference from molecules able to cross-

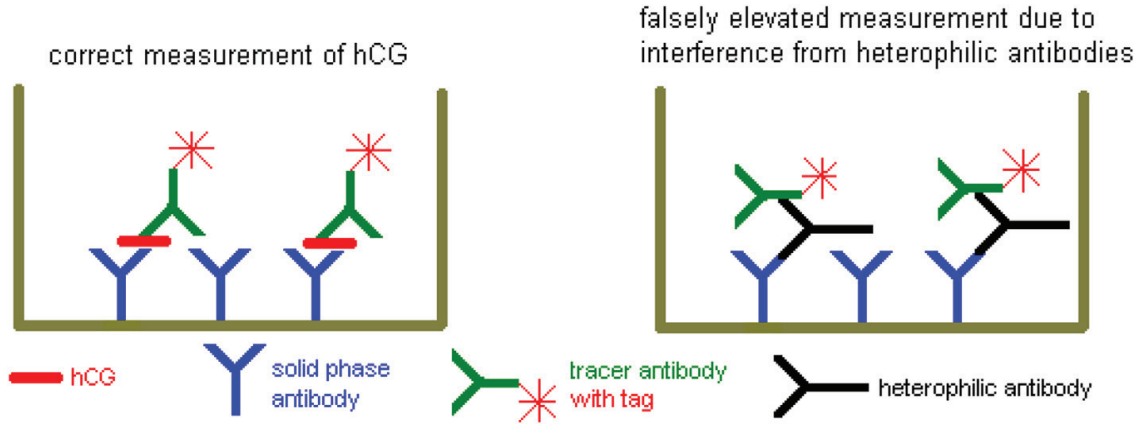

Fig. 1. Left: A schematic illustration of an immunometric assay for hCG. Right: Falsely elevated result caused by interference from heterophilic antibodies.

link the assay antibodies. Several publications described how interfering antibodies could be demonstrated and neutralized, providing the immunoassay industry with tools to make their assays more resistant to heterophilic antibodies.[12–17]

Despite this knowledge, the turn of the millennium saw several publications reporting interference from heterophilic antibodies in commercial assays for hCG.[18–20] In some of these cases, the false results had detrimental effects on patient treatment (case 1), and focus on interference intensified.

Case 1: The hCG-scandal. (Previously described by Rotmensch and Cole[20])

In the late 1990s, a 22-year old woman with irregular menstrual bleeding had repeatedly elevated measurements of serum β-hCG on the Abbott AxSym platform. Pregnancy was excluded, and she received methotrexate for suspected trophoblastic disease. This had no effect on the β-hCG-result. She was then given several courses of combination chemotherapy, and eventually underwent hysterectomy and bilateral salpingo-oophorectomy. A PET-scan indicated a possible pulmonary metastasis and she underwent a thoracotomy so biopsies could be taken from the suspicious area. Her β-hCG-level remained elevated. Pathologists did not find signs of malignant disease in any biopsies or surgical specimens.

At this point, serum and urine samples were evaluated by Dr. Laurence Cole at the USA hCG Reference Service, who concluded that the β-hCG-results on the Abbott AxSym platform were falsely elevated, most likely caused by heterophilic antibody interference. The woman never had cancer. In a subsequent lawsuit, she was awarded $16 million in compensation for the damages caused by the unnecessary treatment. The jury allocated equal responsibility for the tragedy to Abbott Laboratories, the manufacturer of the test, and the hospital where she was treated. Several similar cases involving the AxSym β-hCG-assay were revealed, and Abbott Laboratories were forced to improve the heterophilic antibody resistance of their assay.

The hCG-scandal forced the immunoassay industry to improve assay protection. Although most modern assays have some level of protection against heterophilic antibody interference, heterophilic antibodies are diverse entities with unpredictable properties, and no assay will be completely invulnerable. In addition, most modern assays are automated hybrid auto-analyzers which routinely perform both clinical chemistries and multiple immunoassays on a single instrument. Concomitant with increasing automation has been the transition from “in-house” methods, performed by assay developers and their highly specialized staff, to an almost total reliance on commercially manufactured kits. Indeed, today’s clinical chemists, physicians and ultimately patients are almost totally dependent on the results from “black box” assays and hence the acumen of the kit manufacturers. Despite this automation, both the assays and the threat of interference remain essentially unchanged.

What is a heterophilic antibody?

In daily laboratory practice, the term heterophilic or heterophile antibody is typically used whenever we suspect a patient sample to contain antibodies that cause false results in immunoassays by binding the assay antibodies. Although their effects on immunoassays can be very similar, these interfering antibodies are traditionally classified into three main groups.

Human anti-animal antibody: known exposure to antigen

As suggested by Kaplan and Levinson,[21] the term human anti-animal antibody should be reserved for human antibodies produced in response to animal antibodies injected for diagnostic or therapeutic purposes. Human anti-mouse antibodies, HAMAs, are of particular concern, as most antibodies used in both clinical medicine and immunoassays are derived from mice. These antibodies can create serious analytical problems, as they can be present in high concentrations and have high affinity. In our experience, using the definition of Kaplan and Levinson, human anti-animal antibodies are rarely

encountered in practice. However, both their frequency and properties could change as (more or less humanized) mouse antibodies are used in more patients for diagnostic and therapeutic purposes.

Heterophilic antibody: unknown exposure to antigen

Most interfering antibodies with affinity to animal antibodies are found in patients without known exposure to animal antibodies. These antibodies are called heterophilic antibodies. Although they are found in individuals without known exposure to antigen, previous studies indicate their production in many cases is an antigen driven process.[22–24] Antibodies with affinity to animal antibodies are very common, reportedly present in up to 40% of the population, but most of these will not create problems in immunoassays. Heterophilic antibodies are often presumed to be low affinity antibodies with broad specificities, but this should be considered a general rule with important exceptions. Several of the heterophilic antibodies we have encountered have displayed impressive affinity to the Fc-region of mouse IgG1-antibodies, the most common isotype used in immunoassays, but little or no affinity to $F(ab')_2$-fragments, mouse antibodies of other isotypes or other animal antibodies.[25]

Rheumatoid factors with crossreactivity to assay antibodies

Rheumatoid factors, identified by Erik Waaler in 1940,[26] are patient antibodies (usually IgM) with affinity to the Fc-region of the patient's own IgG-antibodies. Depending on the test, these anti-self antibodies are found in 5–10% of the general population, and approximately 70% of patients with rheumatoid arthritis.[27] Crossreactivity with animal antibodies is not uncommon, as there is significant homology between Fc-domains in human antibodies and Fc-domains in antibodies from several animal species. Rheumatoid factors have much in common with heterophilic antibodies, with some previous studies suggesting they have common immunological origins.[28,29] Rheumatoid factors are traditionally perceived as low affinity antibodies with broad specificities, but as with heterophilic antibodies, exceptions to this rule exist.[30]

When should we suspect interference?

Interference should always be considered when a laboratory result is unexpected or discordant with the clinical picture. Communication and collaboration between clinicians and laboratorians are absolutely essential to discover interference, but the questioning of all immunoassay results is incompatible with the realities of clinical practice. Below we list situations where it is of particular importance to consider heterophilic antibodies as a potential source of interference in immunometric assays.

Patients who have previously had false or dubious results in immunometric assays

The antibodies used in commercial immunometric assays are usually mouse monoclonal IgG1, most often derived from the inbred Balb/C-strain. The constant regions, including the Fc-regions, of these antibodies are very similar. Since the majority of problematic heterophilic antibodies target the Fc-region of assay antibodies, patients that have previously had false results in one assay will have a relatively high probability of getting a false result in other assays.[25]

Patients previously exposed to animal antibodies similar to assay antibodies

In particular, we must expect that patients injected with unmodified mouse monoclonal antibodies (for therapeutic or diagnostic purposes) to make antibodies that can interfere in immunometric assays that use mouse antibodies.[31] Thankfully, most modern "biologicals" are chimeric (mouse Fc-region replaced with a human Fc, suffix *-ximab*) or have been humanized (>95% of mouse sequence replaced with human sequence, suffix *-zumab*). These modifications certainly lower the risk of patients developing problematic anti-mouse antibodies,[32] but with the increasing number of patients receiving these modified antibodies, we think it is likely that some patients will make (anti-drug) antibodies that could interfere in commercial immunometric assays. In particular, assays designed using humanized or

chimeric mouse antibodies could be vulnerable to these patient antibodies.[33] To our knowledge, only a few such assays are marketed today.

Patients with seropositive rheumatic disease

Although most immunoassays have some form of protection against interference from rheumatoid factors, patients with seropositive rheumatic disease deserve particular attention and critical evaluation of immunoassay results. The likelihood of incorrect results caused by interfering antibodies is increased in this patient group for several reasons:

- Rheumatoid factors are common, and although they in most cases do not interfere in immunoassays, pernicious interfering antibodies are relatively speaking more common in patients with rheumatoid factors than in the general population.
- Rheumatoid factors may be present in healthy individuals, but high concentrations are typically seen in patients with active disease, making interference more likely in this patient group.
- Patients with rheumatic disease are increasingly treated with chimeric or humanized mouse antibodies.
- These patients have chronic diseases, often with complicated clinical presentations, which tend to generate large numbers of laboratory tests over time, further increasing the probability of generating false results.

Patients where the laboratory result could have a strong impact on treatment

In some cases, laboratory results are decisive for the diagnostic workup or treatment of patients, particularly when confirmatory tests are unavailable or lead to unacceptable delays. In these situations, particular care is required because of the potential consequences. Examples include

- troponin (T or I) measurement when suspecting acute myocardial infarction.
- tumor markers, particularly hCG when suspecting trophoblastic disease.
- serology tests, particularly if the immunoassay results cannot be confirmed by other tests or test modalities, e.g. PCR in virus diagnostics.
- hormone measurements, particularly if clinical and hormonal axis evaluations are inconclusive.

In all these situations, it is important that clinicians are educated on the inherent vulnerability of the immunoassay technology, so they are more likely to question the laboratory results when not supported by the clinical picture.

Confirmatory measures, interference tests

The scrutiny of immunoassay results is often initiated by clinicians when assay results do not match the clinical presentation of the patient. In general, sometimes to an undeserved degree, clinicians trust laboratory results. When critical questions are raised, these are most often justified, and laboratorians should invest the necessary time and effort to ensure the quality of the laboratory service. A collaborative approach to explore discordant results can be educative and rewarding to both laboratorians and clinicians. Ultimately, communication and collaboration are necessary to ensure satisfactory quality of patient care. Persistently choosing fence sitting or defensive tactics can only be harmful, both to collegiality and patient treatment.

Before discussing different interference tests, we would like to stress that many cases can be resolved, and tragedies avoided, using common (medical) sense. In order to limit the number of false results, the total number of blood tests performed should be limited. Most physicians will agree that a blood test should only be ordered when indication is present, and that uncritical screening of patients with blood tests is considered bad medicine. This is particularly relevant for assays where the results can be decisive for patient management, such as tumor markers, markers of cardiac disease or markers of infectious diseases.

For some analytes, not limited to our examples, knowledge of physiology and metabolism can help us evaluate suspicious results:

- Individual hormone results must be evaluated in relation to the other hormones in the endocrine axis they are part of.
- Elevated serum hCG, potentially indicating malignant disease in men and non-pregnant women, should always be confirmed by measurement of hCG in urine.[20] Heterophilic antibodies (like other antibodies) are not found in urine, and a normal urine hCG strongly implies interference as the cause of elevated serum hCG.
- If a patient with an elevated troponin T or I does not have other findings indicative of heart disease, the other troponin should be used as a control test. Information from CK-MB, ECG and imaging must also be considered.

Laboratories should choose interference tests that can be executed and interpreted consistently and correctly. In order to avoid confusion and false conclusions, most laboratorians should probably choose one or two methods they can become familiar with. It is also important to have some knowledge of, and experience with, the analyte and assay in question. Two general rules apply to interference testing:

- A sample without interference, where the concentration of the analyte is known and preferably in the same range as reported for the sample to be tested, should be included as a control in the interference test.
- A negative interference test does not exclude interference, while a positive interference test, provided it is performed correctly, usually indicates interference.

Below we list the most common approaches and briefly discuss their advantages and associated challenges.

Reanalyzing with the same assay

As an initial control, unexpected immunoassay results should first be reanalyzed by the same method to exclude analytical errors such as pipetting inaccuracies, inefficient wash, tracer aggregates or other contaminants.[6] Some samples with heterophilic antibody interference can give varying results with repeated testing, and great variation can increase the suspicion of interference. However, lack of variation upon retesting does not exclude interference. It is important to know what variation can be expected in the concentration range of the immunoassay in question. This information should be retrieved from the laboratory's quality control records, not from the manufacturer of the assay.

Reanalyzing with different immunoassay or alternative methodology

In cases of suspected interference, reassay with an alternative assay or methodology is highly recommended. Usually the sample is sent for confirmation to another laboratory which uses a different immunoassay. Particularly difficult patient samples may cause interference in both assays, but even in these rare cases the effect of the interfering antibodies are usually different in the two assays. While this approach is accessible to most laboratories and can be very informative, alternative assays do not always exist. In some cases, logistic challenges related to sample transport or instability may also hinder this approach.

Dilutions

Diluting samples, e.g. using the kit diluent, and reanalyzing is a common strategy when faced with spurious test results. This approach is available and familiar to most laboratorians, as it is often used to retest all samples with extremely elevated analyte concentrations.

In interference testing, dilutions can be useful if the result is sufficiently high. We look for non-linearity upon dilution to indicate interference. However, results after dilutions can be difficult to interpret correctly in interference testing, particularly because

- Some samples with heterophilic antibodies give linear responses upon dilution, meaning that dilutions may give a false confirmation of the original result.
- Some analytes or assays do not give linear responses upon dilution, potentially causing a false confirmation of suspected interference.

Results are easier to interpret correctly if a parallel sample with a truly elevated concentration of the analyte, preferably at roughly the same level as the suspicious sample, is included in the dilution test. The potential pitfalls of using dilutions have previously been discussed in depth by Ishmael et al.[34]

Blocking

The addition of irrelevant animal immunoglobulin to the sample prior to reassay is a commonly used strategy to neutralize interfering antibodies. Logically, if a patient sample contains heterophilic antibodies that cross-link the mouse IgG1 assay antibodies, the addition of mouse IgG1 to the sample can neutralize the heterophilic antibodies and prevent interference. Aggregated antibodies, either heat-treated or chemically aggregated, are more potent blockers than non-aggregated antibodies.[15,22] This is most likely explained by the improved ability of antibody aggregates to form stable complexes with heterophilic antibodies, typically with 2 (IgG) or 10 (IgM) antigen-binding sites, due to the number and proximity of epitopes on the aggregates. If, for example, a heterophilic antibody has high affinity for the Fc-region of mouse antibodies, an aggregate of mouse antibodies with large numbers of Fc-regions close together provides an irresistible target for the heterophilic antibody. As a rule, we let the sample and blocker incubate for 10–15 min prior to reassay to allow complete binding of heterophilic antibodies to antibody aggregates.

In assays using polyclonal rabbit or goat antibodies, polyclonal rabbit or goat IgG should be added to the sample, but higher concentrations of polyclonal antibody (0.5–1 mg/mL) are often necessary to achieve efficient blocking compared to monoclonal antibodies (0.1–0.2 mg/mL). It is our experience that blocking effectiveness is increased when the polyclonal immunoglobulin is aggregated.

Immunoassays that combine antibodies from two species, e.g. a mouse monoclonal as the solid phase antibody with rabbit or goat polyclonal as tracer antibody, can be difficult to block. We have had most success blocking with aggregated antibodies similar to tracer antibodies.[35] This is perhaps not surprising, as we usually get samples referred for interference testing because the result is unexpectedly elevated. In these cases, more tracer antibody is bound than expected, potentially because the sample contains heterophilic antibodies with affinity for the tracer antibody. Since the blocking is meant to neutralize these heterophilic antibodies, we are most likely to succeed using a blocker similar to the tracer antibody. In theory, if negative or blocking interference is suspected (i.e. less tracer antibody is retained than expected) a neutralizing antibody from the same species (and of the same isotype) as the solid phase antibody might be the best candidate.

As an alternative to animal immunoglobulin (although to our knowledge, most of these reagents also contain animal immunoglobulin), several blocking reagents and blocking tubes are commercially available. The blocking tubes, which contain a pellet of blocking reagent, provide an attractive and safe alternative to clinical laboratories in need of an easy-to-perform interference test.[36] For laboratories with particular focus on interference testing, the lack of flexibility of ready-made blocking tubes makes them less attractive alternatives. It is important to remember, like with all interference tests, lack of effect using commercial blockers does not exclude interference.

Depletion of antibodies in the sample

Methods used to remove heterophilic antibodies from samples are usually based on precipitation, affinity extraction or size-exclusion. When antibodies, including interfering antibodies, are removed, the true analyte can be measured in the antibody-free sample. Any contribution from antibodies can

also be confirmed by assaying the reconstituted or eluted antibody fraction. While these methods can be very useful tools, they are demanding and potentially deceptive interference tests. Thus, a control sample should always be tested in parallel to ensure correct interpretation.

Antibody precipitation: When present in sufficient amounts, ammonium sulfate, $(NH_4)_2SO_4$, and polyethylene glycols (PEGs) precipitate proteins by lowering their solubility in aqueous solutions such as serum or plasma. Protocols that effectively precipitate human immunoglobulin are available,[6,37,38] but laboratories should conduct in-house experiments for the analyte, sample material and salt or PEG in question to validate the protocol prior to using it in interference testing. It is quite possible that even though the precipitate is 99.99% pure immunoglobulin, clinically relevant proteins present in low concentrations have also been completely cleared from the sample. In addition, precipitation may denature proteins, and complete renaturation of the precipitated protein upon reconstitution is not always achieved. If the precipitated antibody does not return to its original form upon reconstitution in an interference test, we might not get the confirmatory result from the antibody fraction, which can complicate interpretation.

Affinity extraction: Several molecules are known to bind immunoglobulin, and some can be used to purify or extract human immunoglobulin from serum or plasma.[39] We have experience using Protein G (or Protein A) columns for affinity purification of animal antibodies, and have modified these protocols for use in interference testing. When buffers and temperature are optimized, Protein G columns efficiently clear human IgG from serum or plasma. An advantage of using Protein G to isolate the IgG in the sample, is that antibodies are eluted from the column with acidic buffers (pH 4–5), which they usually tolerate very well. Thus, the procedure does not denature the antibodies and their properties are conserved so they can be used in confirmatory testing.

Size-exclusion: Antibodies are large molecules, with molecular weights ranging from ~150 kDa for IgG to ~950 kDa for IgM. Most analytes are smaller than antibodies, and when the size difference is large enough, we can use this to our advantage in interference testing. If the suspicious sample is passed through (usually be centrifugation) an ultrafiltration device with a defined cutoff, smaller molecules (analytes) will pass through while larger molecules (antibodies) will be retained. We usually spin the sample until we have roughly equal volumes on either side of the filter. Measuring the two fractions can then reveal if an elevated result is caused by large molecules such as heterophilic antibodies, or small molecules such as the analyte the assay is intended to measure. Importantly, the cutoffs given for the filter units are never absolute, and their ability to separate molecules is affected by sample matrix and quality. For this reason, we only use this approach if we can choose filters with size cutoffs with comfortable margins to both analyte and antibodies. In practice, we use filters with cutoffs smaller than half of the antibody size (~75 kDa) and at least double the size of the analyte. This effectively limits the use of this approach to analytes with molecular weights below 30 kDa. Similarly, gel-filtration chromatography can be used to separate large antibodies from smaller analytes. Although simple in theory, there are several drawbacks to this approach, making it less attractive to use in interference testing. Most importantly, the sample (and analyte) will usually be diluted significantly, so gel-filtration is only applicable when results are sufficiently elevated. Also, the sample is separated into a large number of fractions that need to be measured.

Interference assays, measurement of heterophilic antibodies

While these assays, strictly speaking, can never prove heterophilic antibody interference as the cause of an elevated result, they are discussed here because they can be useful tools in interference testing and research. However, because they require equipment and knowledge (unfortunately) deemed obsolete in most modern laboratories, these assays are only available to laboratories that still work with antibodies and in-house assays.

Interference assays, sometimes referred to as non-sense assays, are deliberately designed not to measure anything except interference caused by heterophilic antibodies able to cross-link the assay antibodies.[12] The assay antibodies have non-corresponding reactivities, meaning that proteins with epitopes for both assay antibodies do not exist. Most commercial assays are designed combining two mouse monoclonal IgG1-antibodies. In our standard interference assay, we use a mouse IgG1 anti-CEA antibody on the solid phase in combination with a mouse IgG1 anti-AFP antibody as tracer antibody.[25]

Since proteins that contain both the CEA-epitope and the AFP-epitope do not exist, any signal in this assay is caused by a moiety (usually a heterophilic antibody) able to cross-link the two assay antibodies. The antibodies used in the interference assay can be replaced to provide more targeted testing. If the patient sample is under scrutiny because of a suspicious result in an assay that uses polyclonal rabbit or goat antibody, or say, a combination of mouse monoclonal IgG1 on the solid phase and polyclonal rabbit as tracer antibody (a fairly common combination), we can mimic this assay by combining a mouse monoclonal IgG1 and rabbit polyclonal in our interference assay.[35] While we do not prove that the suspicious result is false, we can demonstrate the presence of heterophilic antibodies in the sample that have the ability to cross-link antibodies similar to those used in the assay that produced the suspicious result.

An advantage of interference assays, particularly important in research, is that the assays can be modified not only by combining different antibodies, but also different antibody fragments. Using this approach, we have previously shown that most cases of interference can be avoided by using antibody fragments, such as $F(ab')_2$, Fab′ or single-chain (scFv), in our immunometric assays.[22,40]

Further modifying the interference assay, we can also establish assays that can quantify HAMAs. By using mouse antibody on the solid phase, we can capture any HAMAs in the sample. After a wash, we can use a tracer antibody specific for human immunoglobulin to quantify the HAMAs in the patient sample. Several similar assays are available commercially, and are often used to monitor patients treated with mouse antibodies, or derivates of mouse antibodies.[41]

Case 2: Blocking. (Previously published as a clinical case study[35])

After a period of fatigue and non-specific abdominal symptoms, a 53-year old man consulted his physician, who ordered a broad range of blood tests. The laboratory reported a grossly elevated result for adrenocorticotropic hormone, ACTH, while cortisol levels were normal. These results were confirmed on repeat measurements. As part of the extensive workup, conventional radiological investigations failed to demonstrate any pathology. However, a PET/CT-scan using a relatively new radiotracer (^{68}Ga-DOTATOC) indicated a possible neuroendocrine tumor (diameter 3.3 cm) in the pancreas. Based on the consistently elevated ACTH and the PET-finding, surgeons at three European hospitals recommended surgical removal of the tumor. The patient wanted minimally invasive surgery, and was offered laparoscopic resection of the tumor at the Intervention Centre at Oslo University Hospital. Preoperative CT could not identify the tumor, and surgery was postponed. ACTH, measured on the Siemens Immulite 2000 instrument in our hospital, remained grossly elevated at 203 pmol/L (ref: $<$10.2 pmol/L). An endocrinologist did not find any clinical signs consistent with the laboratory result, and suggested it was caused by heterophilic antibody interference.

The Immulite ACTH-assay combines a mouse monoclonal antibody on the solid phase with rabbit polyclonal antibody as tracer antibody in an immunometric "sandwich" format. Interference assays demonstrated the presence of heterophilic antibodies in the sample able to bind both mouse and rabbit antibodies. Blocking with aggregated mouse antibody had no effect on the elevated result, but blocking with aggregated rabbit antibody normalized the ACTH-result. This indicated that the ACTH-results were falsely elevated due to heterophilic antibody interference. The 3.3 cm tumor identified on PET/CT could not be visualized using conventional CT or endoscopic ultrasound, and was most likely an artifact caused by physiological accumulation of radiotracer in the uncinate process of the pancreas. The patient was discharged without treatment and had a completely normal CT-scan six months later.

If the confirmatory measures described above do not indicate heterophilic antibody interference as the cause of immunoassay results clearly discordant with the clinical presentation, the manufacturer of the assay in question should be consulted. In very rare cases, high concentrations of biotin[42] (particularly relevant in dialysis patients) or antibodies to tracer molecules[43,44] can interfere

in immunoassays. These cases are difficult to resolve in clinical laboratories, but assay manufacturers usually have access to reagents and knowledge required to identify the source of interference.

Immunoassay design

Heterophilic antibody interference will always be a challenge in laboratories using immunoassays, the endless variation and unpredictable affinities of human antibodies means that invulnerable immunoassays can never exist. However, the incidence of interference can be minimized by using assays with sufficient specific protection against interfering antibodies. Although design details are not always provided in marketing brochures or kit inserts, it is our experience that most companies are happy to share this information with customers who ask for it.

Clinicians and laboratorians can limit the damage caused by heterophilic antibody interference by staying vigilant and performing effective interference tests. However, the truly effective tools to reduce the incidence of such interference are in the hands of the immunoassay industry. Specific measures to improve protection of commercial assays have to be taken during assay development.[45,46] For assay manufacturers, the investment in interference protection is a case of priority.

Antibody fragments and purity

Most immunometric assays are designed using mouse monoclonal antibodies, usually of IgG1-subtype. This means that it is very likely that the solid phase antibody and tracer antibody share common epitopes, thus increasing the chance that antibodies able to cross-link the antibodies are present in patient samples. Since most interfering antibodies target the Fc-portion of assay antibodies, the removal of Fc is probably the single most important protective measure.[16,22,47] For this reason, most modern commercial immunometric assays are designed using $F(ab')_2$- or Fab′-fragments. Recently, recombinant single-chain (scFv) antibodies have been demonstrated to be promising alternatives to antibodies or antibody fragments in assay design,[48,49] with excellent resistance to heterophilic antibodies.[40] As of yet, they are not used in commercial immunoassays. This is most likely related to higher production costs, but also to the difficulty of developing high-affinity scFvs.

Unfortunately, using fragments increases assay production costs. The necessary cleavage and purification procedure usually leads to some antibody loss, we expect 20–30% loss in our in-house procedure. This is obviously a concern to the immunoassay industry, as the purchase or production of assay antibodies usually constitutes a major share of total production budgets. Despite the added costs, most companies now rely on fragments when designing their assays, a testament to their effectiveness in limiting interference. Unfortunately, some older assays, designed before fragments became common, are still marketed today.[25] These assays are undoubtedly more prone to interference than most modern assays and deserve particular vigilance.

Some immunometric assays combine antibodies from two species, commonly a mouse monoclonal as solid phase antibody and rabbit polyclonal as tracer antibody. The solid phase and tracer antibodies are less likely to share common epitopes, making these assays potentially more resistant to cross-linking antibody interference than assays using two monoclonals from the same species. However, falsely elevated results can occur through interference from antibodies that do not cross-link the solid phase and tracer antibodies. Heterophilic antibodies with affinity for the tracer antibody can enhance assay signals by forming antibody complexes with tracer antibodies. We have experienced that likely falsely elevated results (in assays combining antibodies from two species) are only normalized when blocking with aggregated antibodies from the same species as the tracer antibodies, and not with aggregated antibodies similar to solid phase antibodies. We believe these blocking experiments indicate that the falsely elevated results in these cases are caused by heterophilic antibodies that primarily bind tracer antibodies. These heterophilic antibodies capture additional tracer on top of the antibody-antigen-antibody “sandwich”, creating a falsely elevated signal.

An additional concern is related to the purity of assay antibodies. Antibody-producing hybridomas are often grown in media containing animal serum, predominantly fetal calf serum. The monoclonal mouse antibody will thus be purified from a supernatant that also contains bovine immunoglobulin. If

the purification protocol does not separate mouse and bovine antibodies, the mouse monoclonal antibody may be contaminated with as much as 10% bovine immunoglobulin. In assays containing contaminated mouse antibodies, we might see bovine immunoglobulin on the solid phase and bovine immunoglobulin labeled with tracer. Endogenous antibodies with affinity for bovine antibodies are extremely common in the general population, perhaps related to the consumption of beef and dairy products, meaning that assays contaminated with bovine immunoglobulin might be particularly vulnerable to interference. Serum-free cell media are available and should be used when producing antibodies for clinical immunoassays, even though they are more expensive than traditional serum-containing media.

Assay buffers and additives

The composition of assay buffers, and the choice of additives, is vital in successful assay design. In addition to providing a beneficial environment for the interaction between antibodies and antigens, appropriate buffers help ensure stability of reagents, limit non-specific binding and may minimize interference from endogenous (e.g. bilirubin) and exogenous (e.g. drugs) interfering substances. While some general rules apply, the buffers often have to be optimized for individual assay formats and analytes.

Specific additives are used to improve resistance to heterophilic antibody interference, but the effectiveness depends on both on the type and amount of additive, the assay format and the analyte in question. Unlike the more general assay constituents, additives that specifically limit heterophilic antibody interference confer a considerable cost to assay production. In the manufacturing of some assay kits, most commonly homogeneous assays (discussed below), companies may invest more money in additives to limit interference than they spend on the actual assay antibodies (personal correspondence).

Previously, non-immune animal serum was added to assay reagents, both to increase protein content (less non-specific binding) and to limit interference from heterophilic antibodies. Today, purified proteins have largely replaced serum and are used both to increase protein content and to improve resistance to interference. As a basis, bovine serum albumin, BSA, and bovine IgG are used in fairly high concentrations in most assay buffers. In addition, most manufacturers will add immunoglobulin from the same species as the assay antibodies to block potentially harmful heterophilic antibodies. This means adding monoclonal mouse IgG1 to assays using monoclonal mouse IgG1-antibodies, polyclonal rabbit antibody to assays using polyclonal rabbit antibodies etc. Several studies have shown that aggregated immunoglobulin is a more potent blocker of interference than non-aggregated immunoglobulin.[15,22] Some manufacturers add antibody fragments or commercially available heterophilic blocking reagents to improve protection.

Assay formats

Several different assay procedures are marketed today, and there has been a clear trend to limit the number of wash steps. Less washing means quicker assays and increased instrument capacity. Reducing the number of wash steps, even avoiding washing altogether, naturally affects assay vulnerability to heterophilic antibody interference.

Traditional immunometric assays, sometimes called 2- or 3-step assays, rely on sequential addition of assay reagents and sample with a wash step before the next reagent is added. In these assay formats, heterophilic antibodies in the sample have to bind the solid phase antibody in order to be exposed to the tracer. Thus, assays can be made more resistant to heterophilic antibody resistance by modifying the solid phase antibody. This usually means removal of Fc through enzymatic cleavage and using $F(ab')_2$- or Fab′-fragments.[50] While traditional 2- or 3-step assays are easier to protect from heterophilic antibodies, they are not very popular in modern clinical laboratories. This is primarily related to lower throughput since they are usually batch assays, and assay duration as they typically take 3–5 h to perform.

Most immunoassays are presently performed on automated random-access platforms and use beads as the solid phase. The flexibility and speed (10–60 min) of these assays means they are preferred to traditional assays in most laboratories. A “sandwich”-complex (antibody-antigen-antibody) is allowed to form

in liquid phase, and is only immobilized when the magnetic/paramagnetic beads (solid phase) are added to the mixture. The sample is thus incubated with both assay antibodies, optimizing binding conditions for the assay antibodies and allowing short reaction times. However, the binding conditions are equally beneficial for any interfering antibodies in the sample, and buffer composition and antibody modification is critical. While the assays use less buffer due to small volumes and fewer wash steps, the buffers often contain a lot of protein and blocking reagents and increase production costs. In these assays, both assay antibodies should be modified to limit interference. In theory, since the tracer antibody generating the signal is exposed to heterophilic antibodies in the sample, it might be particularly important to modify it.

Recently we have seen the commercial release of several homogeneous immunoassays that do not contain any wash steps.[51,52] The measured signal, usually a light emission resulting from energy transfer from a donor molecule on one antibody to an acceptor molecule on another antibody, is generated only when the donor molecule and acceptor molecule are within a certain distance from each other. Thus, the quantification of analyte depends on the positioning of the two assay antibodies when they are bound to their epitopes on the analyte. Because there is no wash step, but also because of the refined detection method, extreme demands are put on buffers used in these assays.

In the increasingly popular multiplex assays, which are known to be vulnerable to heterophilic antibody interference,[53] an interference (non-sense) assay could be included in the multiplex.[54,55] This would not improve the resistance to heterophilic antibody interference in the assay, but the test could alert us to likely interference in a sample and help limit the consequences.

Case 3: Abbott hCG revisited 2009. (Previously described[25])

In 2009, a decade after the hCG-scandal involving β-hCG on the Abbott AxSym platform, a woman in her 30s with irregular menstrual bleeding had repeatedly elevated measurements of serum β-hCG on the Abbott Architect platform. Pregnancy was excluded, she was given several courses chemotherapy and underwent three surgical procedures for suspected trophoblastic disease. The hCG-result remained unchanged despite the treatment, and heterophilic antibody interference was considered as the cause of the elevated hCG-result. Reanalysis with another assay for β-hCG on the Roche Cobas e601 platform, and reanalysis on Abbott Architect after blocking with aggregated mouse IgG, both showed she had normal, low levels of β-hCG. The elevated results, which in turn lead to harmful and unnecessary medical and surgical treatment, were caused by heterophilic antibody interference in the Abbott Architect assay.

For some analytes, such as steroid hormones and vitamin D, immunoassays are gradually being replaced by mass spectrometry. In these cases, the analytical advantages might legitimize the additional costs, even in routine clinical laboratories. However, we must rely on immunoassays for the foreseeable future for quantification of most clinically relevant proteins and peptides. Exemplified by the relatively recent and utterly avoidable tragedy described in case 3, we emphasize the importance that both clinicians and laboratorians update their knowledge and remain vigilant to the threat of heterophilic antibody interference.

Practice points:

- The risk of confusing results increases when more blood tests are ordered, and clinicians should only order tests that are indicated for the patient.
- Laboratory results that are discordant with the clinical presentation must be dealt with in collaborative efforts between clinicians and laboratorians.
- A negative interference test does not exclude interference, while a positive interference test usually indicates interference.
- Heterophilic antibody interference is always a risk in immunoassays, but the incidence can be reduced through specific protection. Poorly protected assays should be replaced with better alternatives.

Research agenda:

- The incidence and properties of interfering antibodies could change with the increasing therapeutic and diagnostic use of modified mouse antibodies, and different strategies for interference detection and protection might be necessary.
- Novel methods for detection of interference should be explored and incorporated in immunoassay systems.

Summary

Heterophilic antibody interference will always be a threat in immunoassays, but the incidence and damage can be reduced through vigilance and collaborative approaches from clinicians, laboratorians and immunoassay manufacturers. Clinicians should limit the total number of ordered blood tests, and consider interference and communicate with laboratorians when results are discordant with the clinical picture. Laboratorians should choose assays that are well protected, and if possible, replace poorly protected methods. They must be open to questions from physicians regarding the validity of immunoassay results, and be confident and skilled with one or two interference tests. Immunoassay manufacturers should use antibody fragments and aggregated blocking immunoglobulin when designing assays, and improve interference protection in existing assays.

Conflicts of interest

None.

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