Protein biomarker-based screening for detection of recombinant bovine somatotropin abuse in dairy cows

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Thesis

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General introduction

Recombinant bovine somatotropin (rbST) is a proteohormone, which can be used to increase milk production in dairy cows. While its use in food production is approved in several countries, such as the United States (US), it is banned in the European Union (EU). Therefore, appropriate monitoring methods are required to control for its abuse. In this thesis, biomarker-based screening methods have been developed to detect rbST abuse in serum and milk of dairy cows. The general introduction shall give an insight into the history of rbST development and the corresponding regulatory mechanisms in different countries. The molecular structure and physiology of the hormone are described and the effects of rbST onto the cow's metabolism are outlined. From this follows a view on the controversial public debate about rbST use in food production. Different analytical detection strategies for monitoring rbST abuse that were followed so far are described. Finally, the immunoassay detection methods for biomarker-based screening used in this thesis are shortly outlined.

1 History and legislation of rbST

In 1937 it was described that an extract from bovine pituitaries can increase the milk yield after it was injected into lactating cows [1]. Because of the general food shortage during the Second World War, British scientists investigated the effects of pituitary extracts on milk yield and milk composition. But since the supply of somatotropin (ST), also called growth hormone (GH), was limited to that extracted from bovine pituitaries, no general beneficial effect on milk supply was seen [2]. In 1949 ST was found to be responsible for this galactopoietic effect [3] and in the 1970s, the primary structure of the protein hormone was revealed [4]. In the beginning of the 1980s, the recombinant DNA technology became available, and rbST could be produced in *E. coli* on large-scale [2].

In the US, the Food and Drug Administration (FDA) approved the use of rbST for increasing milk yield in 1993. In 1994 rbST became available on the US market and within 6 years, approximately 40 % of the US dairy farmers had adopted it [5]. Also other countries, such as South Africa, Brazil and Korea, approved the use of rbST for increasing milk production [6]. However, there are consumers' concerns about hormone use in food production, and therefore, some US retailers and dairies indicated not to use or sell products, which contain milk from rbST-treated cows.

In contrast in the EU, a moratorium was put on rbST use in 1990 to perform further research on potential negative effects on human health and animal welfare. Eventually, the administration and sales of rbST were banned for animal welfare reasons since January

2000 [7, 8]. RbST use has also been forbidden in other countries, such as Australia, New Zealand and Canada [9]. Since there is a ban on this veterinary drug, monitoring of its possible abuse is required. So far, no method has been implemented EU-wide for rbST abuse monitoring.

2 Endogenous and recombinant bovine somatotropin

2.1 Molecular structure

Bovine ST (bST) is a proteohormone and is produced by the anterior pituitary gland. Naturally, bST occurs in four different forms consisting of 190 or 191 amino acids (the 190 amino acid form lacks the N-terminal phenylalanine) and having either leucine or valine at position 126 or 127 respectively [10, 11]. It is a single polypeptide chain that is folded and stabilized by two internal disulfide bonds [4] (**Figure 1**).

During production of rbST, the N-terminal amino acid alanine is replaced by methionine, leading to a 60 Da difference in molecular weight (**Figure 1**) [12]. Despite the amino acid exchange, 99 % of the molecular structures are identical for the endogenous and recombinant forms of the hormone. Other forms of rbST having different N-terminal ends have been produced and they were shown to be fully bioactive *in vivo* [13].

2.2 Physiology

After ST is produced in the anterior pituitary gland, it is released into the blood stream under the control of several different hormones, such as growth hormone-releasing hormone (GHRH), somatostatin and ghrelin (**Figure 2**) [14]. In blood, ST binds to binding proteins (GHBPs) and is transported to target tissues. In general, ST exerts direct effects on target tissues and indirect effects via the mediator insulin-like growth factor-1 (IGF-1) [15]. Direct effects of ST are for instance increased protein synthesis in muscle, increased lipolysis and the release of IGF-1 from liver tissues. Indirect effects via IGF-1 are for instance increased protein synthesis in bone and organs [15, 16]. As illustrated in **Figure 2,** also IGF-1 is bound to binding proteins (IGFBPs) in the blood stream, which regulate its bioavailability [14].

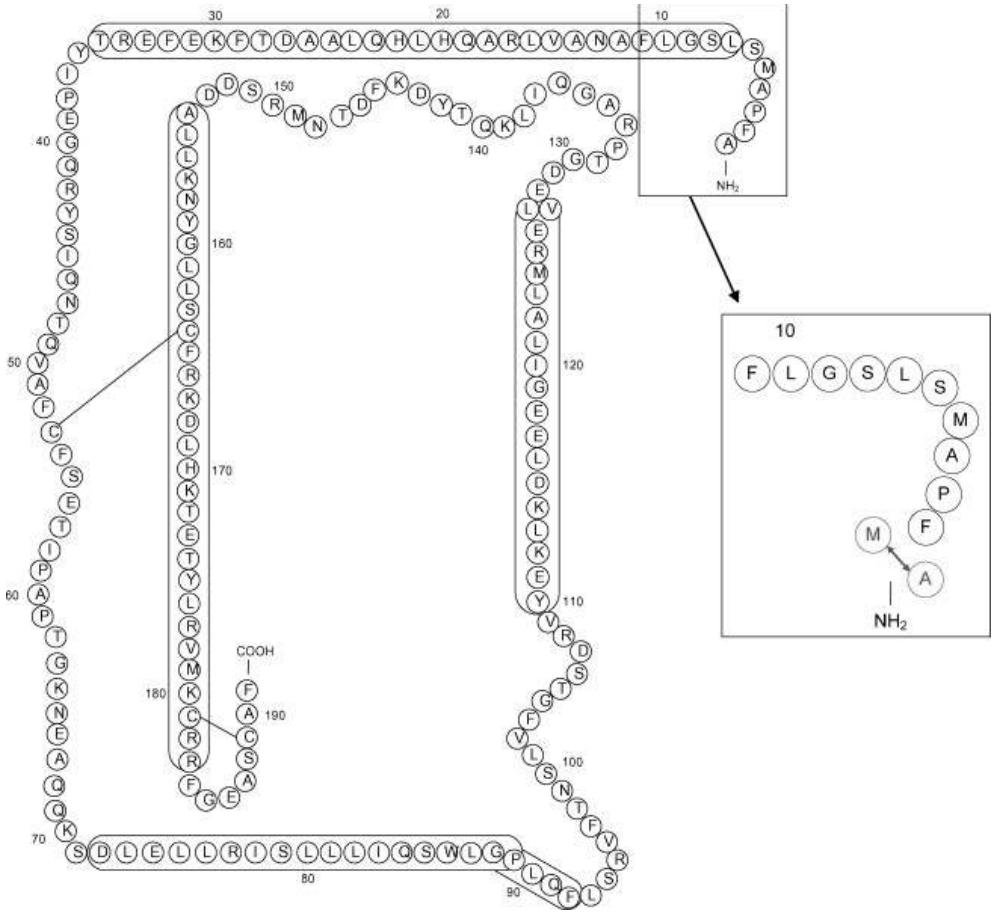

Figure 1: Amino acid sequence of endogenous and recombinant bST (reproduced with permission according to Le Breton *et al*. [12]). The endogenous bST carries an alanine at the N-terminus whereas the recombinant form of the hormone carries a methionine.

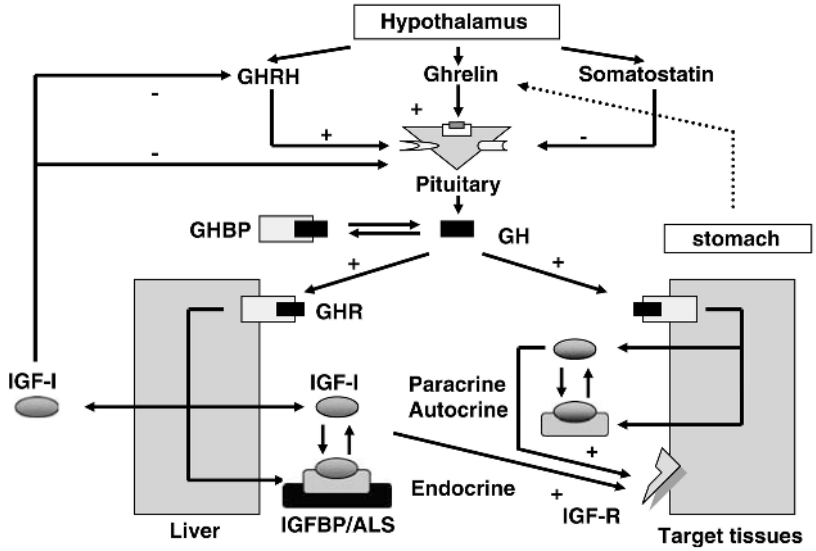

Figure 2: GH regulatory circuit including feed-back loops (reproduced with permission from Holt *et al*. [14])

Many studies have been performed on the kinetics of bST in circulation. Toutain *et al*. compared plasma bST concentrations after intravenous bolus injection of pituitary bST or rbST [17]. They found a slightly shorter half-life time for the tested rbST of 54.8 minutes compared to pituitary bST, which showed a half-life time of 61.8 minutes. Le Breton *et al.* studied the kinetics of rbST administrated in a slow-release formula [12, 18]. Maximum rbST concentrations were found in serum approximately one day after administration and ranged from 9 up to 120 ng mL^{-1} between individual cows. In all tested animals, the concentrations dropped to half of the maximum concentration within 2-3 days after the last administration. Moreover, Breton *et al.* reported that they were able to find rbST for 13 days in the cow's circulation. However, that study was performed with cows who received a double rbST injection within , which is not suggested for regular practice.

2.3 RbST administration as a veterinary drug

When rbST is administered to dairy cows, milk yield on average increases by 11.3 % for primiparous and 15.6 % for multiparous cows [19]. According to the suggestions by the manufacturer, rbST should be injected starting from the ninth or tenth week in lactation (57-70 days) when the lactating performance of the cow is at its maximum [20]. If rbST administration is repeated every 14 days until the end of lactation, the decrease in milk

yield, which is usually observed in untreated cows, is slowed down (**Figure 3**). Therewith, the milk production of rbST-treated animals is increased over the entire lactation period. Increased lactation performance was found to be due to higher milk synthesis rates of the mammary gland and increased maintenance of mammary gland cells [2]. Moreover, nutrients required for milk synthesis are taken up more efficiently and the blood flow in the mammary gland increased after rbST administration [2].

Even though rbST increases milk yield in dairy cows, hardly any effect on milk composition was observable [19]. In a meta-analysis comparing 18 rbST treatment studies, very little increases in milk butter fat were found and a slight increase in protein concentration in multiparous cows was described [19]. Dohoo *et al.* concluded that these small effects had no practical consequences for the dairy industry [19].

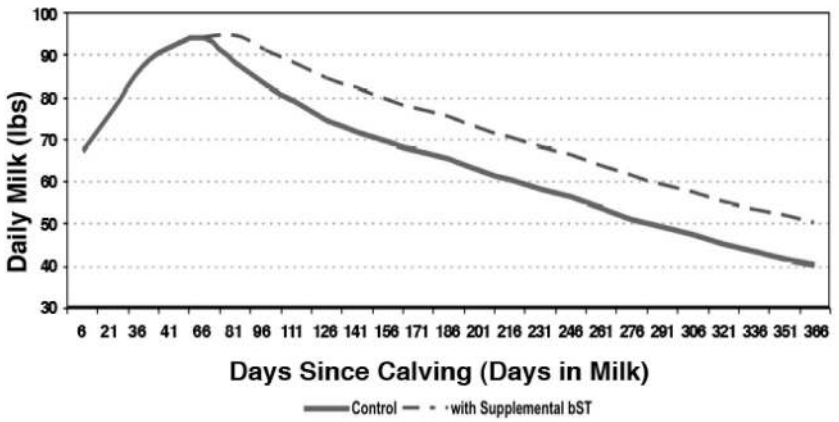

Figure 3: Typical lactation curves for control cows (solid line) and cows administered with rbST (dotted line) (obtained from Elanco website [21]).

Administration of rbST does not only increase milk yield but also has general effects on the cow's body and physiology. Similar to the physiologic actions of endogenous bST, rbST leads to elevated lipolysis and glycogenolysis to provide the body with energy and resources for the increased milk production and protein synthesis [2, 15]. The increased energy requirement also leads to an increased feed uptake in dairy cows of approximately 1.5 kg day^{-1} [19]. The increases in milk production are, however, not proportional to the increased feed intake, which means that rbST also causes an increased production efficiency (more kg milk per kg feed intake) [2].

Apart from the effects on milk production, rbST was also shown to have effects on bull

calves for beef production. Observed effects were increased protein and decreased fat content in muscle tissue and a general growth enhancement [22].

2.4 Controversial debate about rbST use in milk production

Ever since rbST has been placed on the market, there has been a public debate about its potential negative effects on human and animal health.
The concerns relating to human health mainly focus on rbST presence and potentially increased IGF-1 levels in milk. The rbST molecule is only 66 % identical with the human form of ST and non-primate STs have been shown to be non-active in humans regarding growth and anabolic actions [13, 21, 23-25]. However, Froesch *et al.* found effects of rbST on the carbohydrate metabolism in men, indicating a selective effect of the bovine hormone [25]. The amount of the administrated rbST was, however, much higher (150 mg per day) than what would be expected to be ingested by milk consumption [26]. Note that rbST levels in milk are in the ng mL^{-1} range. Additionally to that, it has been shown that rbST levels in milk are decreased by more than 85 % after pasteurisation and is further broken down in the human digestion system [27]. Therefore, it is not expected that rbST residues in milk exert quantifiable effects on human.
IGF-1 levels in milk were shown to be significantly increased after rbST administration [28] and the molecular structures of bovine and human IGF-1 are identical [29]. Based on studies performed on behalf of Elanco and Monsanto (rbST-producing companies), the FDA concluded that increased IGF-1 levels found in milk from rbST-treated cows are in the range of IGF-1 levels usually found during lactation of untreated cows [24]. A discussion paper criticized that this statement might be scientifically correct but misleading, as colostrum (milk produced during first days after calving comprising the highest IGF-1 concentrations during the lactation period [28]) is not used for the dairy market anyway [30]. Furthermore, IGF-1 was shown to have oral bioactivity in rats and effects on the gastrointestinal tract in piglets and calves [24, 31-33]. However, orally ingested IGF-1 was hardly taken up into the blood stream in piglets [34]. Despite these controversial findings, the Codex Committee on Residues of Veterinary Drugs in Foods (CCRVDF) defined the maximum residue limits and acceptable daily intake of rbST as ‘not specified’, which means that milk from rbST-treated cows is declared as being safe for human consumption. This opinion is shared by more than 50 countries including the EU [21].

However, there are many concerns relating to the effects of rbST on animal welfare. Even the accompanying package insert of rbST preparations provides information on possible side effects for the injected cow. These include reduced pregnancy rates, increased

risk of mastitis, injection site reactions, udder oedema and foot and leg disorders [35]. Furthermore, it is indicated that the incidence of antibiotic treatments might be increased to treat the mentioned disorders. A meta-analysis performed by Dohoo *et al.* summarized adverse health effects observed after rbST treatment in dairy cattle. They found a 25 % increased risk of clinical mastitis and 40 % increased risk of unsuccessful conception. Additionally to that, rbST-treated cows had a 55 % increased risk of lameness [36]. Also the EU published a "Report on Animal Welfare Aspects of the Use of Bovine Somatotrophin", in which similar effects are described [37]. Based on this report, rbST use has been banned in the EU because of animal health and welfare reasons [8].

Despite potential adverse health effects for the cows, rbST use is approved in the US and other countries. However, consumers were and are concerned about the use of artificial hormones in food production. Therefore, US dairy companies and retailers started labelling milk that has been produced without the use of rbST [38]. The 'rbST-free' labelling relies on a form signed by dairy farmers, wherewith they pledge not to use rbST. So far, since the approval of rbST in 1994, there is no method to control for correct labelling of milk and milk products.

3 Monitoring of milk quality and safety in the European Union and the Netherlands

According to Council Directive 96/23/EC, it is required to monitor unauthorized substances in animals used for food production or their products [39] and the technical requirements for the analytical methods used are described in Commission Decision 2002/657/EC [40]. The control of dairy products in the Netherlands is divided into two parts: the official governmental food safety control and the quality control by the producers.

The EU member states are obliged to perform residue analyses within the framework of the national residue control plan and this residue control plan should adequately assure food safety [40]. This means that efforts must be made to identify potential food safety risks, *i.e.,* the use of banned substances in food producing animals. In the Netherlands, this obligation is implemented by using a multi-level surveillance system. First, there are general inspections, which usually occur on farms directly. During these inspections, packaging or preparations of veterinary drugs might be found, which can indicate a violation of food safety regulations. Second, small surveillance programs are run for the monitoring of a specific drug residue or contaminant to identify a potential new food safety issue. At this stage, up to a few hundred samples are measured preferably using

a simple screening method. Results of such surveillance studies are also used to provide the European Food Safety Authority (EFSA) with data necessary for risk assessment. Member States of the EU have the obligation to assist EFSA with this task [41]. If suspicious samples are found during screening, then a confirmatory method is required to detect and quantify the residue or contaminant itself. Third, if a new food safety issue has been identified, the specific drug residue or contaminant is included in the national residue control plan. This national residue control plan is a yearly-defined program based on EU legislation, which is performed at official laboratories and the results have to be reported to the EU. According to Commission Decision 97/747/EC, the minimum amount of milk samples analysed should be 1 per 15,000 tons of the annual milk production, but at least 300 samples [42]. Milk is tested for substances that belong to group A6 (certain pharmacologically active substances) and several of the B sub-groups (*e.g.*, antibacterials, anthelmintics, non-steroidal anti-inflammatory drugs and environmental contaminants) [39]. In the Netherlands, approximately 1500 samples are analysed annually by the Food Authority [43]. Fourth, if non-compliant results occurred during the national residue plan, follow-up actions are undertaken at EU level or national level and specific policies might be developed to decrease the use of the veterinary drug or to better enforce regulations in the future. Furthermore, in case of a food crisis, large-scale testing is performed on all relevant matrices at all relevant locations.

Apart from the official food safety control, the Dutch dairy sector developed a comprehensive control system for safety and quality control to maintain its strong position as a milk-exporting country. This quality assurance system for raw milk comprises controls on farm level, on truck level as well as tank level at the dairy plant. These controls mainly focus on protein and fat content, somatic cell count, the presence of antibiotics residues and microbial flora. They are performed by a private central controlling station [44]. For instance, there are 2.3 million quality analyses performed each year in the Netherlands already during transport of the milk from the farm to the dairy plant [45].

For most of the described food safety analysis levels, screening methods are required, which are defined by the Commission Decision 2002/657/EC as “methods that are used to detect the presence of a substance or class of substances at the level of interest. These methods have the capability for a high sample throughput and are used to sift large numbers of samples for potential non-compliant results. They are specifically designed to avoid false compliant results.” [40]. After such a screening method has been developed, it needs to be validated, which means that it must perform according to specific performance criteria. The “Guidelines for the validation of screening methods for

residues of veterinary medicines" emphasised the key requirement for screening methods: if the analyte in question is present in the sample, the analytical method must identify the sample as screen-positive with a false-compliant rate of <5 % [46]. Minimum performance criteria for screening methods are in general recovery, specificity (discrimination between the analyte and closely related substances) and the above-mentioned false-compliant rate below 5 % [40].

Note that the validation of the analytical performance of the developed method needs to be discriminated from biomarker validation. Biomarker validation assesses the specificity of the biomarker for the applied treatment as described in **Chapter 2** in this thesis.

4 RbST detection strategies

For detection of rbST abuse, different approaches are possible and have been followed so far. Basically, these approaches can be classified in direct and indirect detection methods. Direct detection methods detect the presence of the rbST molecule itself and indirect detection methods detect the effect of rbST in the cow's body.

4.1 Direct detection methods

Direct detection methods can be divided in immunological methods, which employ specific antibodies, and mass spectrometric methods for detection of rbST in a sample. In general, rbST detection has always been challenging because of the similarities of the endogenous form and the recombinant form of the hormone and the expected concentrations in blood and milk in the ng mL^{-1} range [26].

4.1.1 Immunological methods

Immunological methods developed for rbST detection in biological samples employed two different antibodies specific for rbST [47]. A monoclonal capture antibody and a polyclonal detection antibody in a sandwich ELISA format yielded an EC_{50} of approximately 5 ng mL^{-1} and a limit of detection of 0.15 ng mL^{-1} [48]. Even though strongly increased rbST concentrations could be measured after rbST treatment, no discrimination between the endogenous and recombinant form of the hormone was possible [48]. Recently, polyclonal antibodies have been produced against the two N-terminal amino acids of rbST, which showed a higher affinity to rbST compared to the endogenous bST and these might be promising in future immunological detection approaches [49].

4.1.2 Mass spectrometric methods

Because of the differences in the N-terminal end of rbST and the endogenous bST (**Figure 1**), rbST can potentially be detected in the cow's circulation. A method based on LC-MS was developed, which allowed the detection and quantification of rbST in serum obtained from rbST-treated animals [26, 50]. RbST residues were detectable until 4 or 11 days after treatment, depending on the treatment protocol [12, 18]. However, the observed concentrations were below the limit of quantification of the developed method already three days after treatment. Moreover, for milk, rbST itself could only be detected at unrealistic high spiking levels and not in milk samples from rbST-treated animals [51]. Therefore, in the future, better extraction procedures and more sensitive confirmatory analysis methods shall be developed that enable direct rbST detection in biological samples.

4.2 Indirect detection methods

The principle of indirect detection methods is the analysis of the effect of rbST onto the cow's body; hence, rbST-dependent biomarkers are measured. A biomarker is defined as "a factor that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacological responses to a therapeutic intervention" [52]. These can include metabolomic biomarkers (*e.g.*, altered blood or urine parameters), transcriptomic biomarkers (*e.g.*, changed mRNA levels) and proteomic biomarkers (*e.g.*, increased or decreased protein levels).

The work described in this thesis focusses on the analysis of protein biomarkers of rbST treatment, thus up- or down-regulated levels of proteins in easily accessible biological matrices, such as blood and milk. A thorough consideration of protein biomarkers in sports doping and veterinary control is presented in **Chapter 2** of this thesis. Here, complementary explanations of the specific protein biomarkers analysed in this thesis work are given.

4.2.1 Insulin-like growth factor-1 (IGF-1)

IGF-1, also called somatomedin C, is a 7.6 kDa growth factor with high sequence similarities to insulin [53]. It is mainly produced by liver tissues and is involved in the growth and function of almost all organs. Its synthesis in liver and release into the blood stream are promoted by ST binding to its hepatic receptors (**Figure 2**). IGF-1 is found in

circulation bound to binding proteins, which modulate its actions. After treatment with rbST, IGF-1 levels were found to be increased in bovine serum and milk [28, 48]. Since IGF-1 is bound to binding proteins, it has to be released for quantification of total IGF-1 using immunoassays. In this thesis, a specific pretreatment including an acidification step was used for IGF-1 release, which has been published previously by our group [54].

4.2.2 IGF binding protein 2 (IGFBP2)

IGFBP2 is an approximately 30 kDa protein produced by hepatic tissues and binds IGF molecules in circulation in a binary complex [55]. Its affinity to IGF-2 is fourfold higher than to IGF-1. IGFBP2 levels are inversely correlated to ST levels [55]; IGF-1, however, increases IGFBP2 concentrations.

4.2.3 Osteocalcin

Osteocalcin, also called bone γ-carboxylglutamic acid-containing protein (BGLAP), is a biomarker of bone turnover [56]. This 5.8 kDa protein is produced by mature osteoblasts [57], is the most abundant non-collagenous protein in the bone matrix and its levels in blood were found to be influenced by hormone treatment, such as by ST [58, 59]. Thus, monitoring osteocalcin levels can give an indication whether rbST was administered.

4.2.4 Anti-rbST antibodies

After administration of rbST to dairy cows, an rbST-specific immunological response has been observed by the development of anti-rbST antibodies [60, 61]. These antibodies are only present after administration of rbST and are therefore very specific biomarkers for rbST use or abuse.

4.2.5 Other possible biomarkers for rbST treatment

As described above, ST has growth-promoting effects on many tissues including bone, muscle, soft tissue, and others. Therefore, also growth-related biomarkers from these tissues might be indicative for rbST treatment. Potential protein biomarkers shown to be ST-responsive in other mammalian species are N-terminal propeptide of procollagen III (PIIINP) [59, 62], C-terminal cross-linked telopeptide of collagen I (CTx) [59], acid-labile subunit (ALS) [63], myostatin [64], and insulin [65].

For all biomarkers, it has to be considered that they might be affected by other factors than the drug treatment itself and therefore, the candidate biomarkers need to be thoroughly validated for their capability to pinpoint drug abuse as discussed in **Chapter 2** of this thesis.

5 Analytical methods applied in this work

To be able to measure several protein biomarkers at the same time in a single sample, multiplex immunoassay methods were applied in this thesis. Mainly two different approaches were used, the first one is based on microspheres and the second one is based on a protein microarray, which both will be explained below.

5.1 Microsphere-based immunoassay methods

Three different microsphere-based immunoassay methods are used for the work in this thesis, namely a flow cytometric immunoassay (FCIA), a planar imaging array and a cellphone fluorescence immunoassay. For all methods, proteins are covalently immobilized on the surface of carboxylated microspheres by using similar chemical protocols. Then, similar sandwich or inhibition immunoassays are performed in a microsphere suspension. There, a detection antibody is employed that carries a fluorescence label for quantification. For the final fluorescence detection, different platforms namely a flow cytometer, a planar imaging array platform or a cellphone with a dedicated cellphone attachment, are used.

5.1.1 Microsphere coupling

Different microspheres were used for all the microsphere-based methods as outlined in **Table 1**. The colour-encoded microspheres are commercially produced by mixing red and infrared dyes in specific ratios, thereby creating more than 50 different microsphere sets with distinct colour codes. For the FCIA, four different microsphere sets were employed; one for each of the four different protein biomarkers. For the planar imaging array platform, only one microsphere set was used. When using paramagnetic microspheres, experimental handling is easier, because only a simple magnet is required to sediment the microspheres, whereas non-magnetic microspheres need to be centrifuged. Protein immobilization was done equally for all, using a two-step carbodiimide reaction employing N-hydroxysulfosuccinimide sodium salt (NHS) and N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC). In the first step, the carboxylic groups on the

microspheres were activated by EDC and NHS and in the second step, an amino group of the protein is reacting with the activated carboxylic group and an amide bond is created. The detailed experimental protocol can be found in the Materials and Methods sections of **Chapters 3 - 7**. During microsphere coupling, the protein biomarkers IGF-1, IGFBP2, osteocalcin or rbST were covalently attached to the surface of a specific set of microspheres respectively (left column in **Figure 4**).

Table 1: Overview of the flow cytometric immunoassay, the planar imaging array and the cellphone fluorescence immunoassay employed in this thesis.

	Flow cytometer-based analysis	**Planar imaging array-based analysis**	**Cellphone-based analysis**
Microspheres	Non-magnetic, carboxylated and colour-encoded (Ø 5.6 µm)	Paramagnetic, carboxylated and colour-encoded (Ø 5.6 µm)	Paramagnetic and carboxylated (Ø 8-10 µm)
Biomarkers simultaneously detected	4	1	1
Discrimination of microspheres	By internal colour code	By internal colour code	Not demonstrated
Microfluidics for microsphere separation	Flow channel in flow cytometer	Microspheres dispersed on a planar magnetic surface	Sample sandwiched between microscope cover slides
Fluorochrome	R-Phycoerythrin (excitation wavelength: 532 nm; emission detection: 565 – 585 nm)	R-Phycoerythrin (excitation wavelength: 511 nm; emission detected by CCD imager)	Quantum Dots (excitation wavelength: 380 nm; emission detection: >610 nm)

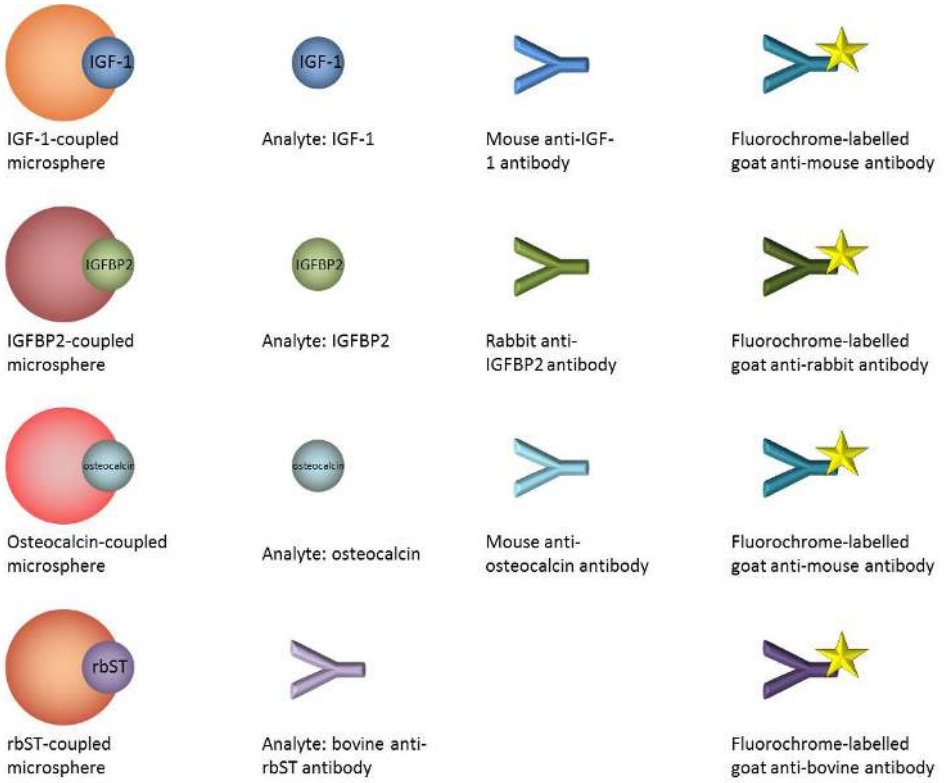

Figure 4: Assay formats for all protein biomarker (analyte) detection immunoassays. For IGF-1, IGFBP2 and osteocalcin, free analyte in the sample and the immobilized standard protein compete for antibody binding (inhibition format). The analyte anti-rbST antibody is sandwiched between rbST standard protein on the microsphere surface and the anti-bovine detection antibody.

5.1.2 Sandwich and inhibition immunoassay formats

Depending on the analysed protein biomarker, sandwich or inhibition immunoassay formats were used. Traditionally in the sandwich format, the biomarker is sandwiched in between two biomarker-specific antibodies. For the detection of anti-rbST antibodies (**Figure 4**), however, the biomarker anti-rbST antibodies was sandwiched in between rbST on the microsphere surface and the labelled goat anti-bovine detection antibody. In this case, the fluorescence signal coming from each microsphere is positively correlated with the biomarker in the sample (**Figure 5A**).

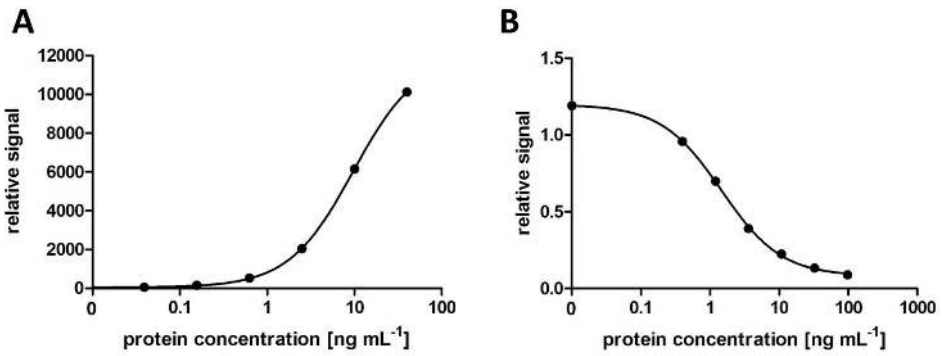

Figure 5: Exemplary standard curves for (A) the sandwich immunoassay format and (B) the inhibition immunoassay format.

For all the other biomarkers, IGF-1, IGFBP2, and osteocalcin, the inhibition format was chosen. In this format, a standard protein of the biomarker is covalently coupled to the microsphere surface (**Figure 4**). When the biomarker is present in the sample, the free biomarker and the biomarker standard on the microsphere surface compete for the biomarker-specific antibody used in the immunoassay. Consequently, the fluorescence signal coming from each microsphere is negatively correlated with the biomarker concentration in the sample (**Figure 5B**).

For both immunoassay formats, it is important to account for day-to-day variations, which can occur due to temperature shifts or other small interferences. For the sandwich immunoassay format, normalization can be done by dividing all obtained sample signals by the signal of a control sample or set of control samples, which are measured every time. For the inhibition immunoassay, all sample signals can be normalized to the signal obtained from the standard curve sample with zero protein concentration. Using this normalization strategy, inter-assay variations can be corrected.

5.1.3 Fluorescence detection

As mentioned above, three microsphere-based approaches are used in this thesis. The FCIA approach combines the simultaneous detection of four protein biomarkers; the planar imaging array and the cellphone microsphere fluorescence immunoassay are used to detect the biomarker anti-rbST antibodies (**Table 1**). In all approaches, it is important that the microspheres are separated from each other to enable the analysis of individual

microspheres.

For the FCIA, the Luminex technology is used, in which a flow cytometer is required to analyse the microspheres. In the flow channel of the flow cytometer, the microspheres pass by the lasers individually and are analysed one by one. The flow cytometer has two lasers; a red laser for identification of the microsphere colour code (*i.e.*, the assay type) and a green laser for quantification of the fluorescence on the microsphere surface (**Figure 6A**). In this Luminex approach, R-phycoerythrin (R-PE) is used as a fluorochrome, which is excited with a 532 nm laser and its fluorescence emission is detected in the range of 565 – 585 nm (**Figure 7**). The light signals are detected by a photo multiplier tube and shown as median fluorescence intensities (MFI).

For the planar imaging array, the MagPix instrument is used, in which the paramagnetic microspheres are dispersed on a magnetic planar surface (**Figure 6B**). The fluorescence in the microspheres is excited by a 621 nm LED. A CCD camera images the emission and from the image, the microspheres are located and classified by software. A second LED is used for exciting the fluorescence label; in this case also R-PE (**Figure 7**), on the microsphere surface at 511 nm. The fluorescence is quantified after imaging with the internal CCD camera.

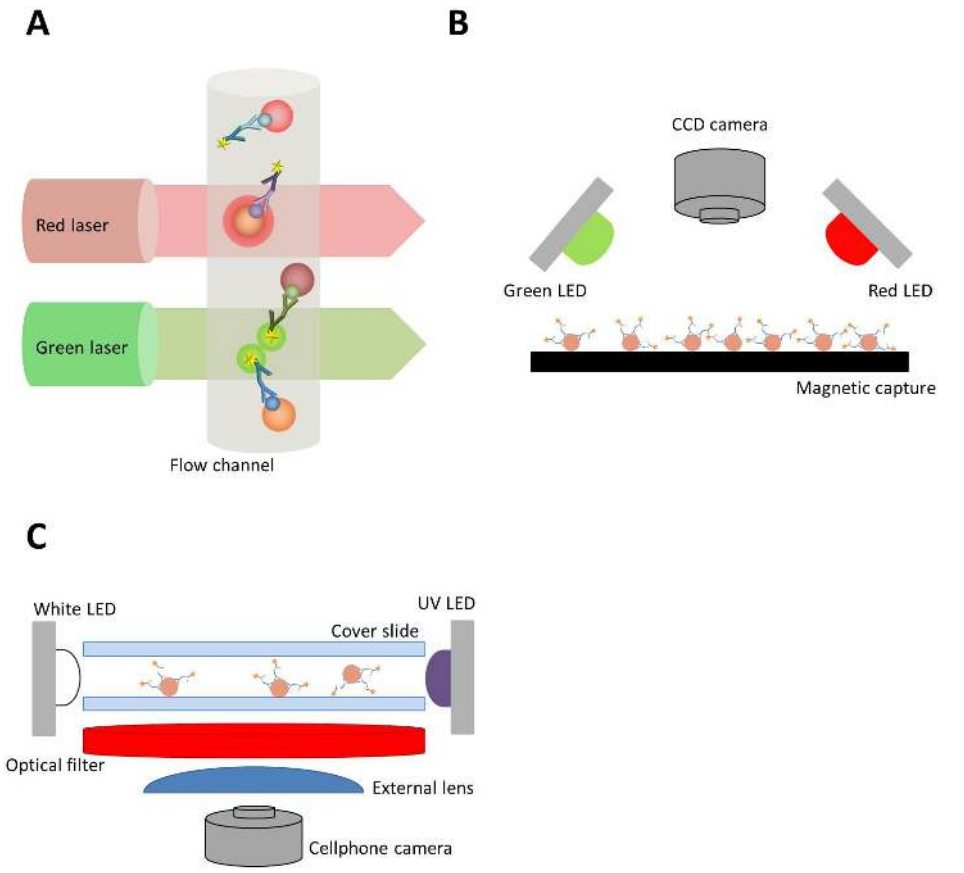

Figure 6: Detection setups of (A) the flow cytometric immunoassay, (B) the planar imaging array and (C) the cellphone fluorescence immunoassay.

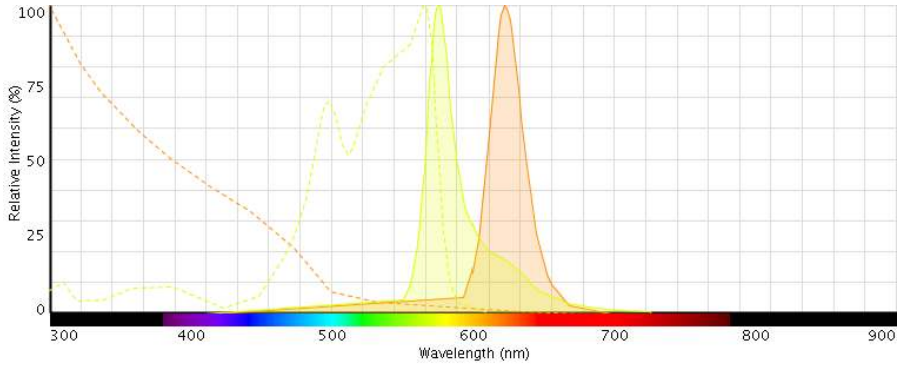

Figure 7: Excitation (dotted line) and emission (solid line) spectra of R-phycoerythrin (yellow) and Quantum Dots (orange). Obtained from www.lifetechnologies.com [66] .

In the cellphone fluorescence immunoassay approach, a dedicated cellphone attachment is required, which houses light-emitting diodes (LEDs) for excitation of the fluorochrome and an optical filter to only allow the fluorescence light to reach the cellphone camera. In this approach, Quantum Dots (QD) are used because of their preferable excitation and emission characteristics. The emplozed QDs are excited in the UV range of light and emit at 625 nm wavelength (**Figure 7**). Therefore, UV LEDs (380 nm) and a simple 610 nm long-pass filter are used to image the fluorescence light coming from the QDs on the microspheres (**Figure 6C**). To ensure that the microspheres are separated from each other, the microsphere suspension is sandwiched between two microscope cover slides. In this approach, all microspheres have the same colour and therefore, no discrimination based on a colour code is possible. That means that only a single biomarker can be analysed using the cellphone fluorescence immunoassay format. Multiplexing can be achieved in the future by using differently sized microspheres or colour-encoded microspheres. For the latter, different LEDs for excitation are required. For each excitation event, a separate image needs to be taken and analysed. Therefore, it has to be ensured that the microspheres remain at their specific location for microsphere identification and fluorescence quantification in a way that the fluorescence results can be assigned to a specific microsphere set.

5.2 Microarray-based immunoassay approach

The second immunoassay approach used in this thesis does not employ microspheres, but is based on a protein microarray. In a microarray, each spot is assigned to a specific protein to be analysed and because of the known fixed spot locations, multiplexing is

straightforward. Another advantage is that on-chip positive and negative controls can be incorporated to correct the fluorescence signals for background and day-to-day variations. For biomarker detection, proteins are covalently coupled to activated glass slides carrying carboxylic groups using the same chemistry as described before (**sub-section 5.1.1**). In this microarray approach, only sandwich immunoassays are performed. The developed protein microarray immunoassay is analysed using the same cellphone and cellphone attachment, and therefore, the same QDs were used as fluorochrome. Two different rbST-dependent biomarkers, IGF-1 and anti-rbST antibodies, are simultaneoulsy analysed using the microarray platform.

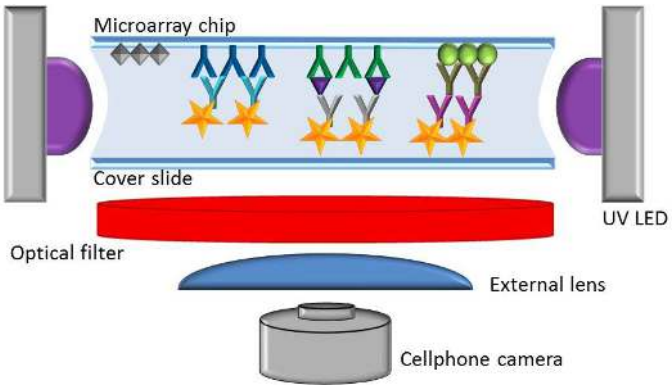

Figure 8: Detection setup of the protein microarray immunoassay approach.

6 Scope and thesis outline

The main purpose of this thesis is the development of protein biomarker-based techniques for the detection of rbST administration to dairy cattle. Existing rbST detection methods mainly lack sensitivity, reproducibility or selectivity for rbST and are therefore not widely applied. Even though rbST use has been banned in the EU since 2000, no method has been implemented so far to monitor its abuse in dairy farming. Therefore, this thesis focuses on an alternative approach using rbST-dependent protein biomarkers. For this end, laboratory-based and on-site testing platforms for serum and milk are developed to meet the needs of current testing procedures. In **Chapter 2**, a thorough review about protein biomarker-based methods used for sports doping and in veterinary control is given to provide the required background on protein biomarker-based approaches, their development and validation criteria.

The experimental chapters of this thesis are divided in two parts based on the type of

sample, which was analysed: the first part focuses on blood biomarker profiling including serum and plasma samples and the second part focuses on milk biomarker profiling.

For the first part of the thesis (blood biomarker profiling), the development of a multiplex assay combining the detection of three different rbST-dependent biomarkers is described in **Chapter 3**. The biomarkers analysed were IGF-1, IGFBP2 and anti-rbST antibodies. This triplex FCIA was applied to serum samples from an rbST treatment animal study to evaluate its capabilities to identify rbST abuse. In order to increase the rbST detection capabilities of the existing triplex FCIA, an additional biomarker (osteocalcin) was added to the biomarker panel and this resulting fourplex FCIA was applied to serum samples from two different rbST treatment animal studies. In **Chapter 4**, the performance of each of the individual biomarkers in the fourplex FCIA is assessed. Then, all possible biomarker combinations are evaluated using an advanced statistical model aiming at identifying as many as possible rbST-treated cows and discriminating them from the untreated cows. As protein biomarkers might be indicative for different treatments, the developed fourplex FCIA was also applied for biomarker profiling in plasma samples obtained from steroid-treated cattle. The resulting treatment-specific biomarker profiles are discussed in **Chapter 5**. Since all the methods described in **Chapters 3 – 5** require a flow cytometer, these methods can only be performed in a laboratory.

For the second part of the thesis, milk samples from rbST-treated and untreated cows were used for analysis. **Chapter 6** describes the assay development for the detection of the biomarker anti-rbST antibodies in raw milk, pasteurized milk and tank milk samples using an FCIA approach. This method is also bound to a laboratory, but on-site testing platforms are preferable for on-farm analysis. Therefore, the development of a cellphone microsphere fluorescence immunoassay for anti-rbST antibodies is described in **Chapter 7**. Using this cellphone platform, rbST-treated and untreated cows can be discriminated based on their anti-rbST antibody presence in raw milk. All methods described in **Chapters 3 – 7** are based on immunoassays on the surface of microspheres. In **Chapter 8**, a protein microarray approach for multiplex protein biomarker detection is presented, with which two different rbST-dependent biomarkers are analysed in milk samples.

The research presented in this thesis is thoroughly discussed in **Chapter 9**. Thereby, specific topics regarding this thesis, general scientific implications of the work performed and its societal impact are considered. Moreover, an outlook is given and potential future developments are described.

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2

Screening of protein biomarkers in sports doping and veterinary control

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Abstract

There are several similarities between sports doping and veterinary control. Prohibited substances, *e.g.* anabolic agents and peptide hormones, are similar and analytical methods applied are immunoassays and chromatography mass spectrometry in both worlds. In recent years, protein biomarker-based detection strategies were successfully developed and adopted in sports control. When measuring biomarkers, the window of detection can be extended due to a prolonged biological response. Thus, a whole range of substances may be tackled in an indirect manner. In view of the similarities in intended biological effects, such as increased muscle mass, it is envisaged that biomarker-based detection may be adopted in future veterinary control. In this review, protein biomarker-based detection strategies are discussed against generic challenges in biomarker discovery and method development. The lessons learnt from successfully implemented biomarker strategies into doping regulations, advocate adoption in the future veterinary world and revision of the current restrictive regulations concerning analytical methods.

Abbreviations

2D-DIGE	two-dimensional differential gel electrophoresis
2D-GE	two-dimensional gel electrophoresis
ALS	acid-labile subunit
CERA	continuous erythropoietin receptor activators
EASIA	enzyme-amplified sensitivity immunoassay
ELISA	enzyme-linked immunosorbent assay
Epo	erythropoietin
eST	equine somatotropin
FCIA	flow cytometric immunoassay
GC	gas chromatography
GH	growth hormone
GHRH	GH releasing hormone
GHRP	GH releasing peptide
ICMA	immunochemiluminescence assay
ICTP	C-terminal telopeptide of type I collagen
IGF	insulin-like growth factor
IGFBP	IGF binding protein
kDa	kilo Dalton
LC	liquid chromatography
MALDI	matrix-assisted laser desorption ionisation
MS	mass spectrometry
ORF	open reading frame
PCR	polymerase chain reaction
PICP	procollagen type I C-terminal propeptide
PIIINP	N-terminal propeptide of procollagen type III
rbST	recombinant bovine somatotropin
rhGH	recombinant human GH
r-HuEPO	recombinant human Epo
RIA	radio immunoassay
rpST	recombinant porcine somatotropin
SHBG	sex hormone-binding globulin
SPR	surface plasmon resonance
SRM	single reaction monitoring
TOF	time-of-flight
uHPLC	ultra-high-performance liquid chromatography

VEGF vascular endothelial growth factor
WADA World Anti-Doping Agency

1 Introduction

Increasing muscle mass and enhancing performance or productivity are common aims of athletes and in food production. It is well known that muscle growth is facilitated by the use of certain prohibited substances. Therefore, it is not surprising that during and after major sports events, a substantial number of athletes is accused of doping and also during routine veterinary controls in food production, the presence of prohibited growth promoters is detected. For both, in sports and food production, the use of similar substances is banned, such as anabolic agents (*e.g.,* exogenous and endogenous steroid hormones and clenbuterol), peptide hormones and growth factors (*e.g.,* growth hormone) [1, 2]; hence similar monitoring and detection methods can be used for their detection.

The best proof of drug abuse is obtained if the abused substance itself is found in the athlete's or animal's body. Numerous methods for their direct detection are available for a multitude of different possibly abused substances. These conventional methods are mainly based on chromatographic separation, such as gas chromatography (GC) or liquid chromatography (LC), followed by mass spectrometric (MS) or tandem mass spectrometric (MS/MS) detection of target ions or ion transitions [3, 4]. Also ligand binding assays, such as the enzyme-linked immunosorbent assay (ELISA) can be used for their direct detection [5]. Furthermore, bioassays detecting compounds with androgenic or anti-androgenic activity in urine may be applied [6].

As an alternative to these conventional direct detection methods, indirect analysis approaches can be used. One approach is monitoring certain biomarkers in the body, the levels of which are specifically increased or decreased after administration of a specific active substance. Thus, the biological effects of an illegal substance are measured and therewith, an indirect proof of doping can be delivered. Such an approach has several advantages over the classical direct detection:

(1) Usually, the biological effect of a substance lasts longer than the presence of the substance itself in body fluids and therewith, the window of detection of doping is expanded.
(2) It can be expected that different substances for growth promotion exert similar effects onto the body and therefore, biomarker-based detection methods have the potential to detect a whole class of substances, including designer substances with unknown chemical structure and synthetic versions of natural hormones.
(3) Low-dose mixtures of different banned substances, which might escape from

direct detection of each individual substance used, could be still detected by the combined effect they exert [7].

Biomarkers, which can be used for the described effect monitoring can be either mRNA, metabolites or proteins, which can be analysed using transcriptomic, metabolomic and proteomic techniques, respectively [7-10].

This review, covering the period from 1997 until 2013, focuses on protein and peptide biomarkers that have been and can be used for the detection of abused drugs in sports and veterinary control. Consequently, only endogenous peptides and proteins are considered, the levels of which are specifically changed after drug administration. The described biomarker-based approaches are not limited to the detection of substances enhancing muscle growth, but also include substances improving performance in athletes and milk production in cattle.

In **Section 2**, the protein biomarker discovery pipeline in sports doping and veterinary control is outlined. Some of the protein biomarker-based methods, described in detail in **Section 3**, have already been implemented in routine doping control. In contrast, so far, no protein biomarker-based method has been successfully implemented for veterinary control, because current regulation has not adopted biomarker-based detection approaches yet. Nevertheless, lessons learnt during the biomarker identification process for doping control may support and stimulate the development and acceptance of protein biomarker-based detection methods in future veterinary control programmes, which is discussed in **Section 4**.

2 Techniques used in the development of protein biomarker-based methods

2.1 Biomarker-based method development process

For the development of biomarker-based methods in sports doping and veterinary control, several phases have to be successfully completed before final implementation of the biomarker-based tests is possible (**Figure 1**).

The first phase in the development of biomarker-based methods is the discovery phase, in which candidate biomarkers for substance abuse are identified by either untargeted

or targeted proteomic approaches. In general, well-controlled treatment studies are performed to obtain samples comprising a limited biological variation. Samples from a treated and an untreated group are compared to create candidate biomarker lists. Unlike in clinical biomarker studies, where also cell culture and animal models can be used during the discovery phase, in doping and veterinary control, biofluids or tissues are analysed, which are also the target sample matrix in the final assay. In any case, easily accessible samples are chosen for analysis, which are mainly urine and blood samples in sports doping control and urine, blood and tissue samples, such as muscle or liver, in veterinary control.

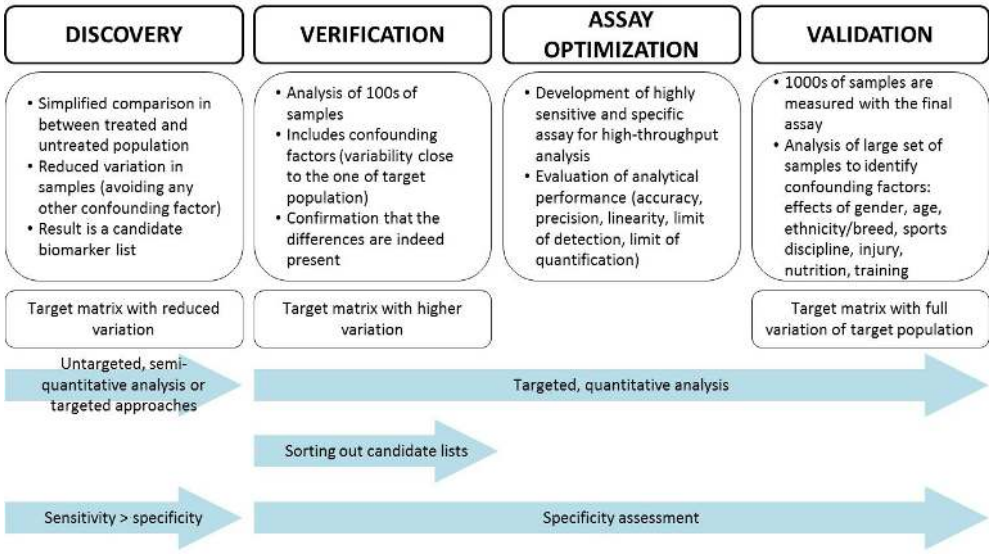

Figure 1: Overview of the protein biomarker discovery, verification and validation process in sports doping and veterinary control.

After completion of the first phase, a separate qualification phase, as done in clinical biomarker studies, is often not necessary in doping and veterinary control. The clinical qualification phase is required for two reasons [11]: First, if cell culture or animal model samples were used during the discovery phase, the discovered biomarkers have to be confirmed in the final sample matrix. Second, an analytical method, which will be subsequently used during the following verification phase, is introduced and evaluated. In contrast, for doping and veterinary control, the final sample matrix was used already during the discovery phase and if necessary, the alternative analytical method is evaluated

in combination with the subsequent verification phase.

During the verification phase, targeted proteomic biomarker analysis techniques are used to confirm the previous findings and to assess the specificity of the candidate biomarkers. From this point, methods are necessary that allow high-throughput measurements of large numbers of samples. Using the untargeted biomarker discovery techniques (**Section 2.2.1**) for rapid screening is not feasible, because of their complex and elaborate nature. Targeted approaches (**Section 2.2.2**), however, are easier and faster and are mainly applied during biomarker verification, assay optimization and validation phases.

During the assay optimization and validation phases, the targeted biomarker detection method is tested on a large set of samples from treated and untreated individuals to assess the biological variation of the biomarkers in a reference population, thereby including as many as possible confounding factors (**Figure 1**). In sports doping control, this usually includes factors such as gender, age, ethnicity, and effects of sports discipline, injury, nutrition and training [12], whereas in veterinary control, these factors would include at least age, gender, breed and feeding regime.

Going from one phase to the next, the number of candidate biomarkers decreases, whereas the required number of samples increases from a few tens to many thousands to reach sufficient statistical evidence for the biomarker [11].

Since the final biomarker-based screening methods often comprise the detection of more than one biomarker, suitable statistical methods are necessary to evaluate the data obtained. For instance for the detection of growth hormone (GH) abuse in athletes, discriminant functions were used to combine the results of two biomarkers and correct their levels according to age and gender [12]. A different approach was chosen for recombinant bovine somatotropin (rbST) abuse detection in cows where the statistical model *k*-nearest neighbours was used to discriminate between rbST-treated and untreated animals [13]. For discrimination of heifers treated with growth-promoting agents and untreated heifers, support vector machines were used to combine the results of 20 measured clinical biomarkers [14].

According to the current 2002/657/EC regulation for veterinary control, an additional phase is necessary upon non-compliant screening results [15]: the abused substance itself has to be confirmed by chromatographic and mass spectrometric methods, thus currently, biomarker-based methods can only be used for the initial screening stage. However, it is

envisaged that eventually biomarker-based approaches will be adopted in the veterinary field as previously done in sports doping and horse racing regulations.

2.2 Approaches for biomarker method development

2.2.1 Untargeted biomarker discovery methods

For a completely unbiased discovery of proteins as candidate biomarkers, untargeted proteomics is the method of choice. The total protein composition in samples from treated and untreated individuals is analysed semi-quantitatively and compared to find significant differences in concentrations of individual proteins, which then may be considered as candidate biomarkers. The unbiased discovery is very meaningful, because also unexpected candidate biomarkers can be found.

The main sample matrix chosen for biomarker studies is blood (**Table 1**). It should be considered that the human plasma proteome covers a wide concentration range from almost 200 mg mL^{-1} down to a few pg mL^{-1} [16]. To be able to also identify and analyse low-abundant proteins, not only highly sensitive methods have to be applied, also good depletion strategies for the high-abundant proteins or selective enrichment methods for the low-abundant proteins are necessary.

Depleting serum from the most abundant proteins can be done by precipitation, ultracentrifugation or commercially available immuno-depletion columns, but it should be noted that depletion will also remove other than the abundant proteins non-specifically [44, 45]. Selective enrichment protocols, on the other hand, are used to extract a specific protein subfraction out of the complex matrix. In that way, glycoproteins can be affinity-enriched by binding to lectins or low-abundant serum proteins can be enriched by protein capture to hexapeptide library columns and therewith, the entire dynamic range of serum samples can be greatly reduced [46, 47]. Also here, a certain bias is introduced since only a very specific subfraction of the proteome will remain for the following analysis and thereby other candidates are possibly excluded at this early step. As a result of depletion or enrichment, however, sample complexity is obviously reduced and therewith the detection of low-abundant proteins by untargeted techniques becomes possible.

2.2.1.1 Two-dimensional fluorescence differential gel electrophoresis

Two-dimensional fluorescence differential gel electrophoresis (2D-DIGE) has been frequently applied in biomarker discovery phases for sports doping and veterinary control [20, 21, 23, 31, 37]. Here, protein compositions of two samples are relatively compared by labelling each sample with a specific fluorescent dye. Before protein separation on a 2D electrophoresis gel, the dyed samples are mixed. The relative abundance of proteins in the samples can be compared by fluorescence gel imaging using light with dye-specific excitation wavelengths [45, 48, 49]. For gel-to-gel comparisons, an internal standard, which was labelled with an additional fluorescent dye, has to be included. After assessment of the significantly different protein spots, the spots are excised, an in-gel protein digestion is performed and the proteins are identified using liquid chromatography coupled to mass spectrometry (LC-MS). Whereas 2D-DIGE really visualizes differences in composition between protein samples, its performance is limited since only three different specific dyes are available and consequently, only two samples can be compared on one gel if an internal standard is necessary. Furthermore, only after removal of interfering high-abundant proteins, either by depletion or selective enrichment of the target proteins, sufficient sensitivity can be achieved for detection of very low-abundant proteins [45].

2.2.1.2 Quantitative mass spectrometric techniques

Instead of using 2D-DIGE for relative comparison of protein compositions, also quantitative MS can be directly applied. Several techniques for absolute and relative protein quantification have been described, which can be classified into isotope label-based and label-free techniques [50]. Despite the fact that untargeted quantitative MS techniques offer a great potential in unbiased identification of protein biomarkers, its application in sports doping control is limited to a very few studies [30].

2.2.1.3 Antibody microarrays

As an alternative, also affinity-based strategies can be followed. In this field so far, untargeted biomarker discovery was not possible due to the limited number of highly specific antibodies. The Human Protein Atlas, however, aims at production of recombinant proteins deriving from all open reading frames (ORF) of the human genome and the production of specific antibodies against all these proteins. So far, more than 10,000 antibodies have been produced within this project, which makes the production of high-density antibody microarrays possible [51, 52]. Because of the high specificity of

the immobilized antibodies, only minimal sample pretreatment would be required. Since during biomarker discovery only a very limited number of samples is tested, the sample throughput of the antibody microarrays is adequate for the purpose. Simplified antibody microarrays, comprising a limited number of specific antibodies, or reverse-phase protein microarrays can then be used during the verification and validation phases or as a targeted biomarker discovery approach (**Section 2.2.2.2**).

2.2.2 Targeted biomarker methods

As an alternative or complementary approach to untargeted biomarker discovery, a more targeted approach can be chosen. This is advantageous if information about the expected outcome is already available, for instance from literature search. Moreover, for the biomarker verification and validation phases, targeted biomarker detection methods, such as affinity-based assay strategies and targeted MS analysis, are highly desired due to their high-throughput potential.

2.2.2.1 Biological pathway investigation

For targeted biomarker discovery, the biological pathway of the administered hormone is analysed by screening available literature for proteins that are up-regulated or down-regulated [7]. As an example, if one is interested in protein biomarkers of growth hormone (GH) administration, the first choice is to investigate candidate biomarkers, which are directly or indirectly regulated by GH. These include mediators of GH action, as well as specifically regulated proteins of the target tissues, where the effect takes place (such as liver, bone and soft tissue) as well as proteins from the endocrine feedback loops (**Figure 2**). Note that the regulation of protein synthesis is tissue-specific and not all candidate biomarkers will show altered concentrations in all target tissues. Therefore, care has to be taken in the choice of the analysed biofluid or tissue, which should, on one hand, be easily accessible for sampling and, on the other hand, show strong biomarker responses after treatment.

The biological pathway investigation has been frequently applied for biomarker searches in sports doping, as for instance for GH biomarker discovery [12, 26-29], and in veterinary control for somatotropin (ST) biomarker discovery [33-35].

Table 1: Overview of abused substances, their candidate biomarkers, which were identified using a specific detection method, and the reported studies, which were performed to validate the candidate biomarkers.

Substance class	Abused substance	Analysed in	Biomarkers	Detection method	Biological validation	Ref.
Steroids	Testosterone	Human blood	Inhibin B	ELISA	15 male body builders, whereof 8 used testosterone	[17]
		Equine blood	Clusterin and leucine-rich α-2-glycoprotein	Strong cation exchange fractionation followed by LC-MS	Two horses undergoing testosterone treatment Forty untreated reference population horses Two horses for longitudinal biomarker study	[18]
	Nortestosterone/ estradiol cocktail	Bovine blood	SHBG, IGF-BP3, PIIINP, ir-inhibin, myostatin	DHT binding assay (SHBG), Western Blot (IGFBP3) and immunoassays	12 male and 12 female calves, whereof 6 each untreated controls	[7]
	Estradiol	Bovine blood	IGFBP2	Multiplexed FCIA	6 estradiol-treated male beef cattle and 6 untreated controls	[19]
	Prednisolone	Bovine blood	IGF-1	Multiplexed FCIA	6 prednisolone-treated male beef cattle and 6 untreated controls	[19]
	Dexamethasone	Bovine blood	PIIINP, osteocalcin, IGF-1, IGFBP2	Multiplexed FCIA (osteocalcin, IGF-1 and IGFBP2), immunoassay (PIIINP, osteocalcin)	6 dexamethasone-treated male beef cattle and 6 untreated controls (IGF-1, IGFBP2, osteocalcin) 12 male and 12 female calves, whereof 6 each untreated controls (PIIINP, osteocalcin)	[7, 19]
	Boldenone and boldione	Bovine blood	Apolipoprotein A1	2D-GE and MALDI-TOF and µLC-ESI-IT-MS	5 calves sampled before and after boldenone/boldione A treatment	[20]
Other anabolic agents	Clenbuterol/ dexamethasone/ estradiol combination	Bovine hepatic cytosols, microsomes	Adenosine kinase, reticuloalbin	2D-GE followed by LC-MS	6 GPA combination-treated calves and 6 untreated calves	[21]

Substance class	Abused substance	Analysed in	Biomarkers	Detection method	Biological validation	Ref.
Erythropoiesis-stimulating substances	Recombinant human erythropoietin (rhEpo)	Human blood	Hematocrit, serum Epo concentration, soluble transferrin receptor concentration, reticulocyte hematocrit and % macrocytes	Flow cytometric analysis (erythrocyte and reticulocyte parameters), ELISA (Epo and soluble transferrin receptor)	Epo treatment study including 27 healthy athletes	[22]
			Specific isoforms of haptoglobin and transferrin	2D-GE and MALDI–TOF	RhEpo treatment study including 8 healthy men	[23]
		Equine blood	Hemoglobin concentration	na	RhEpo treatment study including 8 horses	[24]
			Anti-Epo antibodies	Protein microarray	Rabbit anti-Epo antibody used as standard for proof of principle	[25]
Growth hormone, its releasing factors and insulin-like growth factor-1	Recombinant human GH (rhGH)	Human blood	Different GH isoforms	Differential immunoassays	Tested in 155 serum samples from endogenous GH stimulation or exogenous rhGH administration Ring test with 27 blinded samples analysed in several laboratories	[12, 26]
			IGF-1/PIIINP combination	RIAs	Several studies with large number of treated and untreated individuals Confounding factors, such as age, sex, ethnicity, injury, sports discipline tested Validation of statistical analysis with independent data set	[12]
			IGF-1/IGFBP2 ratio and IGFBP2/IGFBP3 ratio	RIAs	Tested in 8 healthy men	[27]

Substance class	Abused substance	Analysed in	Biomarkers	Detection method	Biological validation	Ref.
			IGF-1/ PIIINP/IGFBP3 combination	Competitive fluorescence immunoassay (IGF-1), RIA (PIIINP), ELISA (IGFBP3)	Blind placebo-controlled study in 15 male athletes Validation of statistical analysis with independent data set	[28]
			IGF-1/ICTP combination	RIAs	Tested in 96 male and female recreational athletes Co-administration of testosterone tested	[29]
			Hemoglobin α-chain	SELDI-TOF	Identified in 120 serum samples obtained from the GH-2000 study	[30]
			Specific isoforms of α-1 antitrypsin and transthyretin, apolipoprotein A-1, hemoglobin β-chain	2D-GE and MALDI-TOF	rhGH and placebo administration to eight male individuals in a randomized cross-over study	[31]
			IGF-1 and leucine-rich α-2-glycoprotein	uHPLC-MS	22 serum samples from 2 rhGH treatment studies	[32]
	Equine ST (eST)	Equine blood	IGF-1, IGFBP3	RIAs	Tested in 4 eST-, 4 bST- and 4 pST-treated horses Confounding factors, such as training, age and sex on IGF-1 analysed	[33]
			Anti-eST antibodies	ELISA and SPR-BIA	Biomarker identified in two eST-treated horses	[33]
	Recombinant bovine ST (rbST)	Bovine blood	Anti-rbST antibodies/ osteocalcin combination	Multiplexed FCIA	Tested in 11 treated and 5 placebo-treated cows Confounding factor age considered by testing reference population	[13]
		Bovine milk	Anti-rbST antibodies	FCIA	Tested in 11 treated and 5 placebo-treated cows	[34]

Substance class	Abused substance	Analysed in	Biomarkers	Detection method	Biological validation	Ref.
		Trout blood	IGF-1, anti-rbST antibodies	Immunoassay and Western Blot	Four studies including 285 fish in different treatment groups	[35]
	CJC-1295	Human blood	GH, IGF-1	RIA (IGF-1), ICMA (GH)	20 healthy CJC-1295-treated men	[36]
			Apolipoprotein A1, transthyretin isoform, β-hemoglobin	2D-GE and MALDI–TOF	Biomarker identified in 11 healthy CJC-1295-treated men	[37]
	GHRP-2	Human blood	GH, IGF-1 and IGFBP3	Chemiluminescent assay (GH), RIA (IGF-1, IGFBP3)	Identified during a clinical study	[38]
	IGF-1/ IGFBP3 cocktail	Human blood	IGF-2, IGFBP2, ALS	Immunoassays	26 female and 30 male recreational athletes treated with IGF-1/IGFBP3 combination or placebo	[39]
Metabolic modulators	Insulin	Human blood	Insulin/C-peptide ratio	EASIAs	Untreated reference population study including elite and recreational athletes and sedentary individuals	[40]
		Human urine	Urinary insulin degradation product profiles	Immunoaffinity chromatography and LC-MS	Urine samples from healthy and diabetic individuals	[41]
Gene doping	Myostatin inhibitor gene doping	Human blood and muscle	Follistatin/ myostatin propeptide ratio	Immuno-PCR	22 healthy male individuals of different training status	[42]
	GH gene doping	Salmon muscle	Myostatin protein levels	Western Blot	8 transgenic salmon and 8 wildtype salmon	[43]

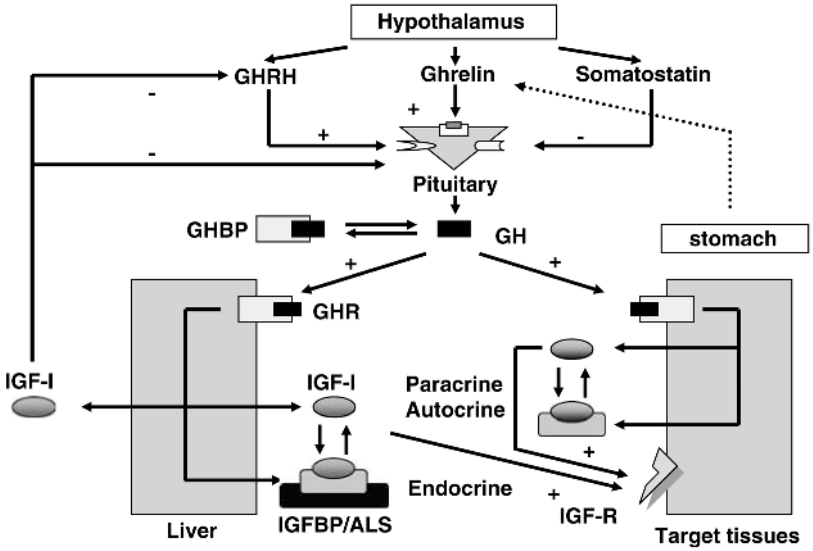

Figure 2: GH regulatory circuit including feed-back loops (reproduced with permission from Holt *et al.* [53])

2.2.2.2 Ligand-binding assay techniques

For targeted biomarker discovery studies and for biomarker verification and validation, ligand-binding techniques, such as immunoassays, receptor assays, transport protein assays and aptamer assays, can be used. Ligand-binding assays have been very frequently applied in sports doping and veterinary control (**Table 1**) [7, 12, 13, 17, 19, 22, 24-29, 33-36, 38-40], because of their simple operation and great high-throughput characteristics as compared to untargeted proteomic approaches. Here, only a protein subset of the entire proteome is analysed in samples from treated and untreated individuals. The successful development of ligand-binding assay techniques always depends on the availability of key bioreagents such as standard proteins and target-specific antibodies, receptors or aptamers of good quality. Several different affinity-based assay techniques are available; the classical immunoassay setup, employing specific antibodies against the target proteins, remains the most commonly used format.

Single-analyte immunoassay formats

Traditionally in diagnostics as well as for targeted biomarker approaches, single-analyte assay formats are used for high-throughput testing. These are mainly radioimmunoassays

(RIA) and ELISAs, which typically employ two specific antibodies against the target protein in a sandwich format. In RIAs, a radiolabelled secondary antibody or standard protein, which competes with the target protein for binding, is used for analysis in a gamma counter and in ELISA, an enzyme, which is coupled to the secondary antibody, causes a colour change of the added substrate, which can be read using a spectrophotometer. Both formats are highly sensitive and can also detect low-abundant serum proteins in the pg mL^{-1} range, but the ELISA format is preferably used because it does not employ radiolabels. Single-analyte formats are very suitable for the late validation phase, when the candidate biomarker list is already reduced to a single or only a few individual biomarkers. This format has been extensively applied across many biomarker studies in sports doping and veterinary control [7, 12, 17, 22, 26-29, 33, 35, 36, 38-40].

Multiple-analyte immunoassay formats

Multiple-analyte formats offer the possibility for combining the measurement of many biomarkers into one assay, which reduces reagent costs and saves sample volume and work load [54]. Usually, they employ specific antibodies against the target proteins and have a similar assay principle as the single-analyte platforms. Several setups are available for the simultaneous detection of several biomarkers in one sample.

For multiplexing, either suspension bead assays, surface-plasmon resonance (SPR)-based biosensors or planar antibody microarrays have been developed [54]. Sets of colour-coded beads can be coupled with specific antibodies and a traditional sandwich ELISA can be performed in suspension. Since a specific antibody is coupled to each bead set, the number of available bead sets and the cross talk between biointeractions define the theoretical limit of how many target proteins can be detected simultaneously. For quantification, the secondary antibody is labelled with a fluorescent protein, which can be measured in a specific flow cytometer employing two different lasers (flow cytometric immunoassay; FCIA). One laser is used to identify the bead set by its colour code, thus the target protein identity, and the other laser is used to quantify the amount of the fluorescent label, thus the target protein abundance in the sample. The FCIA technology has been applied in biomarker-based studies for rbST and steroid hormone abuse detection in veterinary control [13, 19, 34]. SPR-based biosensors detect protein interactions on the modified surface of a gold-coated glass prism by changes in the angle of the reflection minimum. Therewith, the amount of target protein binding to the specific antibody can be quantified without the use of any label. Since many antibody spots can be coupled to a single SPR chip, simultaneous quantification of up to hundreds of proteins is possible [55]. SPR-based approaches were used for protein biomarkers of equine ST (eST)

[33]. A simplified version of the already described antibody microarray (**Section 2.2.1.3**), only comprising the specific antibodies for the target proteins, can be used as a targeted multiplex protein quantification method and has been applied for biomarker analysis after erythropoietin (Epo) doping in racing horses [25].

Reverse protein microarrays

For the verification and validation phases, high sample throughput is required to confirm that the discovered candidate biomarkers can still discriminate treated from untreated individuals in a diverse population. Reverse protein microarrays are designed to measure the concentration of one biomarker in hundreds to thousands of samples simultaneously [52]. Samples (body fluids or tissue lysates) are printed onto a nitrocellulose membrane-coated array chip and the target biomarker is quantified by a specific antibody. The advantage of this technology is that it only requires a minimal amount of sample and that all samples can be tested simultaneously and therewith, technical interassay variability is greatly reduced.

2.2.2.3 Targeted mass spectrometric analysis

As an alternative to high throughput ligand binding assays, targeted MS methods can be applied in biomarker discovery, verification and validation. Here, stable isotope-labelled internal standards for the target protein or peptide are used for protein quantification and comparison across many samples [8]. A tandem mass spectrometer operated in the selected reaction monitoring (SRM) mode selectively analyses and quantifies preselected ion transitions from the target candidate biomarkers [32]. This technique has been often applied for biomarker quantification in sports doping and veterinary control [18, 32, 41].

3 Biomarker-based analysis methods for banned substances in doping and veterinary control

For increasing muscle mass as well as enhancing performance in sports and productivity in food production, similar illegal substances are abused, such as anabolic agents, peptide hormones and growth factors. In both fields, protein biomarker-based approaches were followed to detect drug abuse and similar biomarkers were found to be indicative for the same abused substance (**Table 1**). As explained in **Figure 1**, several phases have to be completed for development of a biomarker-based method for drug abuse detection in sports doping and veterinary control. In all biomarker studies, that are introduced in this chapter, candidate biomarkers were successfully discovered, but only a limited number of

these studies followed up with the verification, optimization and validation phases.

3.1 Anabolic agents

Growth-promoting agents, such as anabolic steroids, are banned in competitive sports and in food production in several countries. Control authorities often face the problem of short detection windows for measuring the abused substance itself. Also mixtures of different substances, so called low-dose-cocktails are used, in which the concentrations of the single substances are well below the detection limits of direct detection technologies. Furthermore, designer drugs, having the same biological effect but different chemical structures than the known substances, cannot be detected by targeted analysis methods. Therefore also here, biomarker-based detection strategies are of great interest.

3.1.1 Steroids

Steroids, such as sex steroids (estrogens, androgens and gestagens) and glucocorticosteroids, have many functions in the body. They are endogenously produced from their common precursor cholesterol and regulate, among others, glucose homeostasis and are responsible for reproductive function. In the World Anti-Doping Agency (WADA) prohibited list, only androgenic steroids are listed as anabolic agents that are banned at all times; glucocorticosteroids are prohibited in-competition and estrogenic steroids are not mentioned at all [2]. In food production, however, steroid administration is prohibited in general [56] and therefore, also estrogenic steroids and glucocorticosteroids are discussed here.

3.1.1.1 Androgenic steroids in sports doping

Testosterone is expected to be abused for its anabolic effects on muscle growth and faster recovery times after exercise. A pilot study analysed the effects of testosterone treatment on several blood biomarkers in weightlifting men. Inhibin B was measured in serum by ELISA and it was found to be significantly decreased after testosterone treatment. Therefore, it may serve as a candidate biomarker for an indirect testosterone abuse test [17]. Currently, testosterone abuse is detected by an altered testosterone/epitestosterone ratio in urine, which is determined by GC-MS-based methods. However, indirect biomarkers such as inhibin B can also be indicative for unknown designer substances having similar biological effects.

In competing race horses, anabolic steroids are expected to be abused for performance enhancement. Two proteins, namely clusterin and leucine-rich α-2-glycoprotein, were increased after testosterone treatment and were mentioned as candidate biomarkers for testosterone abuse detection in race horses [18].

3.1.1.2 Steroids in veterinary control for food production

Steroids exert anabolic effects on the body and are interesting compounds for increasing meat production and quality. Certain steroids also have a water-retaining effect and may be abused short before slaughter to increase the carcass weight. Direct detection of the abused steroids has limited value if synthetic versions of natural hormones are used and therefore, biomarker-based detection strategies offer an advantage over the direct detection. Direct and indirect interaction partners of the administered substance, such as binding proteins, mediators of the hormone action and markers of target tissues may be candidate biomarkers. Several protein biomarkers were identified after treatment with dexamethasone or a cocktail of estradiol and nortestosterone, such as sex hormone binding globulin (SHBG), insulin-like growth factor binding protein 3 (IGFBP3), N-terminal propeptide of procollagen type III (PIIINP), ir-inhibin and myostatin [7, 57]. To measure several of these biomarkers at once, a multiplex method based on SPR biosensor technology was developed [7]. Furthermore, using a fourplex protein biomarker FCIA, originally developed for detection of rbST abuse (**Section 3.2.2.4**), administration-specific biomarker profiles were obtained from cattle treated with estradiol, dexamethasone or prednisolone. These profiles were distinguishable from the profiles from rbST-treated animals (**Table 2**) [19]. These findings suggest that it should be possible to detect a full range of anabolic substances by using a limited number of protein biomarkers. The effects of boldenone and boldione were analysed using 2D gel electrophoresis (2D-GE) and matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF/MS) and LC-MS analysis [20]. These steroids increased plasma apolipoprotein A1 (ApoA1) levels in veal calves.

Table 2: Biomarker profiles after treatment with prednisolone, dexamethasone and estradiol in comparison to treatment with rbST. Biomarker responses are indicated as being significantly increased (+), decreased (-) or stable (Ø) after treatment (reproduced with permission from Ludwig *et al.* [19]).

	biomarkers			
treatments	**IGF-1**	**IGFBP2**	**osteocalcin**	**anti-rbST antibodies**
rbST[1]	+	-	+	+
prednisolone	+	Ø	Ø	Ø
dexamethasone	+	-	-	Ø
estradiol	Ø	+	-	Ø

[1] adapted from Ludwig *et al.* (2012) [13].

So far, no biomarker-based detection methods have been implemented for official control of steroids in food-producing animals. However, in the future, biomarker-based methods could be used as a fast screening procedure for thousands of samples prior to elaborate MS-based blood confirmatory analysis to identify the responsible component for only the few suspicious samples.

3.1.2 Other anabolic agents

Clenbuterol is a β-adrenergic agonist and illegally used in food production to obtain leaner meat. In sports it appears on the WADA 2014 prohibited list, because it is used by athletes to increase aerobic capacities [2]. When calves were treated with a combination of different anabolic agents (clenbuterol, estradiol and dexamethasone), the concentrations of two proteins, namely adenosine kinase and reticulocalbin, were significantly altered in hepatic cytosols and microsomes as shown using 2D-GE and LC-MS/MS [21]. Therefore, these proteins could serve as candidate biomarkers for detection of anabolic agent abuse including clenbuterol. Future studies should elucidate whether these proteins are specific for the administered steroids or clenbuterol and whether the observed changes in biomarker levels are also affected in serum, because liver sampling is only possible at slaughter and more laborious from a sample preparation point of view. This study design, however, shows that even a combination of several different drugs leads to the discovery of candidate biomarkers, which confirms the potential of biomarker strategies for the detection of cocktails comprising several substances.

3.2 Peptide hormones, growth factors and related substances

In the WADA 2014 prohibited list, this class of substances comprises several different peptide hormones, growth factors and other substances with similar chemical structure or physiological effects [2]. Biomarker-based approaches for the detection of these were so far followed for erythropoiesis-stimulating substances and GH, its releasing factors and insulin-like growth factor-1 (IGF-1).

3.2.1 Erythropoiesis-stimulating substances in sports doping

Erythropoietin (Epo) is an endogenous glyco-proteohormone, which is produced upon low oxygen levels in blood. It regulates and promotes the production of red blood cells in the body. Furthermore, it decreases the plasma volume and therewith increases the total hemoglobin content and the oxygen carrying capacity of blood [23]. Epo can be produced recombinantly and is used for treatment of diseases, such as anaemia. But it is also known to be abused by athletes because of its endurance-enhancing effects. There are direct methods for recombinant human Epo (r-HuEPO) detection in urine and blood, which are used by WADA to test athletes for possible abuse. These methods detect the different glycosylation patterns of endogenous and recombinantly produced Epo by isoelectric focussing.

Indirect protein biomarker-based methods are used as well, in which different blood parameters are analysed. These parameters include hematocrit, reticulocyte hematocrit and % macrocytes, which are measured by flow cytometric analysis. Moreover, serum Epo concentration and soluble transferrin receptor concentration are analysed using specific ELISAs [22]. All the above mentioned biomarkers increase upon Epo administration (**Figure 3**). When combining these biomarkers by using mathematical models, a final score is obtained which gives information whether recombinant Epo was used. A combination of this indirect approach and the direct Epo detection was used at the Sydney Olympic Games in 2000 for detection of blood doping. But it was decided that blood sampling in-competition is not feasible and nowadays, Epo abuse is detected by the direct urinary isoelectric focussing method [22]. Other potential protein biomarkers, such as haptoglobin and transferrin isoforms, were identified by using 2D-GE and MALDI-TOF [23]. Using biomarker-based detection, also other blood-doping methods, such as blood transfusion, administration of continuous erythropoietin receptor activators (CERA), gene doping or Epo analogues could be detected [22, 23]. Challenges that remain are the inter-individual differences in these blood biomarkers.

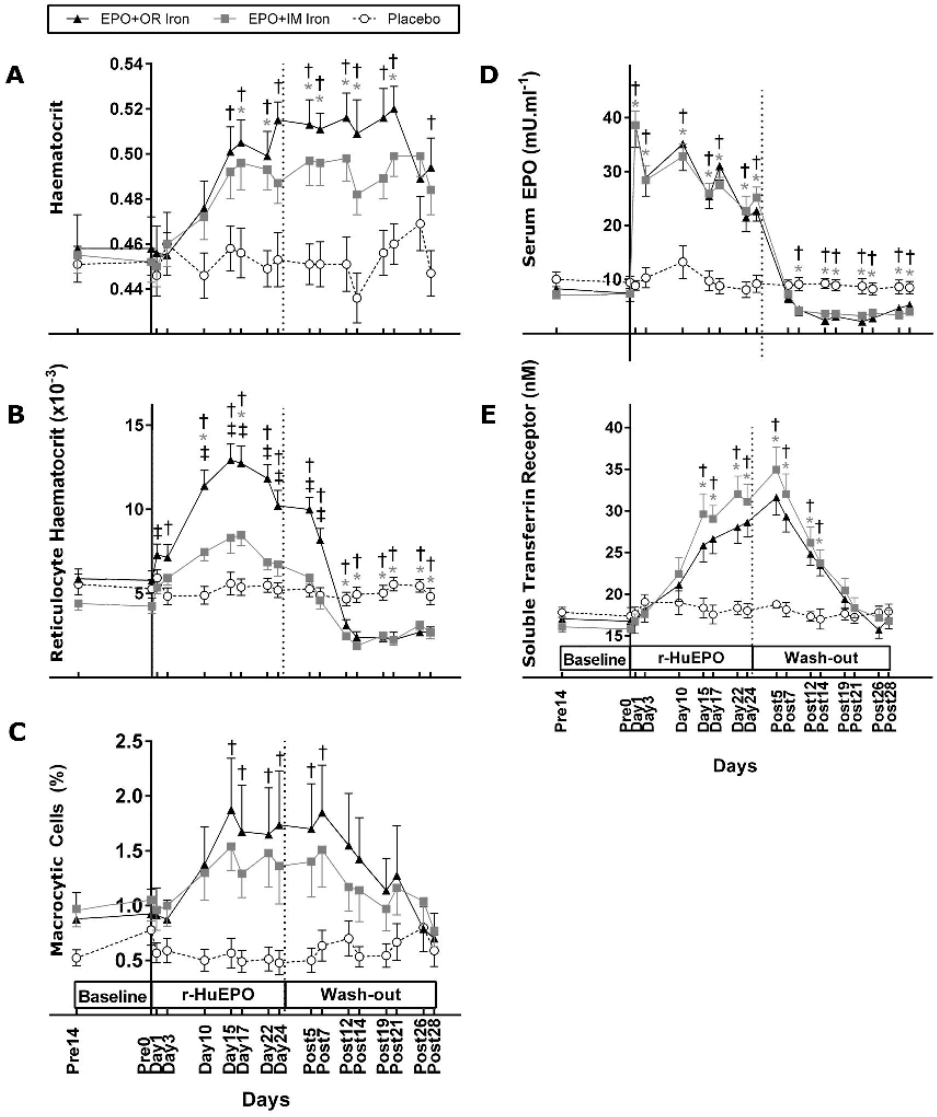

Figure 3: Human blood biomarkers hematocrit (A), reticulocyte hematocrit (B), % macrocytes (C), serum Epo (D) and soluble transferrin receptor (E) after 25 days recombinant human Epo administration. Mean values and standard error of the mean are shown for the different treatment groups: EPO+OR group (r-HuEPO and oral iron treatment), EPO+IM (rHuEPO and intramuscular iron treatment) and placebo group

(sham r-HuEPO injections, sham iron injections, sham iron tablets). Statistically significant differences ($p<0.05$) are: † = EPO+OR versus placebo group. * = EPO+IM versus placebo group. ‡ = EPO+OR versus EPO+IM group (reproduced with permission from Parisotto *et al.* [58]).

To further increase the value of the indirect biomarker test for Epo detection, the biological passport for athletes was introduced recently [59]. By using this passport, an athlete's blood parameters are compared to the own previous parameters and therewith, false-positive or false-negative results are avoided.

Also in horse racing, an increase of red blood cell volume and aerobic capacity was observed after repeated treatment with recombinant Epo and therefore, the increased hemoglobin concentration was identified as a candidate biomarker for Epo administration [24]. Furthermore, horses injected with recombinant Epo develop specific antibodies as an immunological response. These antibodies were detected using a protein microarray approach, where different available recombinant Epo preparations were coupled to a microarray surface and the presence of anti-Epo antibodies was detected by fluorescence-labelled secondary antibodies [25].

Epo is not expected to be abused in food production, because improved aerobic capacity does not have a major impact on increased meat production or quality. However, the concept of the biological passport and the implemented biomarker-based method for Epo abuse detection are great examples for how biomarkers can be used to indirectly pinpoint drug abuse.

3.2.2 Growth hormone, its releasing factors and insulin-like growth factor-1

Growth hormone (GH) or somatotropin (ST) is a proteohormone endogenously produced by the anterior pituitary gland (**Figure 2**). Its release from the gland is triggered by many factors. There are GH-inducing factors, such as GH releasing hormone (GHRH), GH releasing peptides (GHRP) and GH-repressing factors, such as somatostatin and IGF-1 [60]. GH has also many different effects on the body, which are exerted either directly onto the target tissues or indirectly via IGF-1 release from hepatic tissues. The main effects of GH are growth promotion, lipolysis in adipocytes and muscle and bone growth promotion [60]. In all the affected tissues, different proteins are either upregulated or downregulated and can be considered candidate biomarkers for the detection of exogenous GH administration.

3.2.2.1 Recombinant human growth hormone

Recombinant human growth hormone (rhGH) is abused by athletes, because of its anabolic and lipolytic actions on the body. The direct detection of rhGH abuse is very difficult, because of several reasons: First, both, the pituitary-derived and the recombinantly produced forms have identical molecular structures. Second, endogenous GH has a pulsatile release pattern and the hormone has a short half-life in circulation, leading to large concentration fluctuations in blood. To overcome these difficulties, biomarker-based detection methods were considered and two complementary approaches have been followed: the 'isoform' approach and the 'biomarker' method.

The 'isoform' approach

In a normal situation, the pituitary produces mainly two isoforms of GH: 70 % of which are the 22 kDa GH form, 5-10 % are the 20 kDa GH form and the remaining forms are oligomers, modified forms or fragments [12]. If rhGH, which consists only of the 22 kDa form, is injected, first, the total amount of the 22 kDa GH form in circulation increases and second, the endogenous GH production is inhibited by negative feedback loops and therewith, the total amount of the other isoforms decreases. The different GH isoforms are measured by using two immunoassays with different specific monoclonal antibodies. In the 'pituitary' assay, a variety of pituitary-derived GH forms are detected, whereas in the 'recombinant' assay, preferably the 22 kDa GH form is measured [61]. From the results of these two immunoassays, a 22 kDa/non-22 kDa ratio is calculated, which increases upon rhGH administration [53]. Thus, with this test, it can be detected whether rhGH was injected. This method has, however, a rather limited detection window of less than 48 hours and if hGH extracted from pituitaries of corpses or growth hormone releasing agents are used, the ratio will not be affected and the test fails to detect the abuse [12].

The differential isoform test has been first implemented for sports doping control at the Athens Olympic Games in 2004. WADA considers a sample an adverse analytical finding as soon as the pituitary/recombinant isoform ratio exceeds a certain gender-specific decision limit [61]. By doing so, eight adverse analytical findings were reported for hGH in 2012 [62].

The 'biomarker' approach

As described in **Section 3.2.2**, GH exerts effects on many different target tissues and there it causes the up- or downregulation of specific proteins. The proteins with altered concentrations serve as candidate biomarkers for the detection of rhGH abuse. The study

group GH-2000 identified two significant GH-dependent protein biomarkers out of a variety of possible candidates [12]. These were IGF-1 and PIIINP, which are both measured in serum by immunoassays. IGF-1, which is a member of the GH/IGF-axis was measured after acid/ethanol extraction using a RIA and PIIINP, a marker of soft tissue turnover, was measured using a two-stage sandwich RIA [63]. For validation, reference values for these biomarkers were obtained from a multitude of elite athletes and the results were used to correct for age and gender by using discriminant functions [12]. In a follow-up, the GH-2004 study group found out that neither ethnicity nor injury changed the overall outcome of the test. Furthermore, the influence of sports discipline, co-administration of testosterone or acute exercise were analysed and none of them influenced the performance of the test negatively. Other groups also identified candidate biomarkers of GH abuse detection [27-32], which are summarized in **Table 1**. Although a high number of candidate biomarkers has been identified, these candidates still need to undergo a similar validation procedure as done by the GH-2000 and GH-2004 teams including a large set of reference samples and covering all possible confounding factors, such as age, sex, effects of exercise, nutrition and injury. Despite the thorough validation of the biomarker approach for human GH detection using the IGF-1 and PIIINP biomarkers, the test has not been implemented yet for official anti-doping testing. WADA states that prior to implementation, it has to be proven that the test can withstand scientific and legal challenge [64].

3.2.2.2 Equine somatotropin

Somatotropin administration also plays a role in equine sports, where it is abused for performance enhancement. Equine ST (eST) has effects on many target tissues and regulates and modulates different protein concentrations. Thus, changed concentrations of these target proteins are indicative for eST abuse and these candidate biomarkers can be used for abuse detection. Proteomic investigations revealed increased IGF-1 and IGFBP3 biomarker levels after eST administration and also the effects of confounding factors such as age, sex and training are well established [33]. Furthermore, specific antibodies produced by the horse against the injected eST were analysed by ELISA and by a SPR-based biosensor [33].

Whereas RIA quantifications of IGF-1 and IGFBP3 levels are already implemented in routine horse racing doping control, it was suggested that anti-eST antibodies could serve as an additional biomarker when there is still doubt after IGF-1 and IGFBP3 analysis [33]. Anti-eST antibodies are a very selective biomarker for eST abuse, because they are not

present in circulation if no hormone was injected, whereas IGF-1 and IGFBP3 are always present at a certain endogenous level. Therefore, anti-eST antibodies show good potential for improving the sensitivity and specificity of the currently used method.

3.2.2.3 Recombinant porcine somatotropin

In growing pigs, recombinant porcine somatotropin (rpST) can be used for increasing muscle growth and to improve meat quality [65]. Several biomarkers of rpST administration are known [65, 66], but no analytical method has been developed to monitor biomarkers for rpST abuse detection.

3.2.2.4 Recombinant bovine somatotropin

In dairy cows, the administration of rbST increases milk production, but its use is banned in the EU [67]. Note that the topic of rbST abuse detection in cattle is indeed ongoing and remains urgent: recently, a network of illegal rbST distribution was uncovered [68]. Similar to the biomarker-based hGH abuse detection, also markers of the GH/IGF-axis and markers of bone and soft tissue turnover are affected by rbST administration (**Figure 2**) and can therefore be used for rbST abuse detection. Furthermore, cows injected with rbST show an immunological response and form specific antibodies against the hormone. A fourplex candidate biomarker panel, consisting of IGF-1, IGFBP2, osteocalcin and anti-rbST antibodies, was tested on rbST-treated and untreated dairy cows [13]. To be able to measure all four candidate biomarkers simultaneously in one serum sample, a multiplex FCIA (**Section 2.2.2.2**) was developed. The candidate biomarkers were measured in serum samples from two independent rbST treatment studies. **Figure 4** shows the differences in the biomarker profiles of rbST-treated and placebo-treated cows. Based on these data, a thorough biomarker assessment was done and it turned out, that a combination of only two biomarkers, namely osteocalcin and the anti-rbST antibodies is sufficient for detecting more than 95 % of the rbST-treated cows truly positive directly after the second rbST injection until the end of their treatment period and even thereafter [13].

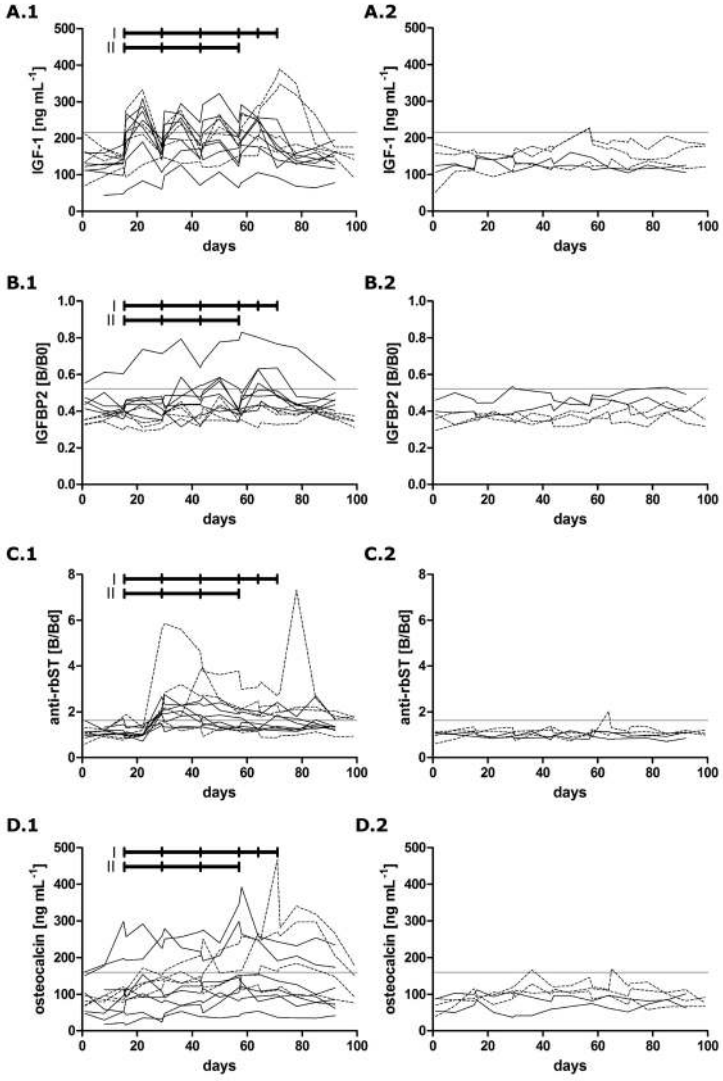

Figure 4: Biomarker profiles of rbST-treated (left; 1) and untreated (right; 2) dairy cows. Biomarkers shown are concentrations of IGF-1 (A), B/B0 levels of IGFBP2 (B), B/Bd levels of antibodies against rbST (C) and concentrations of osteocalcin (D). The rbST treatment schedules are indicated by two black horizontal bars and decision limit per biomarker by the grey horizontal line (reproduced with permission from Ludwig *et al.* [13]).

The same anti-rbST antibodies are found in raw milk from rbST-treated cows and the response was detectable for at least two weeks after the last treatment with rbST [34]. Also when analysing tank milk samples from rbST-treated cows, more than 95 % of the samples were detected as being positive for rbST-treatment. The biomarker-based screening for detection of rbST abuse in dairy cattle performs according to EU legislation [15]. But note that current EU legislation also requires a subsequent confirmatory method to identify the abused substance itself after a sample is found suspect in a screening procedure. Since rbST differs in one N-terminal amino acid from the endogenous bST, it can theoretically be detected as an exogenous compound in the cow's circulation. A confirmatory method based on LC-MS was developed which allowed the detection and quantification of rbST in serum obtained from animals treated with rbST [69, 70]. Residues of rbST remained detectable until 4 or 11 days after treatment, depending on the treatment protocol [71, 72]. The concentrations, however, were below the limit of quantification already three days after treatment. Furthermore, for milk, rbST itself could only be detected at unrealistic spiking levels [73]. Thus, there is an urgent need for better extraction procedures and more sensitive confirmatory analysis methods for detection of rbST in serum and in milk.

3.2.2.5 Somatotropins in fish production

In fish production, rainbow trout and tilapia are potentially injected with ST to increase body weight and length. Because rbST and rpST are easily available and also show growth effects in fish, rbST and rpST are the main forms used as growth enhancers. As a physiological response to exogenous rbST hormone treatment, fish show increased serum IGF-1 levels as well as a specific immunological response [35, 74]. Interestingly, the negative feedback loop of decreased endogenous somatotropin, as observed in mammalians, was not affected by rbST treatment in trout and tilapia. In trout, IGF-1 levels were determined by RIA and specific endogenous trout anti-rbST antibodies were found by Western Blot analysis [35]. In tilapia, anti-rbST antibodies were detected by ELISA [74]. The observed altered protein levels as the physiological response upon rbST treatment are candidate biomarkers for the indirect proof of the hormone use. So far, no biomarker-based method for detection of rbST use in fish production was developed and validated. Aquaculture control is frequently based on feed and water analysis and does not yet include systematic control for somatotropin.

3.2.2.6 Growth hormone releasing hormone

As shown in **Figure 2**, growth hormone releasing hormone (GHRH) and its analogues elevate growth hormone production and release from the pituitary gland by binding to the GHRH receptor. GHRH has a very short half-life of only approximately 7 minutes in circulation. One of its synthetic analogues CJC-1295, however, shows an increased half-life of up to 8 days and is therefore expected to be abused for performance enhancement [75]. As GH levels in circulation are increased after administration of GHRH or its analogues, GH-dependent biomarkers are also expected to be changed. Therefore, research for biomarker discovery focussed on similar biomarkers as for growth hormone abuse itself. Ionescu *et al.* used an immunochemiluminescence assay (ICMA) for GH measurements and a RIA for IGF-1 detection. They found increased basal serum GH levels and IGF-1 levels after administration of CJC-1295 in healthy men [36]. Sackmann-Sala *et al.* analysed the same samples using 2D-GE and MALDI-TOF and identified several significantly altered proteins, such as an ApoA1 isoform, a transthyretin isoform and β-hemoglobin, which were already identified as biomarkers for hGH abuse (**Section 3.2.2.1**) [37]. Moreover, ApoA1 was also affected by steroid treatment in veal calves (**Section 3.1.1.2**), indicating that it is involved in a general anabolic mechanism and is therefore a potential protein biomarker [20]. Before these identified candidate biomarkers can be implemented for detection of GHRH or GHRH analogue detection, they need to be validated on a large reference population including as much as possible confounding factors.

3.2.2.7 Ghrelin and growth hormone releasing peptides

Ghrelin and its exogenous mimetics are growth hormone releasing peptides (GHRP) and stimulate GH release via the growth hormone secretagogues receptor 1a [38]. Since GH concentrations in blood are elevated after ghrelin or GHRP administration, similar biomarkers as for GH detection are expected to be affected. Okano *et al.* tested whether the isoform ratio of GH is affected by GHRP-2 treatment as well (**Section 3.2.2.1**). But this approach was not successful to detect GHRP-2 abuse [76], because endogenous growth hormone release from the pituitary is stimulated and therefore, no change in the isoform pattern was observed. Clinical studies, on the other hand, analysed several candidate biomarkers and indeed showed increased levels of GH by chemiluminescence assay, IGF-1 and IGFBP3 by RIA after treatment with ghrelin or GHRP-2 [38]. In addition to that, a useful next step to detect ghrelin or GHRP abuse, is to also include PIIINP measurements, which proved to be a good biomarker for GH abuse in the biomarker approach (**Section 3.2.2.1**).

3.2.2.8 IGF-1

Since IGF-1 mainly mediates the indirect anabolic actions of GH (**Figure 2**), it is expected to be abused in sports. It is believed that the same biomarkers, which are indicative for GH abuse detection, would be indicative for IGF-1 misuse. Therefore, the GH-2000 and GH-2004 teams investigated markers of the GH/IGF-axis and markers of bone and collagen turnover after administration of a cocktail of recombinant human IGF-1 and recombinant human IGFBP3 to healthy volunteers [39]. They found that some protein biomarkers of the GH/IGF-axis, such as IGF-2, IGFBP-2 and acid-labile subunit (ALS), are affected by IGF-1/IGFBP3 treatment, whereas the markers of bone and collagen turnover remained unaffected [39]. Future studies should elucidate if a combination of the investigated biomarkers can be a reliable indicator for IGF-1 abuse.

3.3 Metabolic modulators

One of the metabolic modulators mentioned in the WADA 2014 prohibited list is insulin [2]. It is released if blood glucose levels rise and facilitates the transport of glucose and amino acids into cells and increases protein synthesis, thereby enhances muscle growth. Besides its anabolic actions, insulin inhibits catabolic pathways and therewith prevents protein breakdown. Its use is banned in sports because of its physiological anabolic effects and the impact on a prolonged endurance and accelerated recovery. During insulin production, mature insulin and C-peptide, a fragment of proinsulin, are released in an equimolar ratio and an altered ratio is expected to be indicative for exogenous insulin. Abellan *et al.* investigated the feasibility of using the ratio of insulin to C-peptide in serum [40]. Specific enzyme-amplified sensitivity immunoassays (EASIA) for insulin and C-peptide were used and it was found that serum baseline levels of insulin and C-peptide differ in between recreational athletes and elite athletes and are also depending on the athlete's discipline. Furthermore, the levels differed according to training season, age, gender and body mass index of the athlete. The same variations were found when insulin and C-peptide concentrations were analysed in urine [77]. It was concluded that extensive future research is needed for considering all possible influencing factors on insulin and C-peptide levels.

Another approach is the detection and quantification of three urinary degradation products of insulin by immunoaffinity chromatography and LC-MS [41]. These insulin fragments were found to be differently abundant in urine depending on whether insulin was endogenously produced or injected. No conclusions were drawn whether these

altered insulin fragment profiles can serve as candidate biomarkers for insulin abuse detection in athletes. Further studies should elucidate whether different insulin fragment profiles are indeed present when comparing healthy untreated individuals and healthy insulin-treated individuals thereby excluding the effect of the disease diabetes.

Insulin administration to food-producing animals also increases muscle growth and this effect can be further enhanced by co-administration of dexamethasone [49]. Therefore, it is expected that insulin is illegally administered to food producing animals. To date, no biomarker studies were done for insulin abuse detection. Considering similar mammalian physiology, similar biomarkers as in human insulin biomarker studies are expected to be indicative for insulin abuse in food production.

3.4 Gene doping

In sports as well as in food production, the use of gene doping is expected to increase in the future. Gene doping is the abuse of gene therapy, in which viral vectors carrying genes encoding certain proteins are introduced into cells of the athlete or food-producing animal [42]. These cells start to produce the encoded proteins and since the protein production uses the endogenous cell machinery, most likely, the produced proteins are indistinguishable from their endogenous forms. Candidate proteins for gene doping are mainly those abused for promoting endurance, such as Epo and vascular endothelial growth factor (VEGF) or for increasing muscle mass, such as myostatin inhibitors, insulin, GH and IGF-1 [7, 42, 78]. IGF-1 gene transfer was successfully performed *in vivo* to muscle of mice and rat and, additional to increased muscle mass and performance enhancement, a local increase in IGF-1 concentration was observed; but no increase of IGF-1 concentration was measured in circulation [79]. This situation is cumbersome for detection of IGF-1 gene doping, since muscle biopsies would be necessary, which is not acceptable for sports doping controls. Myostatin is a protein that inhibits muscle growth. If the biological activity of myostatin or its endogenous production is reduced or inhibited, biomarkers of the whole myostatin regulation pathway, such as follistatin and myostatin propeptide, are affected as well. The levels of these two proteins were analysed using immuno-PCR and the ratio of both proteins was shown to be affected in individuals with increased muscle mass [42]. Thus, this biomarker strategy is a possible detection method for myostatin inhibitor gene doping.

In food production, transgenic approaches are already used to increase growth rates in marine animals, such as carp, catfish, salmon and tilapia. When transgenic salmon

overexpressing GH was compared to wildtype salmon, altered myostatin levels were found in different fish muscle tissues [43]. Moreover, all protein biomarkers related to GH administration are expected to be affected as well by the increased circulating GH levels in fish. Therefore, protein biomarkers are a valuable tool to monitor whether genetic approaches were used to increase muscle growth and productivity.

4 Discussion & Outlook

Biomarker discovery, verification, assay optimization and biomarker validation are the phases that have to be followed for the development of a biomarker-based method for detection of drug abuse (**Section 2.1** and **Figure 1**). A large range of different illegal substances is used in sports and food production for increasing muscle mass and enhancing performance or productivity. As outlined in **Section 3**, similar substances are abused in both fields to achieve these common aims and therefore, the same biomarker-based detection methods may be employed (**Section 2.2**).

Two main approaches are followed during biomarker discovery: targeted methods, mainly applying affinity-based techniques such as immunoassays and untargeted methods, applying 2D gel electrophoresis methods combined with MS analysis or LC-MS. Both targeted and untargeted methods, successfully discovered candidate biomarkers for several different drug treatments in sports doping and veterinary control (**Table 1**). When looking more closely into the data and the expected ranges of specific protein concentrations in serum, targeted methods were much more sensitive compared to untargeted methods (**Figure 5**). So far, untargeted methods mainly discovered high-abundant proteins as candidate biomarkers in the concentration range of around 20 µg mL^{-1} up to 100 mg mL^{-1}. Targeted biomarker discovery methods, however, detected proteins down to the concentration of around 10 pg mL^{-1}. That means that targeted approaches can be a factor of $2x10^6$ more sensitive compared to untargeted methods. Though both, targeted and untargeted approaches, are highly specific, MS techniques are severely hindered by high concentrations of non-specific proteins. Good depletion strategies, however, may allow the detection of low-abundant candidate biomarkers in serum. LC-MS-based techniques used in targeted analysis are highly sensitive methods and offer great potential for detection of several candidate biomarkers [18].

Certain candidate biomarkers were identified in blood for more than one specific drug treatment, such as IGF-1, leucine-rich α-2-glycoprotein or PIIINP, meaning that they are indicative for several different groups of abused substances. It was shown that biomarkers

used to identify rbST treatment in dairy cattle were also affected by different steroid treatments in beef cattle and all different treatments induced a treatment-specific biomarker profile [19]. This finding allowed the preliminary conclusion that a certain biomarker profile can indicate the group to which the abused drug belongs, which clearly highlights the importance of biomarker-based detection strategies.

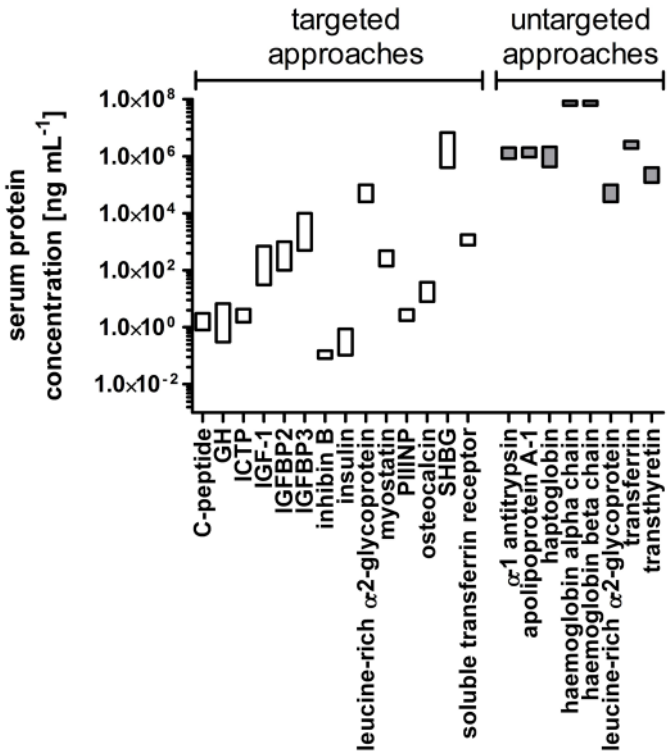

Figure 5: Normal human serum protein concentration ranges of discovered candidate protein biomarkers of different drug treatments, analysed with targeted approaches (for instance immunoassays) and untargeted approaches (MS-based methods) [80-86].

Even though many candidate biomarkers for several different illegal drug treatments were discovered, methods for only a very limited number have been implemented so far. In 2000, the indirect approach based on five blood parameters for the detection of Epo abuse was applied during the Sydney Olympic Games, but thereafter it was decided that only urine sampling is feasible during competition [22]. The blood parameters are still monitored, but mainly out of competition. Longitudinal biomarker results of each tested

athlete are collected in the blood parameter module of the athlete`s biological passport [59]. Using the passport, the blood parameters of one athlete are compared to his or her own previous values and therewith, inter-individual variation does not play a role in the evaluation of the results anymore. Furthermore, the out-of-competition testing is likely to detect more doping attempts, since especially the substances targeting enhanced aerobic capacity and faster recovery are mainly used during training. There are also other upcoming modules going to be implemented in the athlete´s biological passport, namely the steroid and endocrine modules [59]. Since the 2004 Athens Olympic Games, the differential immunoassays for determination of the hGH isoform ratio are used and already several athletes were charged based on the results of this method [62]. During the 2012 Olympic Games in London, the biomarker-based approach for rhGH abuse detection was applied for the first time during a competition.

Several lessons can be learnt from the successful path of biomarker discovery until full method implementation in sports doping control:

(1) Extensive prior knowledge is available from literature on the physiological effects of certain drugs including hormones, growth factors and others. From this, a lot of information can be retrieved on candidate biomarkers for drug abuse. By doing so, elaborate untargeted approaches can be avoided and the time invested in thorough literature studies for targeted biomarker discovery will pay off.

(2) A final method format should be chosen, which is widely accepted and applied in testing laboratories. If specific and expensive equipment and additional training are required to perform the final biomarker-based method, the acceptance chances will be very low.

(3) The availability of bioreagents, such as standard proteins and high-quality antibodies is a key issue for the development of reliable and robust high-throughput biomarker methods. The long-term availability has to be ensured to continuously perform the same high-standard methods for biomarker-based drug abuse testing.

(4) There is an essential need for properly planned comprehensive validation studies to assess the specificity of the discovered biomarkers (**Figure 1**). Consider at an early stage what types of samples are needed to perform biomarker validation and include all conceivable confounding factors (age, gender, ethnicity, *etc*.). From this follows the required number of samples, which can easily comprise many thousands. Therefore, it is advised to establish international collaborations with partners, who can contribute by either providing a fraction of the total samples

and/or performing a part of the validation experiments.

(5) Inter-individual variations observed in the analysed biomarkers seem to be a challenge for method implementation. Therefore, either biomarkers should be chosen that are not influenced by other factors than the drug treatment itself or biomarker panels including several different biomarkers should be analysed, which have to show a specific biomarker profile after drug treatment. The problems arising from inter-individual biomarker differences are avoided, when individual biological passports are used. The same strategy is possible for monitoring longitudinal biomarker profiles of racing horses. In veterinary control, however, the introduction of an animal's biological passport is not feasible, because of the large number of food-producing animals and the random sampling in monitoring programs. On the other hand, note that animal herds for food production are much more homogenous than individual athletes and therefore, inter-individual differences are expected to play a less important role.

(6) During the entire process from biomarker discovery up to biomarker validation, it should be aimed for a close collaboration with control authorities who will decide about the final implementation of the biomarker-based method in the regulations. Therewith, it can be ensured that the biomarker-based method fulfils the requirements established in the field. Moreover, foreseen limitations, such as an inappropriate regulatory background, can be communicated and necessary changes can be discussed at an early stage of the developmental phase.

In contrast to successful implementation of several biomarker-based strategies in doping control, none of the biomarker-based approaches for detecting drug abuse in food-producing animals has been implemented so far in routine veterinary control. There are various reasons for this situation. To be implemented in official veterinary control, an analytical method has to be validated. Until now, no validation approach for performance criteria of a biomarker-based method has been proposed. However, with the upcoming more risk-based veterinary control programmes, in which the proactive search for yet unknown residues is an important part, it is expected that the interest in biomarker-based screening will increase. Eventually, this will result in adjusted validation requirements specific for biomarker-based screening. Note that, in non-statutory surveillance programmes in several EU member states, methods for detecting endogenous antibodies produced upon ST treatment in cattle and horses are already being used [7]. Another important point for implementation in veterinary control is that so far, a subsequent confirmatory analysis for unequivocal identification of the substance of abuse itself or its metabolite is required. Likely, a biomarker-based method can still detect drug abuse while

the drug levels in the animals body fall below the limit of detection of the instrument used for subsequent confirmatory analysis. To solve that issue, there are at least two different approaches: on the one hand, additional efforts can be put on confirmatory methods to be used in combination with biomarker-based screening methods. Improved sensitivity, mass accuracy and mass resolution will be important issues. It is to be expected that future generations of MS equipment will be able to provide that level of performance. On the other hand, the successful implementation in sports doping control can be taken as a guideline to also implement biomarker-based techniques for veterinary control into the EU regulations, which are under revision anyway. In doping control, it was shown that when efforts for method validation are extended in a way that the majority of confounding factors is assessed, then a confirmatory analysis for the abused substance itself is not necessary anymore. Therefore, it can be expected that if biomarker-based detection methods are comprehensively validated, confirmatory methods may become less crucial in future veterinary control.

As seen in the cases of the biomarker approach for hGH abuse detection or the biomarker-based Epo test, a single biomarker is not sufficient to pinpoint drug abuse, because it is also influenced by confounding factors, such as age, gender and nutrition. To circumvent this problem, multiple protein biomarkers are combined in a biomarker panel and then, a specifically altered biomarker profile indicates the drug abuse. For future developments, it is also possible to combine different types of biomarkers into one panel. A lot of work has been done for detection of drug abuse applying metabolomic and transcriptomic methodologies [8-10]. Therefore, additional biomarkers do not necessarily have to be proteins; they can also be mRNA or metabolites, which are significantly altered after drug treatment. Taking together protein (proteomic), mRNA (transcriptomic) and metabolic biomarker profiles can further strengthen the analytical power and increase the sensitivity for finding more samples from illegally treated athletes or animals.

In summary, a lot of effort has been made to discover candidate biomarkers for many different illegal drug treatments in sports doping and veterinary control. Biomarker-based technologies have several advantages when direct detection methods reach their limitations. Nevertheless, proper validation of the candidate biomarker is very complex and method development for ultimate implementation requires the availability of high quality standards and bioreagents. For the detection of GH and Epo abuse, biomarker methods have been successfully implemented in sports control. Even though biomarker-based detection is accepted in sports doping control as sufficient proof of drug abuse, in veterinary control, so far, the unequivocal identification of the abused substance itself

is still required. Lessons learnt from the successful path of biomarker discovery until full method implementation in sports doping control will facilitate the acceptance of biomarker-based detection strategies in veterinary control of food-producing animals in the future.

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Part I

Blood biomarker profiling

3

Multiplex flow cytometric immunoassay for serum biomarker profiling of recombinant bovine somatotropin

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Abstract

Recombinant bovine somatotropin (rbST) is licensed for enhancing milk production in dairy cows in some countries, for instance the United States, but banned in Europe. Serum biomarker profiling can be an adequate approach to discriminate between treated and untreated groups. In this study a multiplex screening tool on a small set of biomarkers for pinpointing recombinant bovine somatotropin (rbST) (ab)use was developed and evaluated: insulin-like growth factor 1 (IGF-1), IGF binding protein 2 (IGFBP2) and rbST-induced antibodies were selected as rbST-dependent markers and combined in one parallel assay format. For this, the colour-encoded microspheres were used in a suspension array, with a dedicated flow cytometer. Serum samples obtained from an animal experiment with rbST-treated and untreated dairy cows were measured with the developed triplex immunoassay and biomarker responses on rbST treatment were evaluated. This resulted in characteristic treatment-dependent responses for all three individual biomarkers. Combining these results with the statistical prediction model *k*-nearest neighbours (kNN), resulted in good discrimination of treated and untreated animals: an overall sensitivity (true-positive rate) of 89.1 % and an overall specificity (true-negative rate) of 97.7 % was reached. Therefore, this is the first multiplex method which can be applied with high confidence for screening of unknown herds of cattle pinpointing at rbST (ab)use.

1 Introduction

Recombinant bovine somatotropin (rbST) can be used to enhance growth and lactating performance in cattle. Within the EU, rbST is banned since 2000 [1], therefore, routine screening methods are urgently needed. A liquid chromatography-mass spectrometry (LC/MS) method for direct rbST detection in blood samples was developed [2], however it showed a small detection window, due to the short half-life of rbST in blood [3]. Moreover, the similarity with the endogenous hormone (bST, also called growth hormone), the low concentrations of bST and rbST in serum, and strong fluctuations of bST hamper the direct detection. Therefore, detection of rbST-dependent biomarkers having a longer half-life offers a promising alternative, as already reported for steroid abuse and in sports doping [4-8]. As rbST strongly influences the growth hormone/insulin like growth factor I (GH/IGF-I) axis, the following biomarkers are considered as indicative for administration of rbST: insulin-like growth factor-1 (IGF-1) and its binding proteins IGFBP2 and 3, rbST-induced antibodies, and several markers of bone and collagen turnover [9-12]. So far, immunoassays detecting single rbST-related biomarkers were developed on different platforms like radio immuno assays (RIA) [9, 13, 14], enzyme-linked immunosorbent assays (ELISA) [15-19], Western Blot techniques (WB) [20, 21] and flow cytometric immunoassays (FCIA) [22-24]. Looking at single biomarkers, by using the above mentioned techniques, an indication of potential rbST abuse might be obtained. However, a much more powerful screening tool can be designed by combining multiple biomarkers into a multiplex assay format. The advantage of biomarker screening in serum of dairy cows using flow cytometry in comparison to surface plasmon resonance (SPR)-based techniques was demonstrated recently [22]. Using colour-encoded microspheres in a suspension array format, in theory 100 different analytes can be detected simultaneously with high throughput in minimal sample volume. In this study, we evaluated the suitability of this technique for multiplex detection of rbST-related biomarkers: IGF-1, its binding protein IGFBP2 and rbST-induced antibodies. This set was selected from literature as it includes two biomarkers with a quick response upon rbST treatment (IGF-I and IGFBP2) and one with a long half-life (rbST-induced antibodies), *i.e.*, together offering the possibility of a prolonged detection window. The development of a biomarker-based method for rbST (ab)use required the analysis of a large population of untreated cow samples to determine endogenous background levels, and the biological variation of each biomarker. Decision limits were then established. Next, the applicability of the developed triplex assay was demonstrated with serum samples from rbST-treated and untreated cows. Using the statistical prediction tool *k*-nearest neighbours (kNN), the origin of serum samples, treated or untreated, was predicted based on single biomarker analysis and combined biomarker analysis. Finally, both biomarker

analysis approaches were compared on their capabilities for pinpointing rbST (ab)use in dairy cattle.

2 Experimental

2.1 Materials and instruments

Posilac® 500 mg single-dose syringes and syringes with only the slow-release formula were purchased from Monsanto company (St. Louis, MO). Hydrochloric acid, potassium phosphate, sodium azide, sodium chloride, sodium hydroxide, sodium phosphate, Tween 20 and the ultrasonic cleaner were purchased from VWR International (Amsterdam, The Netherlands) and glycine was from Duchefa (Haarlem, The Netherlands). N-hydroxysulfosuccinimide sodium salt (sulfo-NHS) was supplied by Fluka (Steinheim, Switzerland) and sodium dodecyl sulfate (SDS) by Serva (Heidelberg, Germany). Bovine serum albumin (BSA), insulin-like growth factor-I (IGF-I; human recombinant), 2-(N-morpholino)ethanesulfonic acid (MES hydrate) and N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) were purchased from Sigma-Aldrich Chemie (Zwijndrecht, The Netherlands). Monsanto rbST standard was obtained from the National Hormone & Peptide Program (NHPP) of Dr. Parlow (Torrance, CA). Insulin-like growth factor binding protein-2 (IGFBP-2; bovine recombinant) was purchased from IBT (Reutlingen, Germany). Mouse anti-IGF-1 was supplied by Spring Bioscience (clone SPM406, Fremont, CA) and the rabbit anti-IGFBP-2 was from USBiological (Swampscott, MA). R-Phycoerythrin (PE)-labelled goat anti-bovine immunoglobulins (GAB-PE) were from Santa Cruz Biotechnology (Santa Cruz, CA) and R-Phycoerythrin (PE)-labelled goat anti-mouse immunoglobulins (GAM-PE) and goat anti-rabbit immunoglobulins (GAR-PE) were purchased by Prozyme (San Leandro, CA). MultiScreen HTS filter plates were purchased from Millipore (Amsterdam, The Netherlands). Protein Lobind Tubes (1.5 mL) and a table centrifuge model 5810R were supplied by Eppendorf (Hamburg, Germany). The Luminex 100 IS 2.2 system consisting of a Luminex 100 analyser and a Luminex XY Platform programmed to analyse a 96-well plate, was purchased from Applied Cytometry Systems (ACS, Dinnington, Sheffield, South Yorkshire, UK). SeroMAP microspheres (sets 025, 050 and 078) and sheath fluid were purchased from Luminex (Austin, TX). The Snijder test tube rotator was purchased from Omnilabo International (Breda, The Netherlands). The microtiter vari-shaker was purchased from Dynatech (Guernsey, UK).

2.2 Sample materials

Eight 5 year old Holstein dairy cows were divided into two groups. After two weeks adaptation, treatment consisted of subcutaneous injections of 500 mg rbST in a slow-release formula for the first group (a-d, referred to as rbST-treated) and the slow-release formula only for the second group (e-h, referred to as untreated). The cows were injected with a two week interval four times and subsequently twice with a one week interval. During the two week adaptation period, blood samples were collected weekly. During the treatment period blood samples were collected a day before, a day after and a week after injection and after the last injection, blood samples were collected weekly for four more weeks. Unfortunately, one untreated cow (denoted e) died in the beginning of the animal experiment, due to swollen hocks, which led to general inflammation and sepsis. Therefore, in this experiment results could be obtained for 4 treated and 3 untreated cows. The experimental procedure was authorized by the ethical committee of the Faculty of Veterinary Medicine, Ghent University.

In addition, blood samples were taken from 20 healthy, lactating cows varying in the age of two to five years, in different stages of their lactating cycle, to reflect a normal population of untreated dairy cows. Based on the origin of these cows the assumption of being untreated with rbST was justified.

After blood collection, all blood samples were placed at room temperature for 4 h to coagulate. Then, samples were centrifuged for 10 min at 3000 g, and serum samples were collected and stored at -80 °C until further use.

2.3 Pretreatment of serum samples

For the generic flow cytometric immunoassay (FCIA) sample preparation procedure [22, 24], serum samples were pretreated by adding 25 µL glycine solution (27.5 mM glycine pH 0.5 (pH adjusted by addition of HCl)) to 25 µL of serum sample or standard solution in a polypropylene tube under constant vortexing. Samples were then incubated at room temperature for 60 min. After incubation, 50 µL glycine-SDS solution (400 mM Glycine, 0.3 % m/v SDS, pH 10 (pH adjusted by addition of NaOH)) was added under constant vortexing. Samples were further diluted with 0.1 % BSA in PBST to a final dilution of 80-times. No further preparation was needed prior to the FCIA.

2.4 Microsphere preparation for the flow cytometric immunoassay (FCIA)

IGF-1, rbST standard and IGFBP2 were coupled to seroMAP microsphere sets 025, 050 and 078, respectively, according to Bremer *et al.* [22]. Briefly, for each microsphere set 2.5 x 10^6 microspheres were coupled with a two-step carbodiimide reaction using 500 µL of a 100 µg mL^{-1} protein solution in MES buffer for IGF-1 and rbST, and 500 µL of a 10 µg mL^{-1} protein solution in MES buffer for IGFBP2. After coupling, the microspheres were stored in blocking buffer (PBS, 0.1 % BSA, 0.02 % Tween 20 and 0.05 % NaN3) at 2-8 °C in the dark until use. Under these storing conditions, microspheres were stable for more than one year.

2.5 FCIA procedure

Standards and sera were pretreated as described (**paragraph 2.3**). One hundred µL of the pretreated and diluted serum samples or standard solutions were added to a filter bottom microtiter plate. Hereafter, 10 µL antibody mixture, containing 1500 times diluted anti-IGF-1 and 25000 times diluted anti-IGFBP2 antibody was added and incubated for 15 min on a microtiter plate shaker. Then, microspheres (10 µL diluted suspension containing about 1250 microspheres per microsphere set) were added to each well and incubated for 1 hour on a microtiter plate shaker. After incubation, the plate was centrifuged (1 min at 130 g) and the microspheres were washed with 200 µL PBST. After washing, 125 µL PE-labelled antibody mixture containing 625 times diluted GAM-PE, 1000 times diluted GAR-PE and 1000 times diluted GAB-PE, was added and incubated for 30 min on a microtiter plate shaker. After this incubation step, the plate was centrifuged and 125 µL of PBST was added per well. Then, the microspheres were detected, according to bead assay type and PE-label in the flow cytometer (1 µL s^{-1} was measured until 100 events per microsphere set were reached with a maximum of 50 µL $well^{-1}$).

2.6 In-house validation study of the developed FCIA

As an in-house validation study, the intra- and inter-assay precision of the individual biomarkers in the triplex FCIA were assessed. The intra-assay variation was calculated by averaging the percentaged standard deviation of each sample obtained by mean fluorescence intensity (MFI) signals of a 10-times repeated triplex FCIA on 8 serum samples obtained from 7 cows. The inter-assay variation was calculated by averaging the percentaged standard deviation of each sample obtained by duplicate measurements of the same 8 sera at 9 different days, using B/B0 results for the IGF-1 and IGFBP2 assay and

normalized values as described in the next paragraph for rbST-induced antibodies.

2.7 Signal normalization for rbST-induced antibodies

Due to the lack of standard for rbST-induced antibodies, the daily variations in assay performance and technical performance of the Luminex 100 IS 2.2 system, a normalization step to enable in between days comparison of signals from rbST-induced antibodies is needed. As the 8 sera used for the in-house validation study were measured in every experiment, MFI signals of these 8 sera were used for normalization: responses of the 8 sera were averaged and responses of the measured samples were divided by that average.

2.8 Assessment of decision limits

To be able to discriminate between treated and untreated dairy cows, a decision limit is needed for each biomarker. For assessing the decision limits with most accuracy, sera from 27 untreated dairy cows (20 untreated cows and 7 from the animal experiment during their adaption period) were measured with the triplex assay. For IGF-1 concentrations were used for the calculations, for IGFBP2, B/B0 values were used due to the absence of a pure standard for obtaining a calibration curve, and for rbST-induced antibodies, median fluorescence intensities (MFI) were used. Concentrations, B/B0 values and MFIs were averaged and the standard deviation determined respectively. Decision limits with 95 % confidence were calculated. Theoretically, IGF-1 concentrations increase due to rbST treatment [10, 12, 25], therefore, 2-times standard deviation were added to the average IGF-1 concentration. IGFBP2 concentrations theoretically decrease due to rbST treatment [12], which results in less inhibition of maximum MFI signals (B0) and consequently in higher B/B0 values. Therefore, 2-times standard deviation was added to the average B/B0 value. RbST-induced antibodies could be formed due to rbST treatment [23, 24], therefore 2-times standard deviation were added to the average MFI signal.

2.9 Statistics

To assess the suitability of the combination of the three analysed biomarkers is already capable to predict rbST abuse, a *k*-nearest neighbours prediction model in the R environment [26] was used. B/B0 values for IGF-1 and IGFBP2 as well as MFI signals for rbST-induced antibodies for every sample from the animal experiment were included in the data analysis. First of all, the whole data set was divided into a training and test set by using a stratified repeated random sub-sampling approach, which means that 70 % of

the rbST-treated and 70 % of the untreated samples were chosen for the training set and the remaining 30 % of both groups for the test set. Subsequently, B/B0 values and MFI signals of the training set were auto-scaled and a kNN model was built using the training dataset. The optimal number of k ($1 \leq k \leq 10$) was chosen based on the bootstrapping approach leaving out 10 % of the training data (randomly with replacement), which was repeated 10-times [27] and the resulting model was tested by predicting the remaining auto-scaled test set data. Correctly and falsely predicted results were evaluated carefully. To obtain an average performance of the kNN model, this procedure was repeated 10000 times; each time different randomly chosen training and test sets were applied and an overall sensitivity (true-positive rate), specificity (true-negative rate) and misclassification rate could be calculated for every sample, for every time point and for the whole animal experiment.

3 Results and discussion

3.1 Development of the triplex FCIA

For the development of the triplex screening assay, three single immunoassays were combined, the previously developed IGF-1 assay [22], the assay for rbST-induced antibodies [24] and a newly developed IGFBP-2 assay. Despite the fact that IGF-1 and IGFBP2 are indirect competitive assays and the assay for rbST-induced antibodies is an indirect assay, one single straight forward approach in terms of pretreatment and incubation times was feasible. Therefore, the colour-encoded microspheres, primary antibodies and secondary antibodies from the individual assays were simply mixed (**Figure 1**). This approach, however, led to an increase in MFI signal of approximately 150 % for serum samples measured in the multiplex IGF-1 and IGFBP2 assays, compared to the same samples analysed in singleplex format, while no increased signal was observed for the standard solution (B0). This phenomenon might be caused by serum antibodies that directly bind to the microspheres unspecifically [28], whereas the standard solution only consisted of a 80 mg mL^{-1} BSA in PBS. No influence of multiplexing was found on the detection of rbST-induced antibodies. To further investigate the source of the increased signal, the influence of the individual primary antibodies and the individual secondary antibodies in combination with or without primary antibodies on all three microsphere sets, were tested. This pointed to unspecific binding of PE-conjugated secondary antibodies to all three microsphere sets, in particular GAR-PE and GAB-PE, as the cause of the increased MFI signals. Therefore, to decrease this background, the PE-coupled secondary antibodies were diluted more until MFI signals were just above 1000 MFI for the

blank standard. Thus, the main modification of the triplex conditions versus the singleplex assays were secondary antibody dilutions for GAM-PE, GAR-PE and GAB-PE of 625, 1000 and 1000 times, respectively, instead of the former 625, 375 and 100 times dilutions. By doing so, the increase in MFI signals upon multiplexing became less than 2 %.

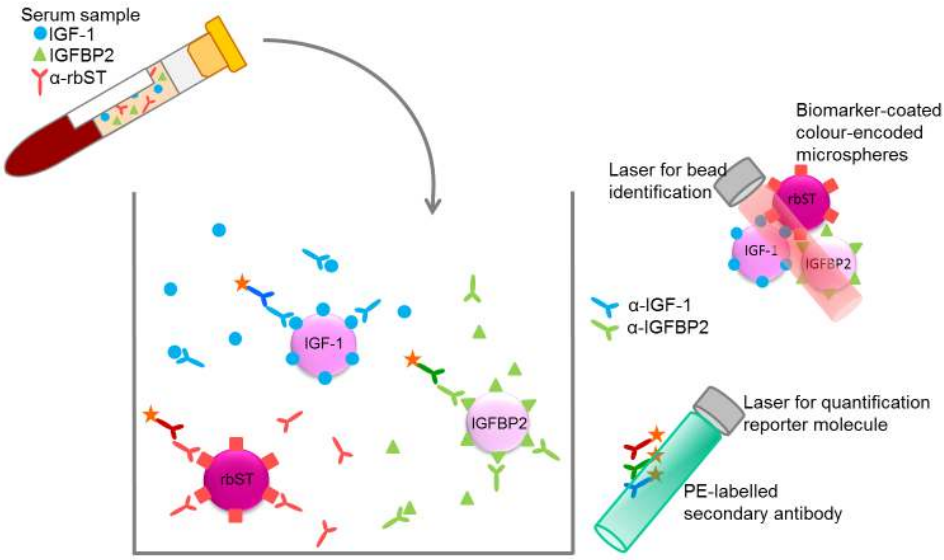

Figure 1: Triplex assay format, the indirect format for IGF-1 and IGFBP2 and direct format for antibodies formed against rbST, all combined within one well.

3.2 In-house validation study of the developed triplex FCIA

For all three assays, high repeatability in both intra-assay and inter-assay variation was assessed. Average coefficients of variation of 5.4 %, 5.4 % and 6.5 % in intra-assay- and 5.3 %, 4.3 % and 7.5 % in inter-assay variation for the detection of IGF-1, IGFBP2 and rbST-induced antibodies respectively were found. This is in very good agreement with the formerly found variations in the IGF-1 singleplex assay.[22].

3.3 Triplex FCIA applicability to real samples

As the ultimate goal is to detect rbST abuse with a biomarker-based method, the applicability of the triplex FCIA was tested.

3.3.1 Establishment of decision limits

For determination of IGF-1 concentrations, a calibration curve was recorded and used to calculate IGF-1 concentrations in sera of the 27 untreated cows (data not shown). Average IGF-1 concentrations of 94 ± 21 ng mL^{-1} were found, resulting in an IGF-1 decision limit of 136 ng mL^{-1}. As no recombinant IGFBP2 suitable for obtaining a good calibration curve was available, decision limit was determined on MFI signals normalized on the maximum MFI signal (B0). For IGFBP2, this resulted in a B/B0 of 0.43 ± 0.04 resulting in an IGFBP2 decision limit of 0.51. For the decision limit of rbST-induced antibodies, an average MFI signal of 193 ± 37 was found resulting in a decision limit of 266 MFI.

For evaluating the applicability of the assays, sera of the animal experiment (4 rbST-treated and 3 untreated dairy cows) were analysed and results were compared to the decision limits as shown in **Figure 2**. As expected, for IGF-1 and IGFBP2, a rapid upcoming and decaying response, and for rbST-induced antibodies a response with a long half-life were observed. All three biomarkers showed specific characteristics in their response upon rbST treatment, together offering a wide detection window with great potential.

3.3.2 Biomarker IGF-1

Following rbST treatment, IGF-1 concentrations were elevated in all four treated cows (**Figure 2A**). This elevation was, however, only for a short period of time in all treated animals. Serum samples taken on the first day after the first treatment already showed an increase in IGF-1 concentration with the highest IGF-1 concentrations seen in sera taken one week after treatment. Then, concentrations declined towards the initial concentration as it can be seen in sera taken two weeks after treatment. In human serum this increase was also observed, however, only for two days after GH treatment [29], whereas similar responses were determined in lactating cows before [30]. Although the increase in IGF-1 concentration is clearly seen in all 4 cows (a-d) after the last rbST treatment, only a part of the serum samples tested showed IGF-1 concentrations beyond the decision limit. Therefore, 37 % of the serum samples were determined as true-positive and subsequently 63 % were classified as false-negatives (**Table 1A**). The untreated cows, as expected, showed no increase in IGF-1 concentration, leading to a true negative rate of 100 %.

Table 1A: Classification of serum samples from the animal experiment based on the single biomarker results in the triplex FCIA assay. **Table 1B**: Average classification based on the single biomarker results.

	A.				B.	
	Untreated		rbST-treated		Average prediction	
Biomarker	True-negative (%)	False-positive (%)	True-positive (%)	False-negative (%)	Classified correct (%)	Classified incorrect (%)
IGF-1	100	0	37	63	64	36
IGFBP2	90	10	31	69	56	44
α-bST	86	14	80	20	83	17

3.3.3 Biomarker IGFBP2

The rbST treatment was also reflected by the results from the IGFBP2 assay. As described in literature, IGFBP2 concentrations decreased due to rbST treatment [9, 31]. Consequently, maximum MFI signals (B0) were inhibited less in sera of rbST-treated animals than in sera of the untreated animals yielding in general an increased B/B0 response due to the decreased IGFBP2 levels. The first and most pronounced IGFBP2 response upon treatment was found one week after the rbST treatment (**Figure 2B**); *i.e.,* later in time than for IGF-1. Two weeks after treatment IGFBP2 signals were back to original signals. In literature, only responses to daily rbST injections were studied, resulting in a return to concentrations within 4 days after rbST treatment cessation [9]. In this study however, responses to the slow-release rbST formula were studied. The IGFBP2 assay showed responses upon rbST treatment in three out of the four rbST-treated cows (a, c and d), but only a part of the serum samples taken from treated animals showed a B/B0 higher than the decision limit. Therefore, 31 % of the serum samples were classified true-positive, whereas subsequently 69 % were tested as false-negative. The sera from untreated animals f and h, were all true negative; only some serum samples of the untreated animal g showed less inhibition, yielding a total of 90 % true-negative samples and 10 % false-positives (**Table 1A**).

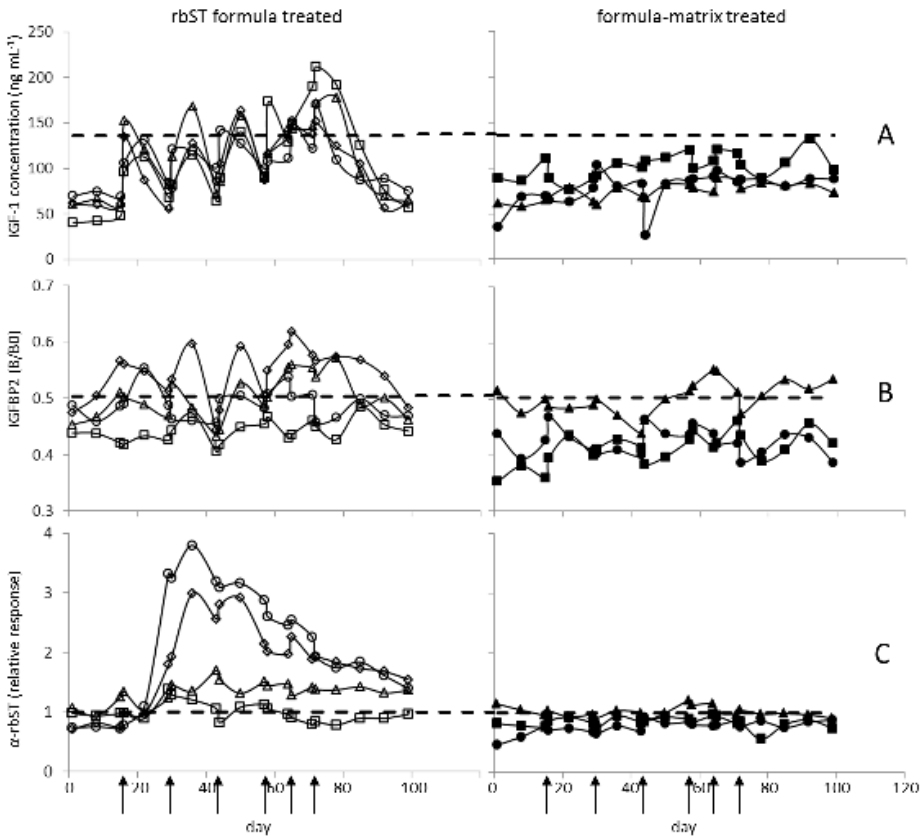

Figure 2: Effect of rbST treatment on serum biomarker levels of dairy cows in time. Time points of treatment (rbST-formula or matrix-formula) are marked by arrows. IGF-1 concentrations (A), IGFBP2 B/B0 values (B) and normalized signals of rbST-induced antibodies (C) are shown for rbST-treated animals a (open diamond), b (open square), c (open circle) and d (open triangle), and untreated animals f (closed square), g (closed triangle) and h (closed circle).

3.3.4 Biomarker rbST-induced antibodies

Two weeks after the first rbST treatment, an increase in antibodies specific for rbST was clearly seen (**Figure 2C**). This increase in rbST-induced antibodies was stable for a longer period of time, *i.e.,* no MFI signal decline to start values in between rbST injections was

seen. Even four weeks after the last rbST treatment, MFI signals for rbST-treated animals a, c and d were still beyond the decision limit, the maximum time point being investigated. This resulted in a true-positive rate of 80 % and subsequently in a 20 % false-negative rate. These results are in agreement with literature [17, 32] where 70-80 % of rbST-treated cows responded with antibody production. The untreated cows, f and h were tested negative and only for cow g some serum samples were found just above decision limit. This resulted in a true-negative rate of 86 % and a false-positive rate of 14 % (**Table 1A**).

3.3.5 Average classification of serum samples from (un)treated cows based on single and multiple biomarkers

Summarizing, for IGF-1, IGFBP2 and rbST-induced antibodies respectively, on average 64 %, 56 % and 83 % of the tested samples were classified correctly (**Table 1B**). According to Commission Decision 2002/657/EC a 95 % true-positive rate is needed for screening assays. None of the single biomarkers on its own can pinpoint rbST abuse with that confidence. Combining results of the three biomarker assays, however, could increase the confidence rate for pinpointing rbST abuse. A statistical prediction model, the *k*-nearest neighbours (kNN) algorithm, was used to discriminate between rbST-treated and untreated animals. After building kNN models on all triplex serum sample data, an overall sensitivity (true-positive rate) of 89.1 % and specificity (true-negative rate) of 97.7 % were obtained (**Table 1C**).

Table 1C. Classification of serum samples based on all three biomarker results following kNN statistics.

	C. **kNN statistic prediction**	
	Classified correct (%)	**Classified incorrect (%)**
Untreated	97.7	2.3
rbST-treated	89.1	10.9

Most of the false-negative results (10.9 %) occurred two weeks after the beginning of the rbST treatment and three and four weeks after termination of the treatment. False-negative results at the beginning of the treatment period could be accounted to IGF-1 and IGFBP2 levels, which declined rapidly after injection, and the antibody titres, which did not increase that much after the first rbST treatment. After multiple treatments, as to

be expected in practice, the false-negative rate became lower. Three to four weeks after termination of the rbST treatment, the prediction power of the model diminished for the same reasons (**Figure 3**). Overall a correct prediction of 93.6 % was observed. Further improvements of this biomarker triplex screening method can be achieved by simply adding additional biomarkers.

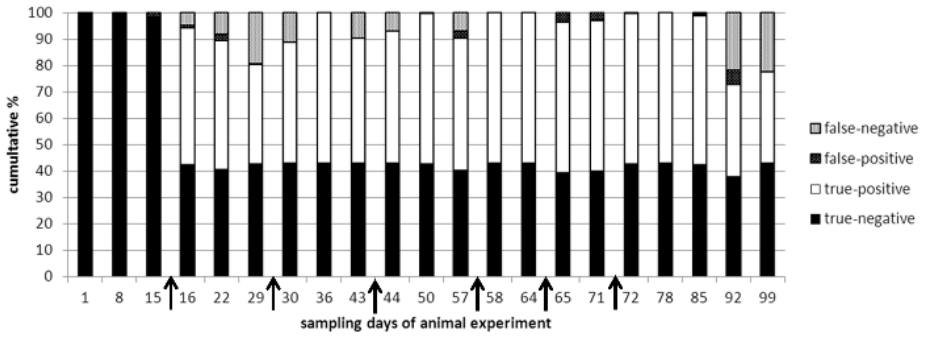

Figure 3: Prediction of correct and incorrect classification, using kNN modelling. Results of all serum samples taken in the animal experiment were categorized by sampling day and used for building the model and predict classification. Time points of treatment (rbST-formula or matrix-formula) are marked by arrows.

4 Conclusion

A unique multiple biomarker FCIA assay has been developed to pinpoint rbST (ab)use in serum samples of dairy cows. Individual immunoassays could be combined into a robust triplex format with only minor modifications. Thus, a reproducible and sensitive platform was obtained. The developed triplex FCIA enables pinpointing rbST abuse by combining results of three biomarkers, IGF-1, IGFBP2 and rbST-induced antibodies. IGF-1 and IGFBP2 biomarkers responded rapidly after the first rbST injection, while responses for rbST-induced antibodies were characterized by a long half-life. For that reason, the combination of these three biomarkers resulted in a very long detection window. The individual biomarkers yielded on average correct classification of 64 % for IGF-1, 56 % for IGFBP2 and 83 % for rbST-induced antibodies in serum samples. A kNN prediction model build on the combined triplex data, enabled even a 93.6 % correct prediction rate. Therefore, this triplex FCIA provides a detailed biomarker profile in serum, ultimately pinpointing rbST abuse in cattle with the highest possible confidence.

Acknowledgements

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4

Multiple protein biomarker assessment for recombinant bovine somatotropin (rbST) abuse in cattle

Ludwig, S.K.J., Smits, N.G.E., van der Veer, G., Bremer, M.G.E.G., & Nielen, M.W.F. (2012). *PLoS ONE*, 7(12), e52917.

Abstract

Biomarker profiling, as a rapid screening approach for detection of hormone abuse, requires well selected candidate biomarkers and a thorough *in vivo* biomarker evaluation as previously done for detection of growth hormone doping in athletes. The bovine equivalent of growth hormone, called recombinant bovine somatotropin (rbST) is (il)legally administered to enhance milk production in dairy cows. In this study, first a generic sample pretreatment and 4-plex flow cytometric immunoassay (FCIA) were developed for simultaneous measurement of four candidate biomarkers selected from literature: insulin-like growth factor 1 (IGF-1), its binding protein 2 (IGFBP2), osteocalcin and endogenously produced antibodies against rbST. Next, bovine serum samples from two extensive controlled rbST animal treatment studies were used for *in vivo* validation and biomarker evaluation. Finally, advanced statistic tools were tested for the assessment of biomarker combination quality aiming at to correctly identify rbST-treated animals. The statistical prediction tool *k*-nearest neighbours using a combination of the biomarkers osteocalcin and endogenously produced antibodies against rbST proved to be very reliable and correctly predicted 95 % of the treated samples starting from the second rbST injection until the end of the treatment period and even thereafter. With the same biomarker combination, only 12 % of untreated animals appeared false-positive. This reliability meets the requirements of Commission Decision 2002/657/EC for screening methods in veterinary control. From the results of this multidisciplinary study, it is concluded that the osteocalcin – anti-rbST-antibodies combination represent fit-for-purpose biomarkers for screening of rbST abuse in dairy cattle and can be reliably measured in both, the developed 4-plex FCIA as well as in a cost-effective 2-plex microsphere-based binding assay. This screening method can be incorporated in routine veterinary monitoring programmes: in the European Union for detection of rbST abuse and in the control of rbST-free dairy farms in the United States of America and other countries.

Introduction

Many different techniques are available for detection of hormone abuse in sports doping and veterinary control, which all have to fulfil the requirements to be reliable, comparably fast and affordable. Biomarker profiling was suggested as a rapid screening approach for detection of doping practices because of its many advantages over the direct detection of the particular abused substances [1]. Biomarker profiles are indicative for more than one administered agent as they reflect the physiological effect, hence, the abuse of unknown compounds can also be detected [1, 2]. Furthermore, in many cases, the analysis of biomarker profiles enables the detection of abused substances for a longer time period, because the biological effect lasts longer than the abused substance itself can be detected in the body [3, 4]. A lot of work was focused on the identification of indicative biomarkers and the development of assays for detection of those [2, 5-10]. But the suitability and discriminative power of each biomarker has to be evaluated in controlled studies where a treated group is compared with an untreated one [11-13].

Extensive studies were done for the biomarker-based detection of recombinant somatotropin (ST; or growth hormone, GH) in sports doping, where ST is abused by athletes for their performance enhancement [14-18]. A similar screening approach can be chosen for the detection of recombinant bovine ST (rbST) abuse in dairy cattle, where the hormone is administered for enhanced milk production [19, 20]. The administration to dairy cattle is approved by the U.S. Food and Drug Administration in the United States of America and allowed in several other countries [21]. But treating cows with rbST is forbidden in the European Union since 1999 because of animal health and welfare reasons [22]. By European regulation, screening and confirmatory methods should be available for the detection of (ab)used veterinary drugs, with for screening, a maximum false-compliant rate of 5 % (β error) [23]. In contrast to the well-established human biomarker-based screening approach, the issue of rbST-dependent biomarker detection is still in its infancy: actually, routine veterinary control for rbST abuse has not been implemented at all, despite the EU ban. So far developed methods which detect rbST directly, such as immunoassays or mass spectrometry-based methods, suffer from the short half-life of rbST. Although biweekly injections containing slow-release formulations are used to prolong the presence of rbST in the cows' body, the protein levels in treated animals cannot be distinguished from the background level throughout the whole two-week inter-injection period and large inter-individual differences in blood rbST levels were reported [19, 20, 24-27]. Furthermore, rbST immunoassays were not capable to distinguish the almost identical recombinant and endogenous forms of bST [19, 20, 24, 25] and mass

spectrometry-based methods on the other hand required very tedious sample preparation procedures [26, 27]. For screening of rbST in cattle, a few biomarker-based methods were developed, but focused on a single candidate biomarker only [4, 9, 28-30]. In a recent study, three candidate biomarkers were combined in one screening tool, but the <5 % false-compliant rate target could not be achieved [31]. Nevertheless, biomarker-based screening for rbST can be considered a very promising start for detecting rbST abuse in dairy cows.

Biomarkers indicative for ST abuse are described in detail in literature and several of them are listed and referenced in **Table 1**. From these, we selected four different candidate biomarkers. These included two biomarkers of the IGF-1 axis, which respond quickly upon rbST treatment, namely insulin-like growth factor-1 (IGF-1) and IGF binding protein 2 (IGFBP2). The other two biomarkers were expected to show a delayed but long-lasting response; these are osteocalcin (marker of bone turnover) and antibodies which are endogenously produced against rbST (anti-rbST-antibodies).

Table 1: Candidate biomarkers for ST abuse and their expected response upon ST treatment in human and cows.

Biomarker	response to ST	described for	reference
Acid labile subunit (ALS)	increase	human	[45]
Anti-rbST-antibodies	increase	bovine	[30, 31, 33, 34]
Apolipoprotein A-1 (APOA1)	decrease	human	[8]
C-terminal cross-linked telopeptide of collagen I (ICTP)	increase	human	[13, 45, 46]
C-terminal propeptide of procollagen I (PICP)	increase	human	[13]
Hemoglobin α-chain (HbA1)	increase	human	[6]
IGF binding protein 2 (IGFBP2)	decrease	bovine	[31, 47]
IGF binding protein 3 (IGFBP3)	increase	human	[45]
Insulin-like growth factor (IGF-1)	increase	human	[45, 46]
Inter-alpha-trypsin inhibitor heavy chain H4 (ITIH4)	decrease	human	[8]
Leucine-rich α-2-glycoprotein (LRG)	increase	human	[9]
N-terminal propeptide of procollagen I (PINP)	increase	human	[12, 45]
N-terminal propeptide of procollagen III (PIIINP)	increase	human	[12, 13, 45, 46]
Osteocalcin	increase	human	[12, 13]
Transthyretin (TTR)	increase	human	[8]
α-1 antitrypsin (AAT)	increase	human	[8]

To be able to screen for these four candidate biomarkers in serum, we developed a 4-plex flow cytometric immunoassay (FCIA) enabling parallel biomarker analysis in a single sample. For IGF-1, IGFBP2 and osteocalcin, a competitive inhibition assay format was chosen, where the respective candidate biomarker is covalently coupled to one set of colour-encoded microspheres. The different microsphere sets can be discriminated by a red laser (**Figure 1**). Biomarker-specific and generic fluorescent secondary antibodies are used for quantification with a green laser. Due to the inhibition format, high sample biomarker concentrations yield low fluorescence signals and *vice versa*. For the anti-rbST-antibodies, a direct assay format with an rbST-coupled colour-encoded microsphere set was used, where the anti-rbST-antibodies bind and can be detected by fluorescently labelled anti-bovine detection antibodies. Here, a high biomarker level leads to a high fluorescence signal.

With this 4-plex FCIA, biomarker profiles were measured in serum samples. Based on the biomarker profiles of 67 untreated animals from different origins, we assessed the inter-individual and physiological variability of these biomarkers within dairy cattle and determined decision limits, beyond which a sample could be classified rbST-treated. Then, we used a large set of serum samples obtained from two independent controlled rbST animal treatment studies to evaluate the discriminative power of each candidate biomarker and of all combinations of biomarkers for distinguishing rbST-treated from untreated cows. Following thorough statistical evaluations, the value of individual and multiple biomarkers was assessed for the prediction of rbST abuse in dairy cows.
The overall aim of the study was the development and validation of a chemical analytical method for rbST-dependent biomarker detection according to European legislation for screening methods [23] and a data analysis approach for identifying biomarker combinations, which can reliably predict rbST abuse. This aim was reached with the help of a statistical prediction model based on the biomarker combination endogenously produced antibodies against rbST and osteocalcin.

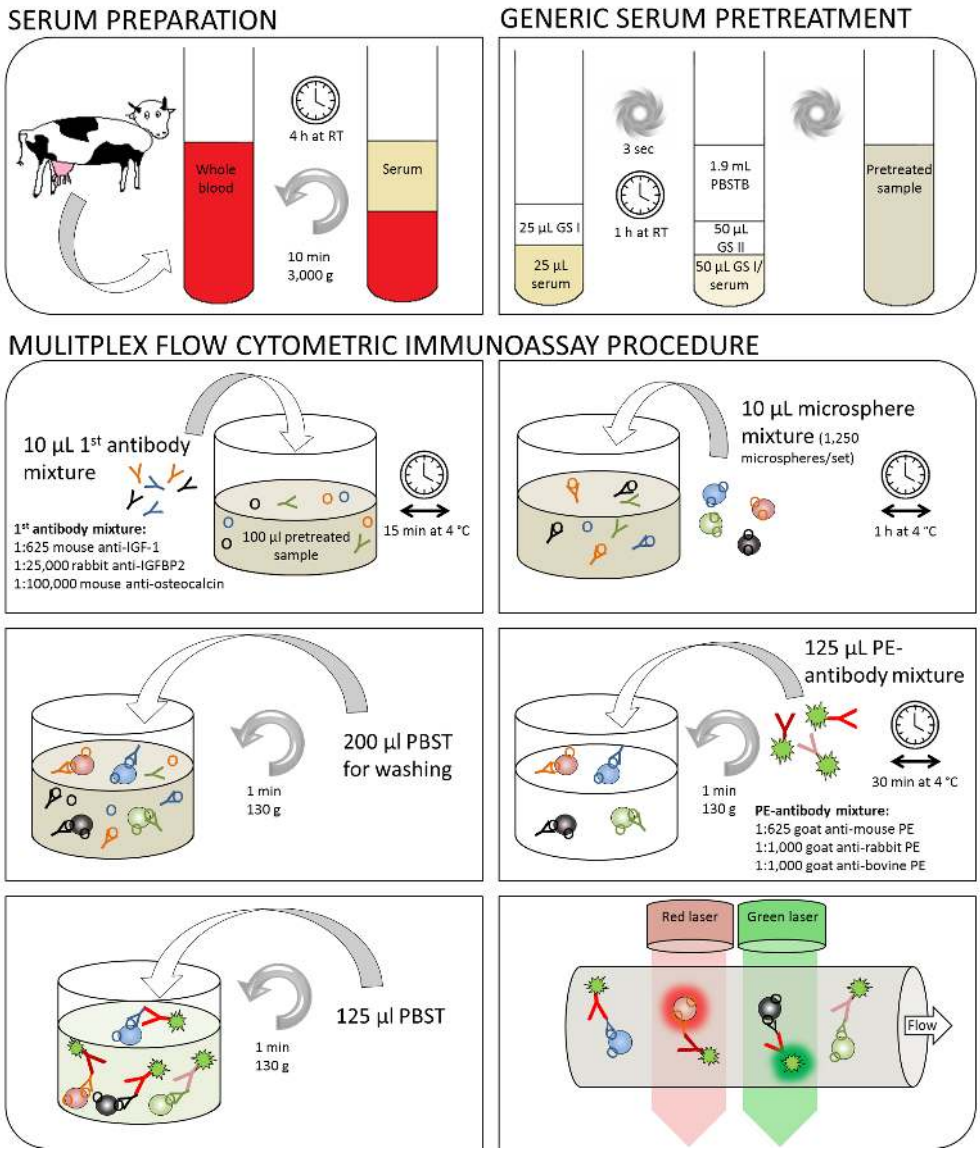

Figure 1: Work flow for serum preparation, generic serum pretreatment and 4-plex FCIA for serum candidate biomarkers. A detailed description can be found in supplementary information. Abbreviations: h – hour, IGF-1 – insulin-like growth factor 1, IGFBP2 – IGF binding protein 2, GS I – glycine solution I, GS II glycine solution II, min – minutes, PBST –

phosphate-buffered saline with 0.05 % (v/v) Tween-20, PBSTB – 0.1 % (m/v) BSA in PBST, PE – phycoerythrin fluorescent label, rbST – recombinant bovine somatotropin, RT – room temperature, sec - seconds; Symbols: incubation time; vortex; centrifugation; ↔ orbital shaking; free IGF-1, mouse anti-IGF-1 antibody, IGF-1-coupled microsphere; free IGFBP2, rabbit anti-IGFBP2 antibody, IGFBP2-coupled microsphere; osteocalcin, mouse anti-osteocalcin antibody, osteocalcin-coupled microsphere; bovine anti-rbST-antibodies, rbST-coupled microsphere; PE-labelled goat anti-mouse antibody; PE-labelled goat anti-rabbit antibody; PE-labelled goat anti-bovine antibody.

2 Results and discussion

For the prediction of rbST abuse in dairy cows, we selected candidate biomarkers based on information found in literature (see **Table 1**). These were markers of the IGF-axis (such as IGF-1 and IGFBP2) and bone markers (such as osteocalcin), known to be influenced by somatotropin and previously examined by the GH-2000 group for detecting somatotropin abuse in athletes [13, 32]. Furthermore, the immune response of cows treated with rbST was examined thoroughly and we used the presence of the specific endogenous antibodies against rbST as a biomarker for its abuse [4, 30, 33, 34]. Although PIIINP, a marker of collagen turnover, is known to show potential in human and bovine hormone abuse detection [10, 18], it has not been included into our biomarker panel yet because of the lack of a suitable commercially available standard protein and antibody.

2.1 Development of a 4-plex flow cytometric immunoassay

For the simultaneous detection of these four candidate biomarkers, we developed a generic sample pretreatment and 4-plex flow cytometric immunoassay (FCIA). To this end, our previously reported 3-plex assay [31] was extended with the biomarker osteocalcin. Adding osteocalcin to the existing triplex FCIA did not result in major interferences of any of the assay components of the four combined biomarker assays (data not shown). IGF-1 and osteocalcin concentrations of tested serum samples were calculated based on the obtained standard curves in serum-matched buffer (**Supplementary Figure 1**). The 4-plex FCIA is capable of determining IGF-1 and osteocalcin concentrations in the relevant range in serum, namely 64 – 400 ng mL^{-1} for IGF-1 and 32 – 320 ng mL^{-1} for osteocalcin (note that serum samples were diluted 80-times prior to analysis, thus the standard curves cover protein concentrations of 0.8 – 5 ng mL^{-1} for IGF-1 and 0.4 – 4 ng mL^{-1} for osteocalcin). For IGFBP2, the standard protein could not completely inhibit the B0 signal, therefore, we

decided to work with normalized responses (B/B0) for the data analysis. For the induced anti-rbST-antibodies, we worked with the responses normalized to a single standard serum (B/Bd).

The generic sample pretreatment was necessary for releasing IGF-1 from its binding protein-complex and preventing nonspecific binding in the detection of anti-rbST-antibodies. The rather harsh pretreatment protocol did not affect the detection quality of osteocalcin, thus it could be adopted for the combined 4-plex FCIA. Note that, adding IGF-2 in excess, as done in commercially available human IGF-1 immunoassays, did not improve the normalized standard curves, nor the detection of biomarker level differences in between treated and untreated animals. The developed assay showed high reproducibility for all measured candidate biomarkers (**Table 2**) and a comparable sensitivity to previous single biomarker methods [35, 36]. However, the newly developed 4-plex FCIA has several advantages, such as the simultaneous measurement of all four markers in one sample from one well of a microtiter plate, which saves sample material, work load and time. Additionally, only one washing step was required compared to an average of six washing steps in a conventional enzyme-linked immunosorbent assay, making the 4-plex FCIA much faster and easy-to-use. The whole assay procedure, starting from a serum sample until the results from the flow cytometer for all four candidate biomarkers, takes 3.5 hours for a whole 96 well microtiter plate. This demonstrates that the 4-plex FCIA is a rapid and promising screening tool for the detection of the four candidate biomarkers in serum.

2.2 Single candidate biomarker profiles of untreated and of rbST-treated cows

After successful development of the 4-plex flow cytometric immunoassay, decision limits for each single candidate biomarker were calculated by analysis of sera from 67 untreated dairy cows (see **paragraph 3.9.1**). Compared to the number of tested athletes in human studies, the number of tested control animals may seem to be rather low, but the variation within the dairy population is expected to be much lower, because of several reasons: First, only female cows have to be taken into account. Second, milking only occurs after first calving (usually at 20-24 months of age), thus after puberty, in which levels of IGF-1, IGFBP2 and osteocalcin are mainly changed due to growth and are more stable thereafter [37-39]. Third, since in this region of Europe mainly Frisian Holstein cows are used for milk production, we focussed on this particular race for the development of the test. And fourth, we do not need to consider sick animals, since their milk will not be allowed for consumption due to the presence of veterinary drug residues and therefore treatment

with rbST is useless for sick dairy cows. Thus, the overall relative variation expected in dairy cows is anyway much lower than in athletes, where gender, different ethnicities, the effect of sports discipline, injury and all age groups need to be considered.

Table 2: 4-plex FCIA assay performance characteristics for the single candidate biomarkers. IC_{50} relates to 80-times diluted samples.

	Candidate biomarker			
Performance characteristics	**IGF-1**	**IGFBP2**	**Anti-rbST antibodies**	**Osteocalcin**
IC_{50} [ng mL^{-1}]	1.5	9.5	-	1.1
Precision and Ruggedness: Interassay variation [%]	15.7	7.9	22.3	17.1
Intraassay variation [%]	6.4	5.7	9.4	9.5
Decision limit	216 ng mL^{-1}	0.52 B/B0	1.62 B/Bd	160 ng mL^{-1}
Stability	The 4plex FCIA can be performed stably over several months by different staff.			
Specificity	No unwanted interaction in between the assays (analytes and antibodies) observed.			

Decision limits were 216 ng mL^{-1} for IGF-1, 0.52 B/B0 for IGFBP2, 1.62 B/Bd for anti-rbST-antibodies and 160 ng mL^{-1} for osteocalcin and are shown as green horizontal lines in **Figure 2**. Results of samples exceeding this limit were considered positive.

Then, biomarker profiles of the dairy cows from both animal studies were measured (**Figure 2**). Results of the cows from animal study I are shown in dotted lines whereas the results of animal study II are shown in solid lines. Note that the animals from animal study I received two additional weekly rbST injections after the biweekly treatment period (the treatment schedules of both animal studies are indicated by the black horizontal bars above the graphs).

IGF-1 levels were found to be elevated directly after rbST treatment (**Figure 2 A.1**) and returned back to baseline before the next treatment. This short response time was observed before in human studies, where IGF-1 concentrations were back to baseline one week after termination of somatotropin treatment [32]. Nevertheless, in athletes, IGF-1 stayed elevated throughout the treatment period. This difference in IGF-1 response to somatotropin treatment could be due to the fact, that athletes were injected daily and, although a slow-release formulation was used in the here presented study, the biweekly treatment schedule does not reflect the same situation of permanently present

somatotropin in circulation. IGF-1 levels of untreated animals (**Figure 2 A.2**) remained below the decision limit. The found IGF-1 concentrations are consistent with previously reported serum IGF-1 concentrations in dairy cows [31, 40].

IGFBP2 levels (**Figure 2B**) are expected to decrease upon rbST treatment [32, 41]. The IGFBP2 assay is of an inhibition format, thus B/B0 levels are inversely correlated with the concentration. Hence, higher B/B0 levels are expected after rbST treatment. For some of the rbST-treated cows, a slight increase in B/B0 levels can be observed after treatment (**Figure 2 B.1**) with a decrease to baseline before the next treatment. But this pattern is not as pronounced as for IGF-1. Furthermore, only occasionally a value exceeded the decision limit. Only the results of one cow were clearly above the decision limit, but these values were observed already during the adaptation period. In humans and despite large inter-individual differences, mean IGFBP2 levels responded quite well upon ST treatment, but the athletes were treated daily on three subsequent days [32]. B/B0 levels of untreated animals (**Figure 2 B.2**) remained below the decision limit at almost all times.

For the antibodies, endogenously produced by the cow as an immunological response upon rbST treatment [30], a delayed increase in signal was observed (**Figure 2 C.1**). Most of the cows developed antibodies approximately 2 weeks after the first rbST injection and a maximum in response could be seen around the third injection (four weeks after start of rbST treatment). Thereafter, the responses declined slowly. Zwickl *et al.* reported an increase of antibody formation within the first three months of rbST treatment and a decline thereafter, but the amount of rbST administered in their study was much higher than recommended by the manufacturer and applied here [34]. For the untreated cows in our studies (**Figure 2 C.2**), only one result was found to be above the decision limit.

For osteocalcin, a slow increase in concentration was observed after rbST treatment (**Figure 2 D.1**) compared to the untreated cows where the concentrations remained below the decision limit at almost all times. A similar effect on osteocalcin levels was observed in the human GH-2000 study [13]. Osteocalcin concentrations in our studies increased consistently in the 8 week treatment period, no gradual decline was observed as for the anti-rbST-antibodies. A slow osteocalcin decrease was noticed after rbST withdrawal but values remained above the decision limit for some of the cows until the end of the animal study.

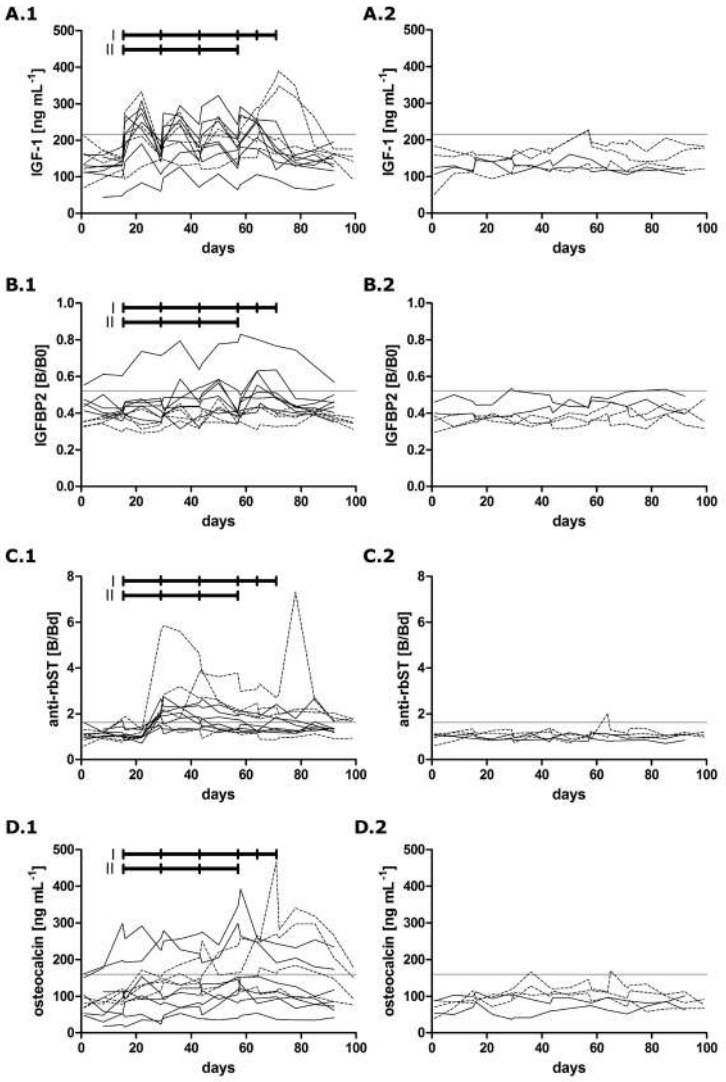

Figure 2: Biomarker profiles of rbST-treated (left) and untreated (right) dairy cows. Profiles from animal study I (dotted lines) and animal study II (solid lines) are shown. Sera from adaption period (3 sera from every cow), treatment period (13 sera per cow from animal study I and 9 sera per cow from animal study II) and withdrawal period (5 sera per cow from animal study I and 6 sera per cow from animal study II) were measured in duplicate. Biomarkers shown are concentrations of IGF-1 (A), B/B0 levels of IGFBP2 (B), B/Bd levels of antibodies against rbST (C) and concentrations of osteocalcin (D). The rbST treatment

schedules for both animal studies are indicated by two black horizontal bars and decision limit per biomarker by the grey horizontal line. Note that cows from animal study II received two additional rbST injections after the biweekly treatment period.

For all of the candidate biomarkers large inter-individual physiological differences in biomarker levels were apparent as for example seen in the adaptation period of the treated animals. IGF-1, IGFBP2 and osteocalcin levels differed quite a lot between individual animals. Biomarker levels are known to be influenced by many factors such as age and state of lactation. Nevertheless, the expected variation is much smaller than in athletes tested for ST abuse as already discussed above. Note that we accounted for the variation in our untreated reference population used to assess the decision limits. Also the response upon rbST treatment differed in every individual cow. Some cows showed a big increase in IGF-1 levels short after injection while others did not show any response above decision limit (non-responders). Also for osteocalcin, some cows hardly showed any response after treatment.

The predictive power of each candidate biomarker was assessed by calculating true-positive rates for all samples from rbST-treated cows in their treatment and withdrawal period (**Figure 3**). False-positive rates were calculated from untreated cows during the whole animal experiment (adaptation period samples from all cows and all the samples from untreated cows). High true-positive rates were reached by IGF-1 already at the beginning of the treatment period. Similar response patterns were observed for both studies. Only the double injections in study I led to a changed IGF-1 pattern. Also for the anti-rbST-antibodies, high true-positive rates of 75 % were seen after the second rbST injection. But the response was study-dependent: while the animals from study I (equal age of 5 years) were found positive after the second injection until the end of the study period, a gradual decrease of the number of positively found animals was observed in study II (age ranged from 2 to 8 years). This could be due to the different ages of the animals in study II. We saw that the antibody response tended to be higher in the older animals. Younger animals also showed antibody response, which declined more quickly than for the older animals. For osteocalcin, as already seen in **Figure 2 D.1**, some of the rbST-treated cows did not show osteocalcin concentrations beyond the decision limit in both studies. The increase of true-positive found samples at the end of the treatment period in study I was due to the double rbST injections. As already expected from the biomarker profiles (**Figure 2 B.1**), IGFBP2 did not show high true-positive rates, *i.e.*, none of the animals from study I and only some animals in study II were found above the decision limit. For all of the candidate biomarkers, false-positive rates were quite low,

indicating a high specificity of all of the biomarkers towards rbST treatment. Nevertheless, none of the candidate biomarkers reached the targeted 95 % true-prediction (<5 % false-compliant) rate at any time point required for a screening method according to Commission Decision 2002/657/EC [23].

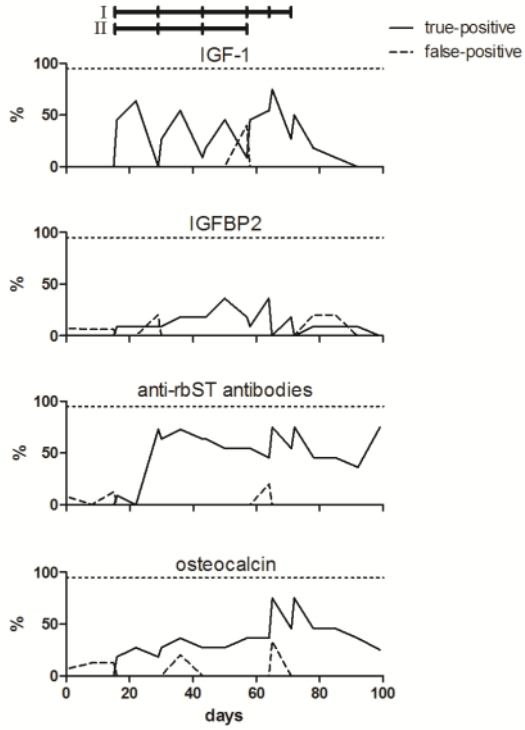

Figure 3: Predictive power of each single candidate biomarker for indicating rbST abuse. True-positive rates were calculated for all samples from rbST-treated cows in their treatment and withdrawal periods of study I and II. False-positive rates were calculated for all samples from untreated cows from the two animal studies (adaptation period samples from all cows and all the samples from untreated cows). Samples were considered positive if their biomarker value exceeded the respective decision limit. The treatment schedules of the two controlled animal studies are indicated by black horizontal bars on top of the graph. The targeted 95 % true-positive (<5 % false-compliant) rate according to 2002/657/EC is indicated by the dotted horizontal line.

2.3 Additive biomarker analysis

Since no single candidate biomarker was capable of predicting 95 % of the rbST-treated samples correctly, we tested different possibilities of combining biomarker results for improvement of the predictive power of our 4-plex FCIA. One approach to do this is the additive biomarker analysis. In **Table 3**, the number of candidate biomarkers responding above decision limit per cow and per time point within the animal studies is shown. As already described in **paragraph 2.2**, there were big inter-individual differences: some cows responded in many markers, others only in one or two for some time points. There were also two extreme cases: one cow responded in all four tested markers above decision limit at one time point and another rbST-treated cow did not show any response above decision limit at any day. On the other hand, there were untreated cows, which showed positive responses in one of the candidate biomarkers. **Figure 4** shows the true-positive rate obtained for the rbST-treated cows of both animal studies considering a sample positive, when at least one biomarker reacted above the respective decision limit.

Table 3: Number of biomarkers reacting above the respective decision limit per cow (in animal studies I and II) and day of the controlled animal studies. Each row represents one individual cow. Vertical dotted lines indicate the treatment time points in both animal studies.

	days	1	8	15	16	22	29	30	36	43	44	50	57	58	64	65	71	72	78	85	92	99
animal study I	rbST-treated	0	0	0	0	0	1	1	1	1	1	1	1	1	1	2	2	2	2	2	1	1
		0	0	0	1	1	0	0	2	0	0	2	1	2	2	2	2	2	2	2	1	0
		0	0	1	2	2	1	3	3	2	2	2	2	3	3	3	3	3	3	2	2	2
		0	0	0	0	0	1	1	1	1	1	1	1	2	1	2	1	1	1	1	1	1
	control	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0
		0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
		0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0
animal study II	rbST-treated		0	0	0	1	1	1	2	1	2	1	1	1	2		1		0	0	0	
		1	1	2	2	2	2	1	2	3	3	3	3	2	2		3		1	1	1	
		0	0	0	0	0	1	1	1	1	1	1	1	1	1		1		1	1	1	
		0	0	0	1	1	0	1	1	0	0	1	0	0	1		0		0	1	0	
		1	1	1	2	2	2	3	4	2	2	3	1	2	3		1		1	1	1	
		1	1	1	1	2	2	2	3	2	2	3	2	2	3		2		2	1	1	
			0	0	0	0	0	0	0	0	0	0	0	0	0		0		0	0	0	
	control	0	0	0	0	0	1	0	0	0	0	0	0	0	0		0		1	1	0	
		0	0	0	0	0	0	0	0	0	0	0	0	0	0		0		0	0	0	

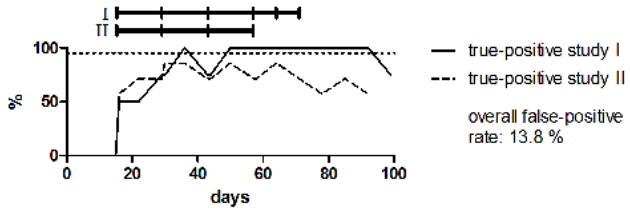

Figure 4: Predictive power (shown as true-positive and false-positive rates) of the additive biomarker analysis. True-positive rates were calculated for all samples from rbST-treated cows in their treatment and withdrawal periods of study I and II. False-positive rates were calculated for all samples from untreated cows from the two animal studies (adaptation period samples from all cows and all the samples from untreated cows). Samples were considered positive if one of the candidate biomarkers exceeded its respective decision limit. The treatment schedules of the two animal studies are indicated by black horizontal bars on top of the graph. The targeted 95 % true-positive (<5 % false-compliant) rate according to 2002/657/EC is indicated by the dotted horizontal line.

Although the true-positive rate obtained with the additive biomarker analysis was much higher than for the single candidate biomarkers, the 95 % true-positive rate required for a screening method was only reached at some time points in study I within the biweekly treatment period. After the double rbST injection in study I, all of the cows were found positive, but this treatment frequency will not be found in real practice. Furthermore, also with the additive biomarker analysis, quite some false-positive results were obtained throughout the whole study.

2.4 Statistical multiple biomarker analysis

Since the single biomarker analysis and additive biomarker analysis, which were both based on decision limits, did not deliver satisfying results for predicting rbST abuse, a different biomarker-combining approach was chosen for analysis of the data. *K*-nearest neighbours (kNN), a statistical prediction tool, was used to build a model from one group of data (Group A: all animals of animal study II and untreated animals from animal study I) and predict the results of Group B (rbST-treated cows of animal study I and 67 independent untreated cows) on basis of the built model. Eleven different models (one for every possible combination of two to four biomarkers) were evaluated to find the optimal biomarker combination for rbST abuse prediction. True-positive rates of Group B data were calculated for every biomarker combination over the time of the whole animal

study and are shown in **Figure 5** (**Supplementary Table 1** shows corresponding data). Six of the eleven different models yielded true-positive rates above the 95 % true-positive rate required for a screening method at several time points. For the biomarker combinations IGF-1 - IGFBP2 - anti-rbST-antibodies (IBA) and IGFBP2 - anti-rbST-antibodies - osteocalcin (BAO), only one time point within the biweekly treatment period was above 95 %. Note that in total, samples from eleven time points were obtained and analysed during the biweekly treatment period of animal study I. For the biomarker combinations IBAO and IA, four and six time points within the biweekly treatment period were above the 95 % target respectively. Seven time points above the 95 % target within the biweekly treatment period were reached by the prediction models based on the biomarker combinations IAO and AO. For the three best performing models (IA, AO and IAO), true-positive rates above 95 % (<5 % false-compliant) were reached following the second rbST injection. For IA, a true-prediction rate of almost 60 % was observed already one week after the first rbST injection, whereas AO only showed 30 %, which is in accordance with expectations since IGF-1 is a quick responding biomarker and osteocalcin has a delayed response time. Since all of the rbST-treated cows were detected by the three best performing models (IA, AO, IAO) at the end of the biweekly treatment period, no further increased prediction rate was observed due to the subsequent two weekly injections. After withdrawal of rbST, the true-positive rate of the models based on IA, AO and IAO remained above 95 % for two more weeks and then declined to 70 % four weeks after withdrawal.

Since we used all of the untreated animals of both animal studies for model building, false-positive rates for the eleven different models were calculated based on the results of the 67 independent untreated cows (**Supplementary Table 2**). For the three best-performing prediction models IA, AO and IAO, false-positive rates ranged from 10.6 % to 14.7 %, which was quite acceptable, since samples screened positive must be analysed by a subsequent confirmatory analysis method according to Commission Decision 2002/657/EC anyway [23]. The confirmation method is based on the detection of an N-terminal peptide of somatotropin, which has a different terminal amino acid in the recombinant form of the hormone [26].

We concluded from the results of the here presented studies that the AO biomarker combination is the preferred model for predicting rbST abuse. It yielded seven out of eleven time points above the 95 % target and if two biomarkers are equally well-suited for prediction as three biomarkers, the more simple version is favoured.

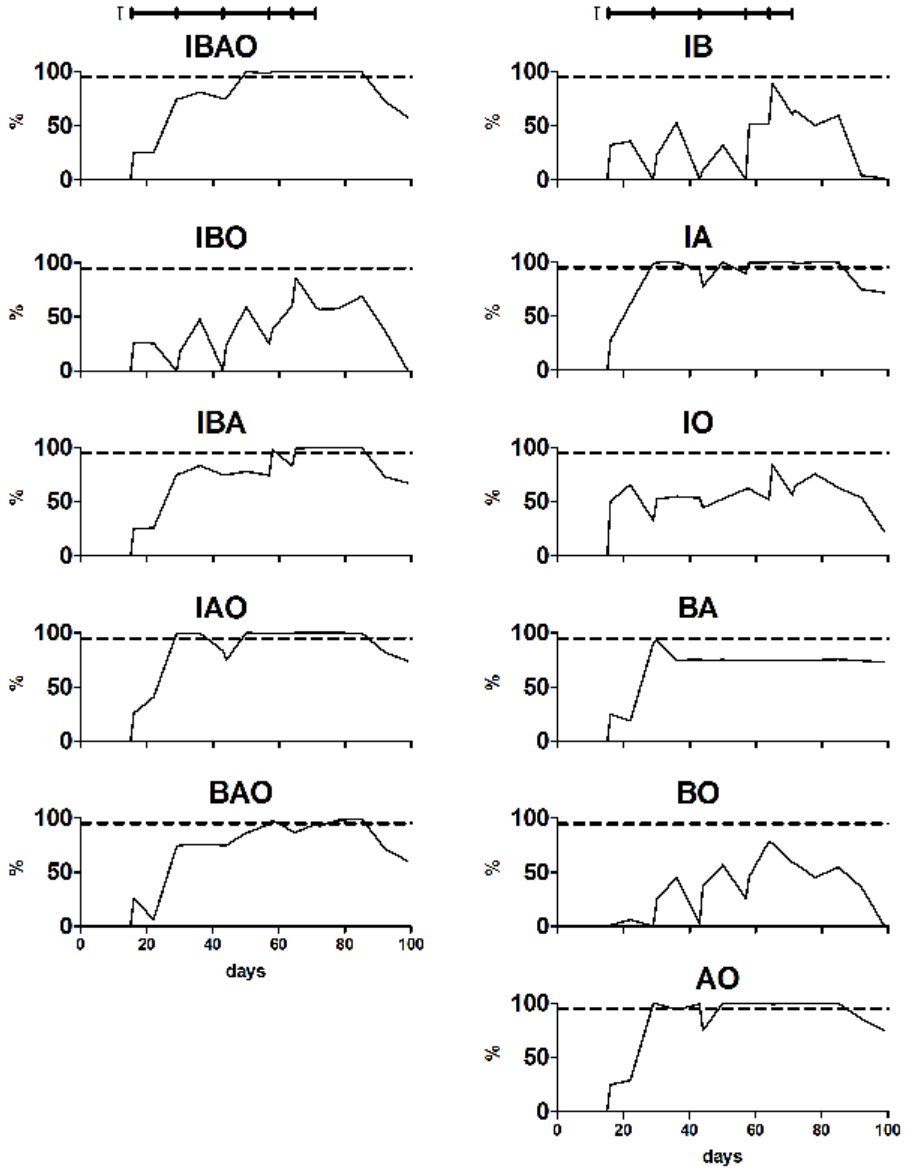

Figure 5: True-positive rates following statistical multiple biomarker analysis. True-positive rates, obtained with the prediction models based on the eleven different biomarker combinations, were calculated for rbST-treated cows from animal study I in their treatment and withdrawal period. The treatment schedules of animal study I is indicated by black

horizontal bars on top of the graphs. The targeted 95 % true-positive rate according to 2002/657/EC is indicated by the dotted horizontal lines.

The results obtained proof that the developed 4-plex FCIA reduced to an AO biomarker combination 2-plex FCIA, applied to an *in vivo* evaluation and combined with a thorough statistical multiple biomarker analysis can detect more than 95 % of the rbST-treated cows truly positive directly after the second rbST injection until the end of their treatment period and even thereafter. This meets the requirements of Commission Decision 2002/657/EC for a screening assay for the detection of banned veterinary drugs such as rbST [23].

When comparing with previously reported results of a 3-plex FCIA combining IGF-1, IGFBP2 and anti-rbST-antibodies [31], the models presented here seemingly perform somewhat less, especially at the beginning of the rbST treatment but the new models are much more realistic: Note that here, two completely independent groups were used for model building (Group A) and prediction (Group B), whereas in the 3-plex experiments [31], sample data used for prediction were from the same cows as the data on which the model was built, leading to an overestimation of true-positive results in that work.

2.5 Discussion

For the first time, to the best of our knowledge, a 4-plex biomarker assay development and data evaluation model is presented for the detection of rbST abuse, which fulfils the requirements of Commission Decision 2002/657/EC for screening assays [23]. Furthermore, the extensive *in vivo* validation with two independent rbST animal treatment studies followed by statistical analysis revealed that a combination of just two candidate biomarkers is actually adequate for detection of rbST treatment. Therefore, even a 2-plex version (namely the combination of anti-rbST-antibodies and osteocalcin) of our assay would already be fit-for-purpose based on the data presented here.

Nevertheless some issues should be considered. First of all, for obvious ethical and cost reasons, the rbST treatment period was limited to 8 weeks in our animal studies, so we do not know yet how the prediction models would perform for long-term treated animals. As can be seen in **Figure 2 C.1**, the antibody biomarker response declined somewhat in course of the treatment period and we do not know whether this would influence the prediction quality in a prolonged treatment. Second, in the presented animal studies, cows were treated with rbST for the first time in their lives and there are no data about

biomarker levels during a second treatment period after calving. According to the manufacturers' treatment schedule, dairy cows are treated starting from 9 weeks after calving until the end of the lactation (typically a biweekly treatment) and the following year again. Eppard *et al.* and Zwickl *et al.* reported that repeated treatment periods did not cause an immunological memory effect with enhanced antibody production in the second treatment period [33, 34]. For both situations, long-term treatment and repeated treatment, the IA, AO and IAO biomarker combinations should be tested and possibly the inclusion of other biomarkers could be considered. Since blood sampling in routine veterinary monitoring programmes might not be justified in some countries, we suggest a tiered approach according to Commission Decision 2002/657/EC. This would consist of three steps: First, a fast screening for anti-rbST-antibodies in tank milk using our previously described milk FCIA [29]. Second, in case of suspicious findings, a more detailed individual bovine serum biomarker profiling using the IA, AO or IAO FCIA presented here will provide additional evidence, since they are based on more biomarkers and data for individual cows. Note, that in practice, a whole stable and not an individual cow is treated with rbST, thus increasing the chance of detecting rbST use. And third, for final confirmation of rbST itself in serum samples of suspect individual cows, a highly sensitive mass spectrometric confirmatory method, which fulfils the 2002/657/EC confirmatory method requirements, is to be used [26].

2.6 Conclusion

In this study a multidisciplinary approach was used for the development of an *in vivo* validated screening assay for rbST abuse in dairy cows. Four candidate biomarkers for rbST abuse were assessed using a newly developed 4-plex flow cytometric immunoassay, *in vivo* validation studies and advanced statistics. Biomarkers indicative for rbST administration were evaluated based on two extensive animal studies with rbST-treated and untreated animals and an additional untreated reference population. Different data evaluation approaches were tested. The prediction tool kNN using a biomarker combination endogenously produced antibodies against rbST and osteocalcin proved to be very reliable and correctly predicted 95 % of the treated samples starting from the second rbST injection until the end of the treatment period and even thereafter. This reduced 2-plex FCIA method (consisting of biomarkers anti-rbST antibodies and osteocalcin) combined with the statistical analysis approach was shown to be a fast, reliable and cost-effective approach to screen for rbST abuse in dairy cattle. These methods and models can be included in routine veterinary control programmes in the European Union for detection of rbST abuse and also in the control of rbST-free dairy farms in the United States of America

and other countries.

3 Materials and methods

3.1 Ethics statement

Permission for animal study I (EC2007/71) was obtained from the Ethical Commission of the Faculty of Veterinary Medicine of Ghent University (Belgium) on basis of the Dutch law on animal studies (Wet op de Dierproeven). For animal study II, permission (EC2010-21) was obtained from the Ethical Commission of the Animal Science Group of Wageningen University and Research Centre in Lelystad (The Netherlands).

3.2 Chemicals and instruments

Ultrasonic bath, monosodium phosphate monohydrate (NaH_2PO_4 x H_2O), potassium dihydrogen phosphate (KH_2PO_4), sodium chloride (NaCl), sodium azide (NaN_3) and Tween 20 were obtained from VWR International (Amsterdam, The Netherlands). Microcentrifuge Model 16K was purchased from Bio-Rad (Veenendaal, The Netherlands). Protein LoBind Tubes, Safe Lock Tubes (amber) and Centrifuge 5810R were obtained from Eppendorf (Hamburg, Germany). 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), 2-(N-morpholino)ethanesulfonic acid (MES) hydrate, ovalbumin and bovine serum albumin (BSA) were obtained from Sigma-Aldrich (St. Louis, MO, USA). MultiScreen HTS filter plates were purchased from Millipore (Billerica, MA, USA). Purified bovine osteocalcin and mouse anti-bovine osteocalcin antibodies were obtained from Haematologic Technologies, Inc. (Essex Junction, VT, USA). Insulin-like growth factor-I (IGF-I; human recombinant) was purchased from Fitzgerald Industries International (North Acton, MA, USA). Insulin-like growth factor binding protein-2 (IGFBP-2; bovine recombinant, receptor grade) was purchased from IBT (Reutlingen, Germany). Mouse anti-IGF-1 was supplied by LifeSpan BioSciences, Inc. (clone SPM406, Seattle, WA, USA) and the rabbit anti-IGFBP-2 was from United States Biological (Swampscott, MA, USA). Monsanto rbST standard was obtained from the National Hormone & Peptide Program (NHPP) of Dr Parlow (Torrance, CA, USA). R-Phycoerythrin (PE)-labelled goat anti-bovine immunoglobulins (GAB-PE) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and R-Phycoerythrin (PE)-labelled goat anti-mouse immunoglobulins (GAM-PE) and goat anti-rabbit immunoglobulins (GAR-PE) were purchased at Prozyme (San Leandro, CA, USA). Donor adult bovine serum was from HyClone (South Logan, UT, USA). Sodium hydroxide (NaOH), disodium hydrogen phosphate dihydrate (Na_2HPO_4 x 2 H_2O) and hydrochloric acid (HCl) were purchased from

Merck (Darmstadt, Germany). SeroMAP microspheres (microsphere sets 025, 050, 078 and 084) and sheath fluid were obtained from Luminex (Austin, TX, USA). The Luminex 100 IS 2.2 system consisting of a Luminex 100 analyser and a Luminex XY Platform was purchased from Applied Cytometry Systems (ACS, Dinnington, Sheffield, South Yorkshire, UK). Snijder Test tube rotator was from Omnilabo International (Breda, The Netherlands). 10 mL polypropylene tubes were obtained from Greiner Bio-One (Alphen aan de Rijn, The Netherlands). Glycine was purchased from Duchefa (Haarlem, The Netherlands) and sulfo-N-Hydroxysuccinimide (Sulfo-NHS) from Fluka (Buchs, Switzerland). Sodium dodecyl sulphate (SDS) was obtained from Serva (Heidelberg, Germany). The microtiter vari-shaker was purchased from Dynatech (Guernsey, UK). Posilac® (rbST) 500 mg single dose syringes and syringes with only the slow release formula were obtained from Monsanto Company (St. Louis, MO, USA) for animal study I and from Elanco Animal Health (Greenfield, IN, USA) for animal study II.

3.3 Buffers and solutions

Buffers and solutions were prepared as follows: phosphate-buffered saline (PBS; 154 mM NaCl, 5.39 mM Na_2HPO_4, 1.29 mM KH_2PO_4, pH 7.4), PBST (PBS, 0.05 % v/v Tween 20), PBSTB (0.1 % w/v BSA in PBST), glycine solution I (GS I; 27.5 mM glycine, pH 0.5 adjusted with HCl), glycine solution II (GS II; 400 mM glycine, 0.3 % w/v SDS, pH 10 adjusted with NaOH), MES buffer (50 mM, pH 5), blocking buffer (PBS, 0.1 % w/v BSA, 0.02 % v/v Tween 20, 0.05 % w/v NaN_3).

3.4 Sample materials

Samples from different sources were used for analysis. Serum samples from two independent controlled animal treatment studies were used. In animal study I, eight Holstein cows were selected. These cows were all about 5 years old, divided in two groups of 4 animals each and treated with 500 mg rbST in slow-release formula or slow-release formula only. After a two-week adaptation period, they received every second week an injection, in total 4-times in accordance with the suggested treatment schedule by the manufacturer (http://www.fda.gov/downloads/AnimalVeterinary/Products/ApprovedAnimalDrugProducts/FOIADrugSummaries/ucm050022.pdf; accessed 2012 Apr 4). Since we did not know for sure whether we would see any response, the cows were thereafter treated two times more but with a weekly interval, followed by a final 4-week withdrawal period. In animal study II, 10 Holstein dairy cows were divided in two groups. In contrast to animal study I, these cows were of different age (2-8 years).

After a 2-week adaptation period, 8 cows were treated every second week with 500 mg rbST in a slow-release formula during 8 weeks and 2 control cows were treated with the slow-release formula only. The biweekly treatment period according to manufacturers' guidelines was directly followed by a 4-week withdrawal period. In both studies, blood sampling was scheduled similarly: During the two week adaptation period blood samples were collected weekly; during the treatment period blood samples were collected a day before, a day after and a week after injection and during withdrawal, blood samples were collected weekly for four more weeks, which yielded 21 serum samples per cow in animal study I and 18 serum samples per cow in animal study II. The treatment schedule and blood sampling time points are shown in **Supplementary Figure 2**. Unfortunately, one untreated cow died in the beginning of animal study I because of swollen hocks, which led to general inflammation and sepsis. Therefore in study I, results could be obtained for 4 rbST-treated and 3 untreated cows. Furthermore, one cow from animal study II fell sick (hock joint inflammation, lung infection and sepsis) in course of the experiment and its biomarker level results were excluded from statistical analysis. For investigation of natural physiological variations in biomarker levels, sera from 67 healthy, lactating cows varying in the age of two to eleven years, from two different locations, in different stages of their lactating cycle were analysed, to reflect a normal population of untreated dairy cows. Based on the origin of these animals the assumption of being untreated with rbST was justified.

3.5 Standard preparation

Protein standards of IGF-1, IGFBP2 and osteocalcin, prepared in serum-matched buffer (80 mg mL^{-1} BSA in PBS), were used for standard curves ranging from 0.08 to 20 ng mL^{-1} for IGF-1 and osteocalcin and from 0.2 to 50 ng mL^{-1} for IGFBP2. Also blank standard samples (80 mg mL^{-1} BSA in PBS without any IGF-1, IGFBP2 and osteocalcin) were measured. Note that no standards are commercially available for anti-rbST-antibodies.

3.6 Sample pretreatment

A generic sample pretreatment procedure which was crucial for removing nonspecific interferences and making the candidate biomarkers accessible for detection was described previously [4, 28, 29], is depicted in **Figure 1** and described in-depth in the supplementary information.

3.7 Microsphere preparation

Covalent coupling of 100 µg mL^{-1} Monsanto rbST standard, 100 µg mL^{-1} IGF-1 and 10 µg mL^{-1} IGFBP2 to seroMAP microspheres (sets 050, 025, 078 respectively) was described before [4, 28, 29]. Coupling 75 µg mL^{-1} osteocalcin to microspheres (set 084) was done following the same procedure.

3.8 Four-plex flow cytometric immunoassay procedure

The assay procedure for detection of three biomarkers was described before [31] and is similar for four biomarkers in the present study and summarized in **Figure 1**. The samples were analysed in duplicate in the flow cytometer at 1 µL s^{-1} until 50 microspheres per set were counted, up to a maximum of 50 µL per sample. A typical analysis of a full 96 well microtiter plate takes 3.5 hours starting from raw serum until the results are obtained.

3.9 Data analysis

Raw median fluorescence intensities (MFIs) were measured by the flow cytometer for every single candidate biomarker. Every sample was measured in duplicate and MFIs were averaged before further analysis. For IGF-1, IGFBP2 and osteocalcin, B/B0 values were calculated per sample by dividing the measured MFI by the MFI of a blank biomarker-free standard. Then, concentrations were recalculated from standard curves (non-linear four-parameter curve fit) using GraphPad Prism program (GraphPad Software Inc., San Diego, USA) for IGF-1 and osteocalcin. For IGFBP2, no complete inhibition could be obtained with the available standard protein, therefore, no actual concentrations were determined and B/B0 values were simply used. For anti-rbST-antibodies, which are endogenously produced by the cow in response to rbST treatment, no standard was available. To be able to normalize, measured sample MFIs were divided by the MFI of one serum sample, which was measured every time (B/Bd). This serum was donor adult bovine serum which was a mixture of sera from many cows from one herd. Since this is produced in large amounts, it can be used for a long time with constant quality.

To assess the 4-plex FCIA quality and compare it to other methods, assay performance characteristics were calculated, such as IC_{50}, interassay and intraassay variation. For IGF-1, IGFBP2 and osteocalcin, IC_{50} was read from standard curves at 50 % inhibition of the signal of the blank. For all candidate biomarkers, interassay variation was determined by measuring 8 different serum samples on 8 days. Mean of results (concentrations for IGF-1

and osteocalcin, B/B0 for IGFBP2 and B/Bd for anti-rbST-antibodies), standard deviation and percentaged standard deviation (% CV) were calculated for every serum. The average of the 8 percentaged standard deviations was the interassay deviation. Intraassay variation was calculated the same way from 8 repetitions of 8 sera within one microtiter plate.

3.9.1 Single biomarker analysis approach

Using a single biomarker for prediction of unknown samples as rbST-treated or untreated, the calculation of decision limits for each biomarker was necessary. These were based on the results obtained from a population of 67 untreated dairy cows being diverse in age, in lactation stage and in origin. For every biomarker, results were averaged and two-times the standard deviation was added to obtain the decision limit. Samples found to show concentrations (for IGF-1 and osteocalcin), B/B0 (for IGFBP2) or B/Bd (for anti-rbST antibodies) beyond the respective calculated decision limit, were considered as rbST-treated (positive). True-positive and false-positive rates could be calculated for every single biomarker from the results of the controlled animal studies.

3.9.2 Additive biomarker analysis

After evaluating biomarker profiles and true-positive rates based on single biomarkers, an additive biomarker approach was tested. Here, a sample was considered as rbST-treated when at least one of the candidate biomarkers reacted above decision limit and also here, true-positive and false-positive rates were calculated.

3.9.3 Multiple biomarker statistical approach

After evaluating single candidate biomarkers and testing the additive biomarker approach, we assessed how well a statistical combination of two to four markers was capable to predict rbST abuse. Therefore, a *k*-nearest neighbours prediction model (kNN) in the R environment [42] and functions available in R package e1071 [43] were used to evaluate all eleven theoretical combinations of two to four biomarkers. As in the single biomarker approach, recalculated concentrations for IGF-1 and osteocalcin and B/B0 signals for IGFBP2 as well as B/Bd signals for rbST-induced antibodies for every sample from the animal studies were included in the data analysis. For obvious ethical reasons, we had only a limited number of rbST-treated animals available. Therefore, all serum sample time points per cow (21 time points in the trial period of 14 weeks for animal study I and 18 time points in the trial period of 13 weeks for animal study II) were used for data analysis,

despite the fact that these are not completely independent. However, only data from independent cows were used for model building and sample prediction.

First, the whole data set was divided into two groups: Group A data were used to build the time-point-independent prediction model. Therefore and to use sufficient sample numbers for the model building, this group contained all data from animal study II (diverse population with biweekly treatment only). Furthermore, since two control animals were not enough to represent untreated cows, Group A also contained the data from the untreated animals of animal study I. In total, 98 samples from treated and 119 samples from untreated cows were used for model building. Group B data were used for prediction based on the Group A model. Group B contained the data from the rbST-treated cows of animal study I (uniform in age with biweekly treatment and two additional weekly injections) and the 67 untreated cows. Note that these are sample data independent from Group A data. For model building of the Group A data, a training and test set were chosen by using a stratified repeated random sub-sampling approach, which means that 70 % of the rbST-treated and 70 % of the untreated samples were selected for the training set and the remaining 30 % of both groups for the test set for internal validation, which is necessary to build a strong model. Subsequently, concentrations, B/B0 and B/Bd values of the training set were auto-scaled and a kNN model was built on the training set data. The optimal number of k ($1 \leq k \leq 10$) was chosen based on the bootstrapping approach [44] leaving out 10 % of the training data (randomly with replacement), which was repeated 10-times. The resulting model was validated with the test set data and thereafter used for predicting Group B data. To obtain an average performance of the model, this procedure was run 10,000 times; every time different randomly chosen training and test sets of Group A data were applied. Correctly and falsely predicted results were evaluated for Group B and a true-positive rate and false-positive rate could be calculated for every Group B sample.

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Supplementary information

Serum preparation

After blood collection, blood samples were kept at room temperature for 4 h to coagulate. After coagulation, samples were centrifuged for 10 min at 3,000 g, and sera were collected. Serum samples were stored at -80 °C.

Generic serum pretreatment

For the generic serum pretreatment procedure 25 µl glycine solution I (27.5 mM glycine pH 0.5) were added to 25 µl serum or standard sample in a polypropylene tube under constant vortexing. After 60 minutes incubation at room temperature, 50 µl glycine solution II (400 mM Glycine, 0.3 % m/v SDS, pH 10) were added under constant vortexing and samples were further diluted by addition of 1.9 mL 0.1 % BSA in PBST (in total 80-times diluted).

Four-plex flow cytometric immunoassay procedure

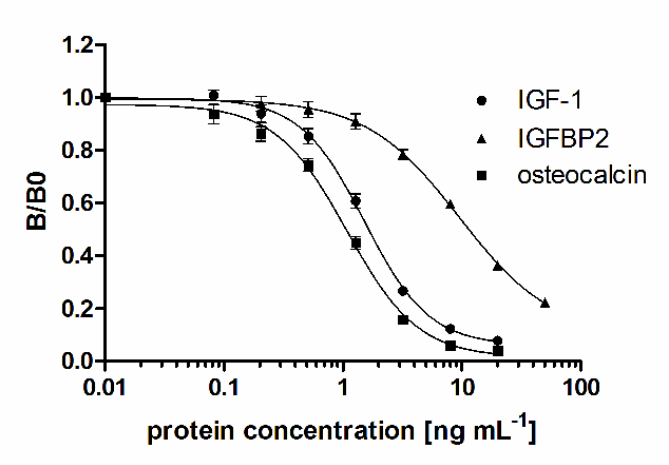
For the 4-plex FCIA procedure, 10 µl primary antibody mixture (1:625 mouse anti-IGF-1, 1:25,000 rabbit anti-IGFBP2, 1:100,000 mouse anti-osteocalcin) were added to 100 µl pretreated and diluted sample in a filter bottom microtiter plate and incubated at 4 °C under orbital shaking for 15 minutes. Thereafter, 10 µl microsphere mixture suspension (containing approximately 1250 microspheres per microsphere set) were added to each well and incubated at 4 °C for one hour under orbital shaking. After centrifugation at 130 g for one minute, everything not bound to the microspheres was removed from the well. For washing, 200 µl PBST were added and the filter bottom microtiter plate was centrifuged again at 130 g for one minute. Then, 125 µl PE-antibody mixture (1:625 goat anti-mouse PE, 1:1,000 goat-anti rabbit PE, 1:1,000 goat anti-bovine PE) were added to each well and the plate was incubated for 30 minutes at 4 °C under orbital shaking. Then, the plate was centrifuged at 130 g for one minute and 125 µl PBST were added. Thereafter, the plate was put into the LX-100 flow cytometer for measurement. Microspheres from every sample were analysed in a flow of 1 µl s^{-1} until 50 microspheres per set were counted up to a maximum of 50 µL per sample. Each microsphere set was identified by its unique colour by a red laser and the fluorescence intensity of the PE attached to each microsphere was measured by a green laser. Median fluorescence intensities were obtained from every analysed sample.

Supplementary Table 1: True-positive rates of the statistical multiple biomarker analysis. True-positive rates, obtained with the prediction models based on the eleven different biomarker combinations, were calculated for rbST-treated cows from animal study I in their treatment (day 16 – 71) and withdrawal period (day 72 -99).

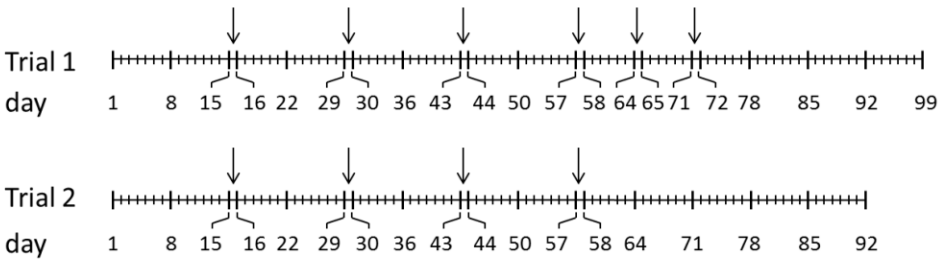
biomarker combination	IBAO	IBO	IBA	IAO	BAO	IB	IA	IO	BA	BO	AO
days	true-positive rate [%]										
1	0	0	0	0	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0	0	0	0	0
15	0	0	0	0	0	0	0	0	0	0	0
16	25.0	26.4	25.0	25.8	25.1	32.2	27.7	50.2	25.0	1.0	25.0
22	25.1	25.4	25.9	40.6	6.5	35.6	61.4	65.8	18.6	6.3	28.9
29	74.5	0.0	75.3	99.7	73.8	0.0	99.1	32.6	90.4	0.3	99.8
30	75.1	18.3	75.9	99.7	75.0	22.9	100.0	52.5	93.5	25.4	99.8
36	81.2	47.6	83.2	99.9	75.3	52.7	100.0	54.7	75.0	44.9	94.3
43	75.0	0.4	75.0	83.5	75.0	0.5	93.5	53.3	75.3	3.0	99.6
44	75.0	24.0	75.0	75.0	75.0	8.9	77.7	44.4	75.0	37.0	75.0
50	100.0	59.2	78.0	100.0	86.0	32.0	100.0	52.8	75.2	56.8	100.0
57	98.1	25.1	74.6	99.9	94.4	0.1	89.4	61.6	74.7	25.7	100.0
58	99.9	38.7	98.4	100.0	97.8	51.9	100.0	61.5	75.0	46.0	100.0
64	100.0	60.3	83.0	100.0	87.8	51.7	99.9	52.0	75.0	78.6	100.0
65	100.0	86.1	99.3	100.0	86.9	89.2	100.0	84.2	75.0	76.4	99.6
71	100.0	59.2	100.0	100.0	94.6	60.6	100.0	56.2	74.7	59.4	100.0
72	100.0	56.9	100.0	100.0	92.5	63.9	99.1	65.0	75.0	58.0	100.0
78	99.9	57.5	100.0	100.0	98.1	50.4	100.0	75.7	75.0	45.2	100.0
85	99.6	69.0	100.0	100.0	99.5	59.4	100.0	62.8	75.5	54.7	100.0
92	72.6	37.5	73.2	82.0	71.7	4.0	74.8	53.6	74.1	36.2	85.8
99	57.8	0.1	67.3	74.0	60.3	1.5	71.8	22.3	73.2	0.3	74.8

Supplementary Table 2: False-positive rates of the statistical multiple biomarker analysis. Results were calculated for 67 independent untreated cows predicted with the eleven different biomarker combination models.

biomarker combination	IBAO	IBO	IBA	IAO	BAO	IB	IA	IO	BA	BO	AO
	false-positive rate [%]										
67 untreated cows	5.5	9.7	6.6	10.6	8.8	25	14.7	24.6	13.4	11.7	11.8



Supplementary Figure 1: Standard curves of the three rbST-dependent biomarkers IGF-1, IGFBP2 and osteocalcin. Each data point is the mean of 8 separate measurements in a serum-matched buffer (80 mg mL^{-1} BSA in PBS solution). All curves relate to 80-times diluted sera.



Supplementary Figure 2: Treatment schedule and sampling time points for animal studies I and II. Arrows indicate the treatment of the cows with rbST in slow-release formula or the slow-release formula only; bold vertical lines indicate blood sampling time points.

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Potential of treatment-specific protein biomarker profiles for detection of hormone abuse in cattle

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Abstract

Targeted protein biomarker profiling is suggested as a fast screening approach for detection of illegal hormone treatment in meat production. The advantage of using biomarkers is that they mark the biological response, thus are responsive to a panel of substances with similar effects. In a preliminary feasibility study, a 4-plex protein biomarker flow cytometric immunoassay (FCIA) previously developed for the detection of recombinant bovine somatotropin (rbST) was applied to bovines treated with steroids, such as estradiol, dexamethasone and prednisolone. Each treatment resulted in a specific plasma biomarker profile for insulin-like growth factor-1 (IGF-1), IGF binding protein 2, osteocalcin and anti-rbST-antibodies, which could be distinguished from the profile of untreated animals. Summarizing, the 4-plex biomarker FCIA is, apart from rbST, also capable of detecting treatment with other growth-promoting agents and therefore clearly shows the potential of biomarker profiling as a screening method in veterinary control. It is proposed to perform additional validation studies covering high numbers of treated and untreated animals in order to support inclusion or adaptation of protein biomarker approaches in future monitoring regulations.

1 Introduction

Protein biomarker profiling has been suggested as a fast screening approach for detection of illegal treatment practices in human sports doping [1], and in veterinary control [2-6] for detection of banned growth-promoting agents (Directives 2008/97/EC, 96/22/EC and 96/23/EC [7-9]). Using protein biomarkers has several advantages: First, they are indicative for any substance having the relevant effect and can therefore give a comprehensive first screening result whether any growth-promoting agent was used. Even the use of designer substances of unknown chemical structure could be detected. Second, biomarker profiles remain changed for a longer period of time than the abused substance can be detected in circulation [10, 11]. And third, the use of fast screening methods prior to an extensive instrumental confirmatory method is beneficial because of its time saving as it already pinpoints suspicious samples and omits compliant samples from the subsequent analysis.

Nevertheless, up till now, protein biomarker analysis tools were specifically developed for only a specific treatment setting [12]. In this study, we investigated if a previously developed 4-plex biomarker flow cytometric immunoassay (FCIA) for the detection of the protein hormone recombinant bovine somatotropin (rbST) abuse [4] can also be used to detect treatments with steroid hormones such as estradiol (E_2), dexamethasone and prednisolone. In contrast to rbST, steroids are not species-specific and also exert hormonal effects in humans if residues are consumed. This highlights the importance of a fast screening method for steroid abuse detection in meat production to maintain the highest possible food safety for the customer.

The biomarker panel of the 4-plex FCIA consisted of insulin-like growth factor 1 (IGF-1), IGF binding protein 2 (IGFBP2), osteocalcin and antibodies produced by cows upon rbST treatment (anti-rbST-antibodies). This biomarker panel was specifically developed for the detection of rbST abuse in dairy cattle and biomarkers were chosen based on information from literature stating specific changes in biomarker concentrations after somatotropin treatment [13-19]. Nevertheless, three out of these four biomarkers, namely IGF-1, IGFBP2 and osteocalcin, are also known to be influenced by steroid hormone treatments such as glucocorticoids and sex steroids [2, 20-27]. IGF-1 and IGFBP2 are members of the somatotropin/IGF-axis, which is usually involved in growth-related mechanisms and is thereby affected by growth-inducing or -repressing substances, such as growth-promoting steroids [2, 20, 22, 23, 25, 26]. One possible target of somatotropin, IGF-1 and steroid hormone action is bone, which reacts with an increase or decrease in bone turnover. Osteocalcin is a marker of bone turnover and therewith, osteocalcin concentrations

respond upon administration of bone turnover altering drugs [21, 24, 27]. Therefore, the selected biomarkers might also be influenced not only by rbST but also by other growth promoting steroids. To proof this, in the here presented study, plasma samples from an animal study with sex steroid (estradiol) and glucocorticoid (prednisolone and dexamethasone) treatments were analysed using the previously developed 4-plex FCIA. The obtained biomarker profiles of the treated groups were compared with the ones from a control group. Based on the results, the potential of targeted protein biomarker profiling is discussed.

2 Materials and methods

2.1 Chemicals

17-β-estradiol-3-benzoate was obtained from Intervet (Boxmeer, The Netherlands) and dexamethasone-21-sodium phosphate from Fort Dodge Animal Health (Bologna, Italy). Prednisolone was from Novosterol (CEVA VETEM SpA, Agrate Brianza, Italy) and the rbST standard from the National Hormone & Peptide Program of Dr Parlow (Torrance, CA, USA). IGF-1 standard was purchased from Fitzgerald Industries International (North Acton, MA, USA) and IGFBP2 standard from IBT (Reutlingen, Germany). Osteocalcin standard and mouse anti-osteocalcin antibody were from Haematologic Technologies, Inc. (Essex Junction, VT, USA) and seroMAP microspheres (sets 050, 025, 078 and 084) from Luminex (Austin, TX, USA). Bovine serum albumin (BSA) and 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) were bought from Sigma-Aldrich (St. Louis, MO, USA) and sulfo-N-Hydroxysuccinimide (S-NHS) from Fluka (Buchs, Switzerland). Glycine was from Duchefa (Haarlem, The Netherlands) and the polypropylene tubes from Greiner Bio-One (Alphen aan de Rijn, The Netherlands). Sodium dodecyl sulfate (SDS) was obtained from Serva (Heidelberg, Germany) and Tween 20 from VWR International (Amsterdam, The Netherlands). Mouse anti-IGF-1 antibody was bought from LifeSpan BioSciences, Inc. (Seattle, WA, USA), rabbit anti-IGFBP2 antibodies from United States Biological (Swampscott, MA, USA) and the filter bottom microtiter plates from Millipore (Billerica, MA, USA). Goat anti-mouse R-phycoerythrin (PE) antibody and goat-anti rabbit PE antibody were obtained from Prozyme (San Leandro, CA, USA) and goat anti-bovine PE antibody from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The LX-100 flow cytometer was from Applied Cytometry Systems (Dinnington, Sheffield, South Yorkshire, UK) and the software Prism 5 from GraphPad Software, Inc. (San Diego, CA, USA).

2.2 Animals and treatment

Since in the here presented study only the feasibility of the chosen biomarkers was tested, Directive 2010/63/EU [28] describing the 3-R-principle in animal testing was considered and no new animal experiment was performed. Instead of that, only a limited number of samples, which originated from a previous study conducted at Università di Torino, was available and used to test the developed method. To assess levels of the four biomarkers after treatment of estradiol (E_2), dexamethasone and prednisolone, plasma samples from an animal study were used. Twenty-four male Charolais bovines (17-22 months) were randomly divided in four groups and received the following treatments: six animals in group E_2 received 0.01 mg (kg bodyweight)$^{-1}$ per week 17-β-estradiol-3-benzoate by intramuscular injection for 6 weeks. All six animals in group DEX were treated with 0.7 mg day^{-1} dexamethasone-21-sodium phosphate per os for 40 days and all six animals in group PRED received 15 mg day^{-1} prednisolone per os for 30 days. The control group consisted of 6 untreated animals. These steroid treatments were chosen because they are expected to be illegally used in meat production as growth-promoting agents [29] and dosages and administration routes were determined based on literature [30, 31]. Blood samples were taken throughout the treatment study; sampling occurred through the external jugular vein with evacuated EDTA tubes to prepare plasma samples by centrifugation at 2000 g for 20 minutes and samples were stored at -80 °C until further use. Plasma samples taken at days 25 (during the treatment) and 43 (after the last treatment) were chosen for analysis. Samples from day 43 reflect the situation of when the animals arrive at the slaughterhouse including a delay after the last treatment (3 days for the E_2- and dexamethasone-treated and 6 days for the prednisolone-treated group). The treatment and sampling schedule is depicted in **Figure S1** in the supporting information. The animal studies were authorized by the Italian Ministry of Health and the Bioethics Committee of the University of Turin.

2.3 Sample pre-treatment, microsphere preparation and assay procedure

For microsphere preparation, 100 µg mL^{-1} rbST standard, 100 µg mL^{-1} IGF-1, 10 µg mL^{-1} IGFBP2 and 75 µg mL^{-1} osteocalcin were covalently coupled to colour-encoded seroMAP microspheres (sets 050, 025, 078 and 084 respectively) by using the sulfo-NHS-EDC coupling chemistry as it was described before [4, 11, 32, 33]. Standard curves were prepared with IGF-1, IGFBP2 and osteocalcin standard proteins in plasma-matched buffer [80 mg mL^{-1} BSA in phosphate-buffered saline (PBS)]. Obviously no standard curves can be produced for the biomarker anti-rbST-antibodies. For the generic plasma pretreatment procedure, 25 µl glycine solution I (27.5 mM glycine, pH 0.5) were mixed with 25 µl plasma

or standard sample in a polypropylene tube under constant vortexing. After 60 minutes incubation at room temperature, 50 µl glycine solution II (400 mM Glycine, 0.3 % m/v SDS, pH 10) were added and samples were further diluted by addition of 1.9 mL 0.1 % BSA in PBST (0.05 % v/v Tween 20 in PBS), which led to an overall dilution of the plasma by 80-times. Detection assays for IGF-1, IGFBP2 and osteocalcin are of competitive format, where free protein in the blood sample inhibits binding of the primary detection IgG to the respective protein-coupled microsphere. The assay for detection of anti-rbST-antibodies is a direct assay, where the antibodies originating from the blood sample, directly bind to rbST-coupled microspheres. The 4-plex FCIA procedure was described previously [4] and the assay principle is depicted in **Figure S2** in the supporting information. Briefly, 10 µl primary detection immunoglobulin G (IgG) mixture (1:625 mouse anti-IGF-1, 1:25,000 rabbit anti-IGFBP2, 1:100,000 mouse anti-osteocalcin) were added to 100 µl pretreated and diluted sample in a filter bottom microtiter plate and incubated at 4 °C under orbital shaking for 15 minutes. Thereafter, 10 µl microsphere mixture suspension (containing approximately 1250 microspheres per microsphere set) were added to each well and incubated at 4 °C for one hour under orbital shaking. Microspheres were washed once with PBST, then 125 µl PE-IgG mixture (1:625 goat anti-mouse PE, 1:1,000 goat-anti rabbit PE and 1:1,000 goat anti-bovine PE) were added to each well, and finally the plate was incubated for 30 minutes at 4 °C under orbital shaking. Then, the plate was centrifuged at 130 g for one minute and 125 µl PBST were added. Thereafter, the plate was put into the LX-100 flow cytometer for measurement. Microspheres from every sample were analysed in a flow of 1 µl s^{-1} until 50 microspheres per set were counted up to a maximum of 50 µL per sample. Each microsphere set (= each biomarker assay) was identified by its unique colour by a red laser and the fluorescence intensity of the reporter PE attached to each microsphere was quantified by a green laser. Median fluorescence intensities (MFI) were obtained from every analysed sample and biomarker concentrations of IGF-1 and osteocalcin were calculated based on standard curves obtained by using the software Prism 5. For IGFBP2, B/B0 values were used, because the slope of the standard curve was too flat to work with calculated concentrations. When using a standard curve with a flat slope for calculating concentrations, large concentration differences will be obtained even though only a small difference in signal was measured. For anti-rbST-antibodies, raw MFI signals were used. The average biomarker levels of the six cows in each group are plotted in **Figure 2** and standard deviations are shown as error bars. Biomarker levels of the different treatment groups were compared to their respective time-matched control group by using the unpaired, two-tailed Student's t-test.

3 Results and discussion

Monitoring biomarker profiles as an indication of possible hormone abuse becomes more and more prevalent in sports doping and veterinary control. Since biomarkers reflect a biological effect and this effect might also be achieved by other hormonal substances, several treatments can be indirectly detected by altered biomarker profiles. A 4-plex protein biomarker FCIA was previously developed to detect rbST abuse in dairy cattle based on an altered biomarker profile. Biomarkers analysed were IGF-1, IGFBP2, osteocalcin and anti-rbST-antibodies endogenously produced by the cow as a response upon rbST treatment. The tested biomarkers were shown to be all responsive to rbST in a previous study: IGF-1 and osteocalcin concentrations were increased, IGFBP2 levels were decreased and anti-rbST antibodies were induced by rbST treatment in dairy cattle [4]. In this work, the 4-plex protein biomarker FCIA was used to test whether beef cattle also shows a change in biomarker levels after steroid treatment. Steroids used were a sex steroid (estradiol, E_2) and glucocorticoids (prednisolone or dexamethasone), which are occasionally used in veterinary practice to illegally improve meat production and quality. Plasma biomarker concentrations for IGF-1 and osteocalcin were calculated based on standard curves prepared in plasma-matched buffer (**Figure 1**). IGFBP2 was analysed using B/B0 values as already described earlier [4]. For the biomarker anti-rbST-antibodies, no standard curve could be obtained, therefore, the presence of these antibodies was expressed as raw median fluorescence intensity (MFI) signals.

Two different time points in all four treatment groups were analysed. One time point on day 25 was in the middle of the treatment period and reflects on-farm testing, whereas the other one on day 43, which was a few days after discontinuation of the treatments (see **Figure S1** in the supporting information), reflects when the animals arrive at the slaughterhouse.

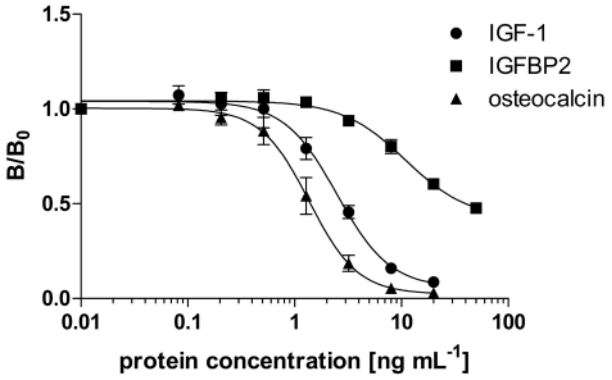

Figure 1: Standard calibration curves for the immunoassays for measuring insulin-like growth factor 1 (IGF-1), IGF binding protein 2 (IGFBP2) and osteocalcin in a competitive format. Standard curves were obtained in plasma-matched buffer.

Compared to the untreated control group, the three different steroidal treatments had specific effects on the biomarker levels. IGF-1 concentrations were significantly elevated after dexamethasone (+30 % IGF-1 on day 25 and +11 % IGF-1 on day 43) and prednisolone (+16 % IGF-1 on day 43) treatment, whereas no significant effect was observed for E_2 treatment (**Figure 2.A**). The effects of glucocorticoids on IGF-1 levels in different life stages were studied before. Acute glucocorticoid treatment of newborn animals resulted in a stimulation of the somatotropic axis and increased IGF-1 levels [25, 34]. In early life stages, glucocorticoid administration had an inhibiting effect on plasma IGF-1 concentrations [20, 22]. Nevertheless, glucocorticoid treatment of adults resulted in increased IGF-1 levels again [23, 26], which is also supported by the results of our study, where 17-22 month old cattle were treated. As already described by Hammon *et al.*, IGF-1 response upon glucocorticoid treatment depends on age, dose and duration of the treatment [22]. Furthermore in untreated cattle, there is a variability in IGF-1 background levels depending on age and sex of the animal [35]. This variability is, however, not a problem as age and sex of a tested animal are known to the veterinary inspection services. Therefore, it can be easily accounted for these fluctuations following full validation covering different age groups.

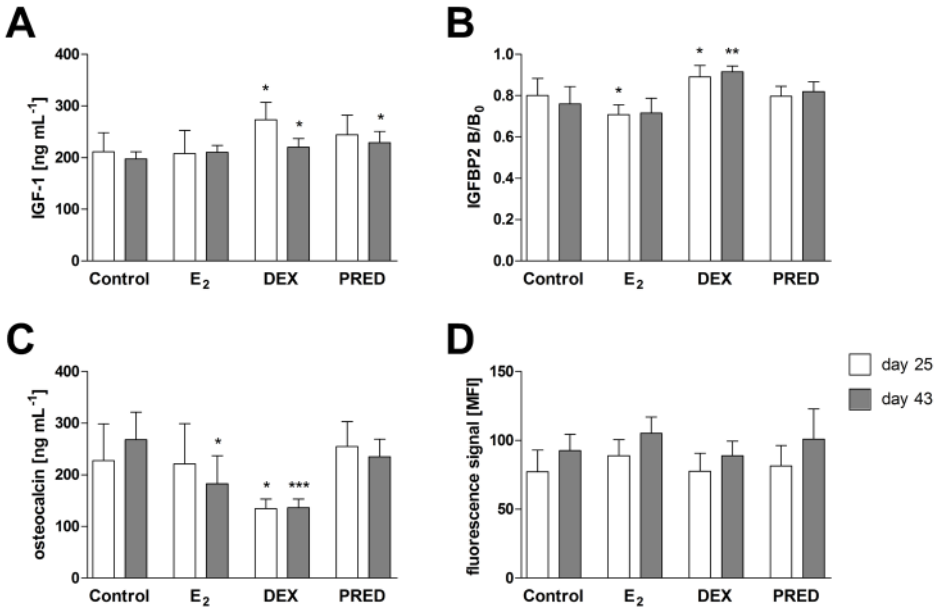

Figure 2: IGF-1 concentration (A), IGFBP2 B/B0 values (B), osteocalcin concentrations (C) and fluorescence signals of anti-rbST-antibody detection (D) obtained from the 4-plex protein biomarker FCIA after treatment with estradiol (E_2), dexamethasone (DEX) or prednisolone (PRED). Each bar represents the average of 6 cows in one treatment group at one time point; error bars indicate standard deviations. Significance levels were: * for P≤0.05, ** for P≤0.01 and *** for P≤0.001 and were obtained by comparing a treated group at one time point to its time point-matched control group using the two-tailed Student's t-test.

For IGFBP2, B/B0 values were used. Since the IGFBP2 assay is of an inhibition format, increased B/B0 values represent decreased plasma IGFBP2 concentrations and *vice versa*. Note that B/B0 values are shown in **Figure 2.B**. Dexamethasone showed a significant IGFBP2-B/B0-increasing effect (+11 % IGFBP2 B/B0 on day 25 and +21 % IGFBP2 B/B0 on day 43), thus a plasma IGFBP2 concentration decreased by dexamethasone treatment (**Figure 2.B**). The studies showing an IGF-1 increasing effect also reported an IGFBP2 decreasing effect due to glucocorticoid treatment [23, 25, 26]. As an exception, Bertozzi *et al.* who reported decreased IGF-1 levels after glucocorticoid treatment, did not see any effect on IGFBP2 concentrations [20]. In rbST treatment studies, an IGFBP2-decreasing effect was observed together with an IGF-1 level increase as well [13, 36], which might

indicate a contrary regulation of these proteins, thus explaining the observed IGFBP2 decrease after dexamethasone treatment in our study. Here, E_2 decreased IGFBP2-B/B0 values (-12 % IGFBP2 B/B0 on day 25), thus increased plasma IGFBP2 concentrations. In contrast to that, IGFBP2 concentrations were not affected by administration of a cocktail of estradiol and nortestosterone in veal calves [2]. Nevertheless, in rat hippocampus cells [37] as well as in bovine granulosa cells [38] it was shown, that estrogen treatment induced the gene expression of IGFBP2. Furthermore, serum IGFBP2 concentrations were increased in barrows treated with estradiol [39], which is supported by the findings of our study. Also for IGFBP2, background levels in untreated cattle depend on sex and age and can be accounted for since these parameters will be known in monitoring practice [40].

Both, E_2 (-32 % osteocalcin on day 43) and dexamethasone (-41 % osteocalcin on day 25 and -49 % osteocalcin on day 43), decreased osteocalcin levels (**Figure 2.C**). Known effects of glucocorticoids are their growth retarding actions on bone. Thus, decreased osteocalcin concentrations were seen before [2] and it was described that glucocorticoids repress the osteocalcin promoter [24]. It is known that estrogens as well as androgens induce bone formation and repress bone resorption [27]. Nevertheless, previous studies described that they did not see any effect in osteocalcin levels of calves throughout a 28-day sex steroid treatment period with combined treatment of estrogens and androgens [2]. Since osteocalcin is released into circulation during bone formation [41] and bone resorption [42], it is a matter of the ratio of bone formation and resorption before and after estrogen treatment, whether increased or decreased levels are observed. Furthermore, osteocalcin turnover is also influenced by the somatotropin/IGF system in an endocrine and paracrine manner [21], which might explain the decreased osteocalcin concentrations observed in our study. As well as for IGF-1 and IGFBP2, background osteocalcin levels in untreated cattle are age-dependent and it can be accounted for this since these parameters will be known to the veterinary inspection services [43].

As expected, none of the steroid treatments affected the endogenous antibody response of antibodies directed against rbST (**Figure 2.D**), which are produced by the animal after treatment with this protein hormone. Thus, this is a biomarker, which selectively discriminates the rbST treatment from steroidal treatment practices in cattle.

The biomarker profile results of every type of steroid treatment are summarized in **Table 1** and are also compared to the biomarker profile of rbST treatment obtained from a previous study [4].

Table 1: Treatment-specific biomarker profiles consisting of significantly increased (+), decreased (-) or stable (Ø) biomarker levels after treatment.

treatments	biomarkers			
	IGF-1	IGFBP2	osteocalcin	anti-rbST antibodies
rbST[1]	+	-	+	+
prednisolone	+	Ø	Ø	Ø
dexamethasone	+	-	-	Ø
estradiol	Ø	+	-	Ø

[1] adapted from Ludwig *et al.* (2012).

In general, some of the changes in biomarker concentrations are rather low, but it always depends on the biological variation in the control group whether an increase of less than 20 % will be detectable or not. If the biological variation in the control group is very low, the less than 20 % change might be sufficient for discrimination. Furthermore, it is not likely that only one biomarker will be indicative for drug abuse. Therefore, always a biomarker profile will be used to identify drug abuse. Combining biomarkers into a profile will be mainly done using statistical models, such as discriminant functions as previously shown for detection of human growth hormone doping in athletes [44], support vector machines as done for the detection of growth-promoting hormones in cattle [45] or the statistical model *k*-nearest neighbours as previously described for rbST detection in dairy cows [4]. If several biomarkers are combined into a statistical model, even a slight change in one biomarker might contribute significantly to the decision making that a sample is considered as being positive for drug abuse.

Obviously, biomarkers of growth-promoting drugs are growth-related and therefore also affected by other factors such as growth in early life stages. Therefore, before implementation of the demonstrated method, the measurement of samples from an extensive control population reflecting all age groups would be necessary. Then, for the ultimate method, age correction of the measured biomarker levels would be necessary to determine whether the animals were treated or not. One possibility to do this is to compare the results of an individual animal to the biomarker profile of its respective age-matched control group. In this case, each age group would have a specific age-dependent threshold level for each biomarker. Another approach would be to establish discriminant functions, in which all relevant biomarker levels and the age of the animal are included

as factors for the calculation of a final test score. If the score then exceeds a set limit, the sample is considered positive. This approach was already followed for the detection of GH abuse in athletes [44].

As can be seen in **Table 1**, each treatment influenced the biomarkers in a unique pattern, which potentially allows the reverse conclusion that a certain biomarker profile is indicative for a specific treatment. Thus, when an unknown sample is analysed by using the 4-plex biomarker FCIA, not only the suspicion that an animal was treated would become clear, but also the possible abused class of substances might be indicated, which is crucial information for the subsequent instrumental confirmatory analysis according to Commission Decision 2002/657/EC [46].

To adapt this 4-plex biomarker FCIA for steroidal compounds in routine veterinary control, a thorough validation needs to be done, as demonstrated for the detection of rbST [4]. First, a large untreated bovine population, representing all age groups, must be analysed to get a more detailed impression of the range of naturally occurring biomarker levels. Then, a comparison of animal treatment study results with control results would allow the calculation of true-positive and true-negative rates thereby considering different steroidal treatment regimes. Furthermore, time course studies need to be done to evaluate whether changes in biomarker levels remain stable after prolonged treatment or whether they decline after a certain period of time in treatment. In **Figure 2.A**, IGF-1 levels are still increased on day 43 after dexamethasone treatment, but they are less pronounced as on day 25. We can only speculate whether this is an effect of a prolonged treatment or whether the observed decrease in concentration versus day 25 is due to that the treatment was discontinued three days before sampling. Furthermore, results have to be evaluated based on the animal's sex and age. If the selected candidate biomarkers would not be sufficient yet for pinpointing the abuse of a certain substance according to EU guidelines (Commission Decision 2002/657/EC [46]), then more biomarkers can be simply added to the developed multiplex screening protocol. The addition of other biomarkers might also increase the potential of the method to detect a treatment with a cocktail of steroids. Additional biomarker candidates for detection of anabolic hormone abuse, such as Ir-inhibin or sex hormone binding globulin (SHBG) [2], were suggested already by Mooney *et al.* and Pinel *et al.* [47, 48].

Summarizing, the feasibility of a 4-plex biomarker FCIA, initially developed for rbST abuse detection, was preliminary demonstrated for detecting treatment with other growth-promoting agents and therefore clearly highlights the potential of biomarker profiling as

future screening methods in veterinary control.

Abbreviations

BSA	bovine serum albumin
E_2	estradiol
EDC	1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide
EDTA	ethylenediaminetetraacetic acid
FCIA	flow cytometric immunoassay
IGF-1	insulin-like growth factor 1
IGFBP2	IGF binding protein 2
IgG	immunoglobulin G
MFI	median fluorescence intensity
PE	phycoerythrin
PBS	phosphate-buffered saline
PBST	0.05 % (v/v)Tween 20 in PBS
rbST	recombinant bovine somatotropin
SDS	sodium dodecyl sulfate
sulfo-NHS	sulfo-N-Hydroxysuccinimide

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Supporting information

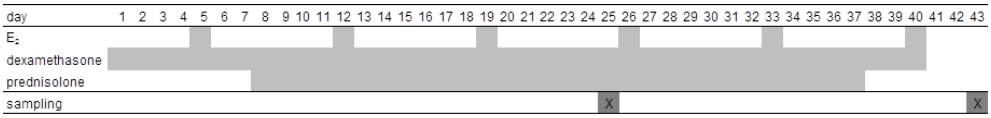

Figure S1: Treatment and sampling schedule for the different treatment groups. Bright grey areas indicate the treatment periods. Dark grey areas indicate the sampling time points.

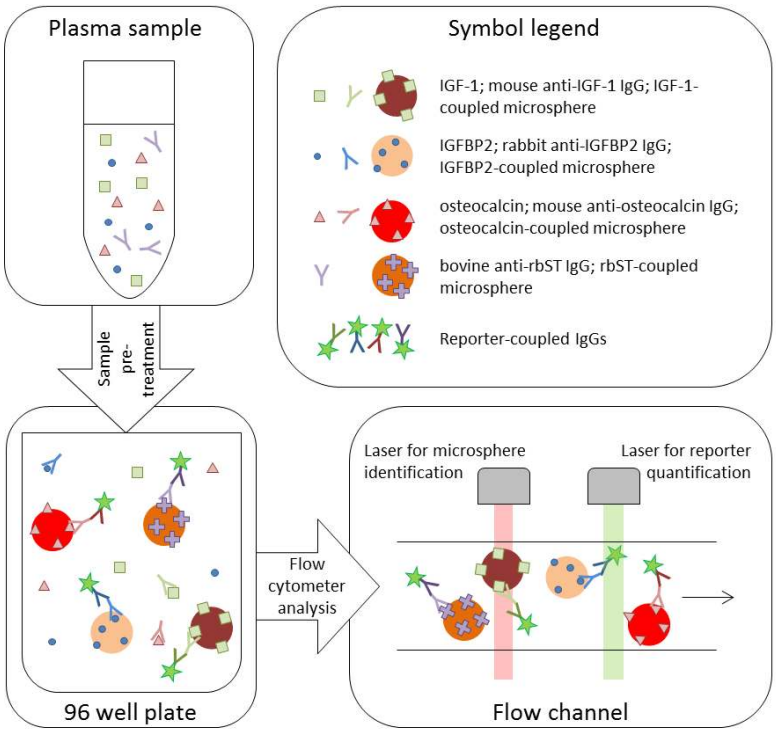

Figure S2: Assay principle of the microsphere-based 4-plex FCIA measuring biomarker concentrations in a plasma sample on basis of a competitive immunoassay for detection of insulin-like growth factor 1 (IGF-1), IGF binding protein 2 (IGFBP2) and osteocalcin and on basis of a direct immunoassay for anti-rbST-antibodies. Microspheres were identified with a red laser and the reporter label was quantified with a green laser in a flow cytometer.

Table S1: P-values of the unpaired, two-tailed Student's t-test corresponding to the results shown in Figure 2 in the manuscript. The treated group at one time point was compared to its time point-matched control group.

	IGF-1		IGFBP2		osteocalcin		anti-rbST antibodies	
day	**25**	**43**	**25**	**43**	**25**	**43**	**25**	**43**
E_2	.8964	.1348	.0356	.3468	.8833	.0211	.1741	.0932
dexamethasone	.0123	.0324	.0484	.0016	.0109	.0002	.9688	.5733
prednisolone	.1598	.0151	.919	.1615	.4553	.2307	.6423	.4313

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Part II

Milk biomarker profiling

6

Monitoring milk for antibodies against recombinant bovine somatotropin using a microsphere immunoassay-based biomarker approach

Ludwig, S.K.J., Smits, N.G.E., Bremer, M.G.E.G., & Nielen, M.W.F. (2012). *Food Control*, 26(1), 68-72.

Abstract

Recombinant bovine somatotropin (rbST) can be used to enhance milk production in dairy cattle. This is permitted in several countries but unauthorized in the European Union. Antibodies, which are produced endogenously in response to rbST administration, can be detected as a biomarker for indicating rbST (ab)use. For the first time, a fast and easy to perform screening assay for anti-rbST antibodies has been developed and applied to raw milk samples. This flow cytometric immunoassay (FCIA) is capable of discriminating between milk from rbST-treated animals and untreated animals. In accordance with literature, 67 % of the rbST-treated animals responded positively with antibody production, whereas 94 % of the untreated animals did not. The analysis of simulated tank milk samples showed more than 95 % of the milk mixtures as truly positive for rbST treatment, indicating that the 33 % physiologically non-responding cows will not be a problem when pooled tank milk samples are considered. FCIA biomarker responses in raw milk were specific for rbST and also obtained in pasteurized milk of rbST-treated animals. Using milk as a sample matrix for detection has the advantages of non-invasive sampling, and for tank milk analysis at the farm only one milk sample is needed to screen the whole farm for rbST (ab)use.

1 Introduction

Increasing milk production by administration of recombinant bovine somatotropin (rbST) to dairy cows [1] is permitted in several countries, such as the United States, but banned in the European Union [2]. Its use, however, cannot be excluded and therefore, a detection method for rbST abuse is necessary. A liquid chromatography-mass spectrometry (LC/MS) method for direct detection of rbST in blood and spiked milk samples [3, 4] was developed, which has the disadvantages of being time-consuming and of having a rather short detection window, because of the short half-life of bST in blood circulation [1]. In contrast, the detection of rbST-dependent biomarkers by fast and easy-to-perform immunoassays, has the advantage of a longer detection window. Several biomarkers are considered to be indicative for rbST treatment, such as markers of the insulin-like growth factor axis, bone and collagen turnover markers and specific antibodies produced against rbST [5, 6]. Since one marker might not be enough to pinpoint rbST abuse, a combination of several markers in one screening tool is considered as well, as already reported for steroid abuse and also in human sports doping analysis [7, 8]. An enzyme-linked immunosorbent assay for the detection of serum antibodies against rbST has been described recently, but showed one out of nine cows reacting false-positively [9]. An optimized sample pretreatment for avoiding false-positive results and a flow cytometric immunoassay (FCIA) for detection of anti-rbST antibodies in serum were developed in our group [10]. However, sampling blood from cows is not justified for routine monitoring in veterinary control, because of its invasive nature. Raw milk, on the other hand, can be used for analysis as well. In this work, we adapted the serum method for milk and present for the first time a rapid FCIA-based screening method for the detection of endogenously produced anti-rbST antibodies in milk. Its applicability is demonstrated in milk of rbST-treated and untreated animals. Moreover, it is shown that tank milk analysis can pinpoint to treated groups since the physiological non-responders' milk will be diluted with the majority of anti-rbST antibody producers' milk and the pooled milk thus obtained will still test positive.

2 Experimental

2.1 Materials and equipment

Monsanto rbST standard was obtained from the National Hormone & Peptide Program of Dr Parlow (Torrance, CA, USA). Posilac® 500 mg single-dose syringes and syringes with only the slow-release formula were purchased from Monsanto company (St Louis, MO, USA). Hydrochloric acid, potassium phosphate, sodium azide, sodium chloride, sodium

hydroxide, sodium phosphate, Tween 20 and the ultrasonic cleaner were purchased from VWR International (Amsterdam, The Netherlands) and glycine was from Duchefa (Haarlem, The Netherlands). N-hydroxysulfosuccinimide sodium salt was supplied by Fluka (Steinheim, Switzerland) and sodium dodecyl sulfate (SDS) by Serva (Heidelberg, Germany). Bovine serum albumin (BSA), 2-(N-morpholino)ethanesulfonic acid (MES hydrate) and N-(3-dimethylaminopropyl)- N'-ethylcarbodiimide hydrochloride were purchased from Sigma-Aldrich Chemie (Zwijndrecht, The Netherlands). R-Phycoerythrin (PE)-labeled goat anti-bovine immunoglobulins were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Multi-Screen HTS filter plates were purchased from Millipore (Amsterdam, The Netherlands). Protein Lobind Tubes and a table centrifuge model 5810R were supplied by Eppendorf (Hamburg, Germany). The Luminex FM-3D flow cytometer, seroMAP microspheres (set 050) and sheath fluid were purchased from Luminex (Austin, TX, USA). The Snijder test tube rotator was purchased from Omnilabo International (Breda, The Netherlands) and the microtiter orbital shaker was purchased from Dynatech (Guernsey, UK). The SW22 water bath was from Julabo Labortechnik (Seelbach, Germany) and Titertubes Micro Tube from Bio-Rad Laboratories (Hercules, CA, USA).

2.2 Buffers and solutions

Buffers and solutions were prepared as follows: phosphate-buffered saline (PBS; 154 mM NaCl, 5.39 mM Na_2HPO_4, 1.29 mM KH_2PO_4, pH 7.4), PBST (PBS, 0.05 % v/v Tween 20), glycine buffer I (27.5 mM, pH 0.5), glycine buffer II (400 mM, pH 10), MES buffer (50 mM, pH 5), blocking buffer (PBS, 0.1 % w/v BSA, 0.02 % v/v Tween 20, 0.05 % w/v NaN_3).

2.3 Sample materials

Serum and milk samples from two animal experiments were used. In the first experiment, 10 Holstein dairy cows of different age (1 to 7 years) were divided in two groups. After a 2-week adaption period, 8 cows were treated every second week with 500 mg rbST in a slow-release formula for 8 weeks and the 2 remaining cows were treated with the slow-release formula only. In the second animal experiment, eight 5-year-old Holstein cows (in groups of 4 each) were treated with rbST in slow-release formula or slow release-formula only in the same manner as in the first experiment. Additionally, they received another injection every week for two weeks directly after the first 4 injections. Both experimental procedures were authorized by the local ethical committees.

In both experiments, blood and milk were sampled weekly. Serum was obtained after

blood samples were kept for 4 hours at room temperature and centrifuged for 10 minutes at 3000 g. Milk was kept at -20 °C and serum at -80 °C until further use. For analysis, the following sampling time points were used: one week before start of treatment and 3, 6 and 8 weeks after start of treatment.

In real practice in rbST-authorized countries, dairy cows are expected to be treated at least 80 % of the milk production period. The remaining 20 %, which is a worst case estimate, consist of the time until the positive energy balance after parturition is restored, which can be 10 weeks. Since, in a worst case scenario, 70 – 80 % of the treated animals are physiologically responding to rbST treatment with antibody production, we expect more than 65 % of the combined milk in a tank load to originate from treated and responding cows. Therefore, milk mixtures for simulating tank milk testing were prepared by combining 10 µL each of three milk samples (randomly two from antibody-responding cows and one from a non-responding cow).

Pasteurized milk was obtained by preheating Micro Tubes to 72 °C in the water bath for 30 minutes. Then, 30 µL of each milk sample (4 milk samples from time point 3 weeks after start of treatment, including 2 samples with a low antibody response and 2 with a high response) were added and kept at 72 °C in the water bath for 30, 60, 120, 300 and 600 seconds. Immediately after, the samples were cooled on ice.

Thirty-four raw tank milk samples from different farms were used to test the applicability and robustness of the anti-rbST FCIA on real samples from an EU country assumed to be negative.

2.4 Bead coupling

Monsanto rbST coupling to seroMAP microspheres was described before [10, 11]. Briefly, 2.5 x 10^6 microspheres were coupled with 0.1 mg mL^{-1} rbST in MES buffer. Microspheres were stored in blocking buffer at 2-8 °C in the dark until further use.

2.5 Pretreatment of serum and milk

Serum pretreatment for removal of non-specific interference of serum proteins was described before [10, 11]. A similar procedure was applied to milk samples. The entire procedures for serum and milk sample pretreatment are summarized in **Figure 1**. Milk pretreatment includes a filtration step to remove interferences of fat micelles with the

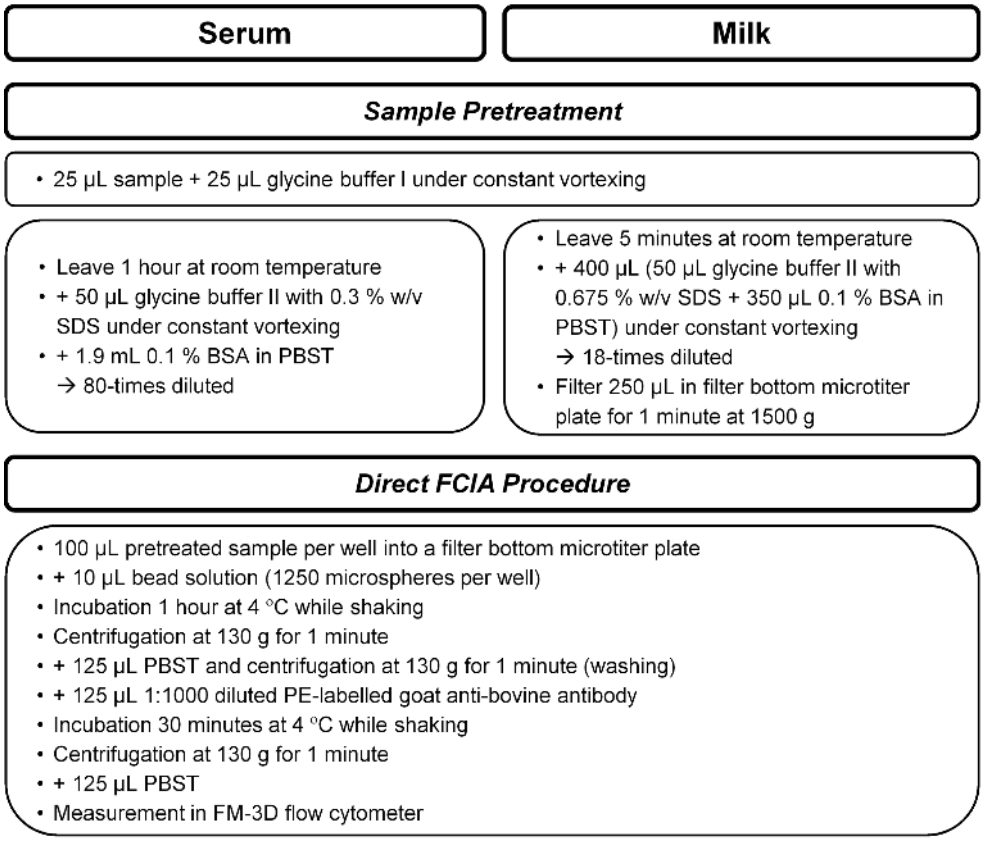

Figure 1: Sample pretreatment and direct FCIA procedure for anti-rbST detection in serum and milk

For comparison of responses in serum and milk, antibody titres were determined by serial dilution of four milk and respective serum samples (2 with high and 2 with moderate MFI responses) after pretreatment and subsequently following the direct FCIA protocol. The titre was defined as the last dilution which still yielded a response above decision limit.

2.6 Direct FCIA

Direct FCIA for measurement of anti-bST antibodies in serum was described before [10]. Median fluorescence intensity (MFI) was measured in response to the amount of antibody bound to the microsphere.

2.7 Inhibition FCIA

To prove the specificity of the induced antibodies upon rbST treatment, different concentrations of rbST were added to inhibit the high fluorescence signal obtained in samples of rbST-treated animals. Therefore, 100 µL pretreated milk samples from four different rbST-treated cows obtained 3 weeks after start of treatment were preincubated for 15 minutes with 10 µL PBST or rbST standard diluted in PBST. Then, 10 µL of microsphere suspension were added and subsequent steps were as described in the direct FCIA (**Figure 1**).

2.8 Data analysis

Decision limit was calculated by averaging the baseline MFI values of all cow samples (taken one week before start of treatment) and adding two times the standard deviation, yielding decision limits of 230 MFI for serum and 202 MFI for milk. Only in case of the inhibition experiment, baseline average of all cows was subtracted and each tested sample from rbST-treated animals was normalized to respective B0 (rbST-treated sample without added rbST standard). For a clear graphical presentation of milk versus serum responses, reciprocal MFI values obtained for every sample are plotted in **Figure 4**.

3 Results & Discussion

3.1 Assay development antibody biomarker against rbST

For detection of antibodies produced in response to rbST treatment in milk, a sample pretreatment was necessary to remove fat micelles from the analysed sample and reduce non-specific binding of other milk proteins to the microspheres. Therefore, the serum sample pretreatment procedure was adapted and by doing this, a shorter incubation time of only 5 minutes could be achieved in contrast to a 60 minutes incubation time in serum (**Figure 1**). This adjustment shortens the whole assay time to 95 minutes incubation in comparison to 150 minutes needed in serum for a whole 96-well microtiter plate. The direct FCIA procedure remained identical for serum and milk.

3.2 Applicability to milk of individual cows

Milk samples from different time points from the animal experiments were tested for antibody biomarker presence. RbST-induced antibody presence was indicated by MFI

(median fluorescence intensity) signals higher than the decision limit (calculation see **section 2.8**). In samples from rbST-treated cows, MFI signals increased and declined slowly but remained above the decision limit throughout the course of the experiment (**Figure 2A**). Ten out of twelve rbST-treated cows (83 %) showed MFIs higher than the decision limit in at least one of the tested milk samples, which is in accordance with literature, where 70-80 % of the cows showed a physiological antibody responses in blood [6, 10, 12]. According to findings from Zwickl *et al.*, some of the treated cows showed very high responses, whereas the responses in others are just above the decision limit. In total, 67 % of all milk samples at any time point from rbST-treated animals displayed an antibody response. That means, that 33 % of the milk samples from rbST-treated animals gave false-negative results, but this percentage includes the 20 – 30 % physiological non-responders indicated in literature. For five out of eight rbST-treated cows from the first animal experiment, antibody responses were still detectable 2 weeks after the last rbST treatment. MFIs of samples from untreated animals remained below the decision limit for all points except two, which are just above the decision limit and considered as false-positive results (one each in **Figure 2A and 2B**). In total, 94 % are correctly found as negative and 6 % are considered false-positive.

To test whether anti-rbST antibodies could also be detected after heat treatment of milk, which is done during milk production process, milk samples from both animal experiments were pasteurized and antibody biomarker responses were analysed. Pasteurization lowered the antibody responses in all four tested milk samples, but MFIs were beyond the decision limit from 30 seconds until 5 minutes of heat treatment. Even after 10 minutes of heat treatment, three out of four samples were still found positive for antibody response. These results indicate that the pasteurization process does not inhibit the detectability of rbST-antibodies in milk completely, whereas detecting rbST itself in spiked milk was considered very heat-sensitive [3].

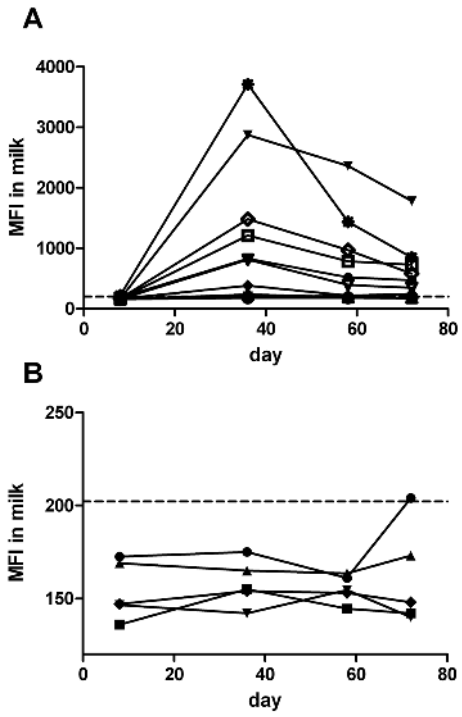

Figure 2: Overlay of MFI responses at different time points in milk of (A) 12 rbST-treated cows and (B) 5 untreated cows. Dashed line indicates the absolute decision limit.

3.3 Specificity of antibodies found in milk

The specificity of the antibody biomarker found in milk was demonstrated by adding different concentrations of rbST standard to four exemplary milk samples from rbST-treated cows. An rbST concentration-dependent inhibition of the MFI response is seen in all of the four milk samples (**Figure 3**). From this result we concluded that the biomarker measured is indeed an rbST-specific antibody.

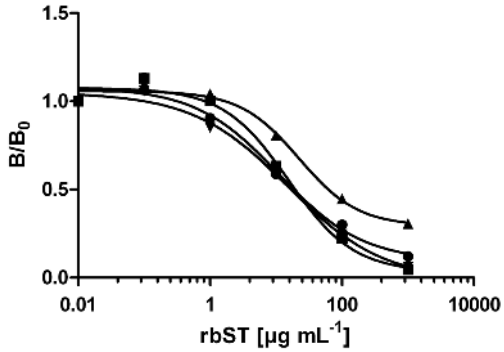

Figure 3: Specificity of endogenously produced anti-rbST antibodies. B/B0 values were inhibited by increasing amounts of rbST in 4 independent cows.

3.4 Comparison of milk and serum levels of rbST antibody biomarkers

To be able to evaluate to which extent antibody presence in milk correlates with serum antibody response, the respective serum samples were analysed as well. Antibody titres were determined for four exemplary serum and respective milk samples and were found to be in average 7-times higher in serum than in milk for three of the tested samples (titres ranging from 18 to 576 in milk and 160 to 1280 in serum). For one tested sample from a pregnant cow, the titre in milk (2304) was 2-times higher than in serum (1280). It was shown before, that antibody titres in milk were much higher in pregnant cows and quickly decreased after calving [13]. Furthermore, it has to be considered that milk and serum matrix might have different influence on the antibody binding to the microspheres and results can not directly be compared. For assessment of the correlation of serum and milk antibody presence in all samples, MFI values were compared. In serum, all rbST-treated cows showed antibody presence in at least two of the tested time points, which gave an overall true-positive rate of 94 %, whereas the false-positive rate was the same as in milk (6 %). Most of the samples showed similar responses in serum and milk, but in some cases, a positive response in serum could not be found positive in milk (**Figure 4**), but in total more samples were found to respond truly positive in serum. Nevertheless, analysing milk has major advantages over serum measurements: sampling milk is a non-invasive method and the assay time is much shorter compared to serum analysis. Furthermore, analysing pooled tank milk can overcome the problem of false-negatively responding serum and milk samples from individual cows; see below.

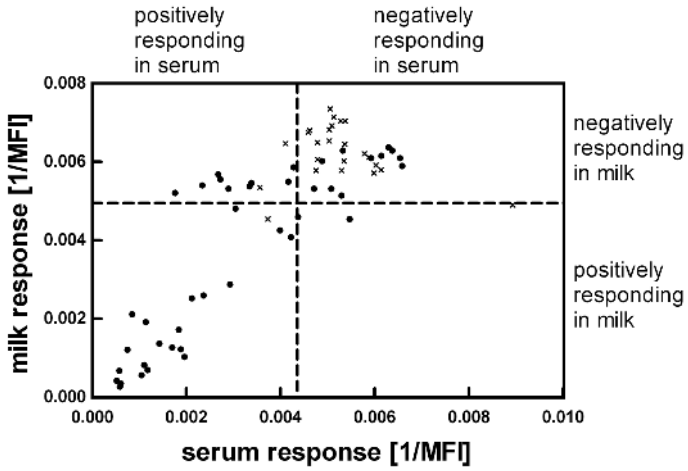

Figure 4: Anti-rbST antibody responses in milk and serum. For clear presentation, reciprocal MFI values of each sample are shown. Dashed lines indicate the absolute decision limits in milk and serum. Dots indicate samples from rbST-treated cows, crosses indicate samples from untreated cows.

3.5 Storage tank milk analysis

In general practice in an rbST-authorized country, it is expected that at least 65 % of the combined milk in a tank load originates from treated and responding cows (see section 2.3), but the pooled tank milk obtained might still yield an overall positive assay result. To proof this we did a simulation experiment, where milk samples from two positively responding rbST-treated cows were mixed with one milk sample from a non-responding rbST-treated cow, *i.e.*, simulating 33 % physiological non-responders and non-treated animals in the mixture. Twenty-four different mixtures were prepared, whereof 96 % showed responses above the assay decision limit, which complies with the <5 % false-negative screening criterion for veterinary drugs in animal products [14]. The advantage of analysing tank milk also applies to the milk of stables where cows were not treated. The very few false-positive responses will be “diluted away” by the many correctly negatively responding milk samples. This was demonstrated by the analysis of 34 tank milk samples from different Dutch farms, of which none responded above the decision limit. Furthermore, analysing tank milk and obtaining an overall positive antibody response for milk of rbST-treated cows offers the future opportunity to develop fast, cheap and easy to use testing devices for on-farm use. The developed FCIA screening method features a

long detection window of at least two weeks after the last rbST treatment. Unfortunately, LC/MS confirmation methods for rbST in blood can only detect rbST for a short period following administration [15] and in milk, no time course studies were done in rbST-treated animals by these authors. Because of this, high frequency blood sampling would be necessary to ensure confirmation of rbST abuse by this LC/MS method. Therefore, for future veterinary control according to Commission Decision 2002/657/EC, we suggest a tiered screening method, which consists of three steps: First, a fast tank milk screening for anti-rbST antibodies using the present FCIA. Second, in case of suspicious findings, a more detailed individual bovine serum biomarker profiling is envisaged, which will provide additional evidence, since that will be based on more biomarkers and data of individual cows. And third, a sufficiently sensitive instrumental method is used for final confirmation of rbST itself in serum samples of the individual cows.

4 Conclusion

An FCIA screening method for anti-rbST biomarker in milk was successfully developed and applied to raw milk, pasteurized milk and raw milk pools (simulating tank milk analysis). The sample pretreatment known from serum analysis was adjusted and shortened for milk analysis, which facilitates the assay procedure and saves time. A preliminary validation of the screening method was performed by testing milk samples from untreated dairy cows, which allowed the calculation of an absolute decision limit. Sixty-seven percent of the tested milk samples from animals treated with rbST showed physiological antibody responses higher than the decision limit, whereas 94 % of untreated animals did not. This resulted in a false-positive rate of only 6 % and a false-negative rate of 33 %. The detection window ranged at least until two weeks after the last rbST treatment, where a positive antibody biomarker response still was found in 63 % of the cows. Antibody responses in milk were specific for rbST and similar to serum responses. Pasteurization did not fully obstruct the detectability of this biomarker, so even processed milk might be tested. In 34 tested tank milk samples from Dutch farms, no single false-positive result was found. Moreover, more than 95 % of simulated tank milk samples from rbST-treated cows showed a positive assay response, which is an adequate true-positive screening rate according to veterinary drug screening regulations.

Acknowledgements

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7

Cellphone microsphere fluorescence immunoassay for the detection of rbST biomarker in milk extracts

Ludwig, S.K.J., Zhu, H., Phillips, S., Shiledar, A., Feng, S., Tseng, D., van Ginkel, L.A., Nielen, M.W.F. and Ozcan, A. (2014). Accepted for publication in *Analytical and Bioanalytical Chemistry*.

Abstract

Current contaminant and residue monitoring throughout the food chain is based on sampling, transport, administration and analysis in specialized control laboratories. This is a highly inefficient and costly process since typically more than 99 % of the samples are found to be compliant. On-site simplified pre-screening may provide a scenario in which only samples that are suspect are transported and further processed. Such a pre-screening can be performed using a small attachment on a cellphone. To this end, a cellphone-based fluorescence imaging platform for the detection of anti-rbST (recombinant bovine somatotropin) antibodies in milk extracts was developed. RbST administration to cows increases their milk production, but is illegal in the EU and a public health concern in the US. The cellphone monitors the presence of anti-rbST antibodies (rbST biomarker), which are endogenously produced upon administration of rbST and excreted in milk. The rbST biomarker present in milk extracts is captured by rbST covalently coupled to paramagnetic microspheres, and labeled by Quantum Dot (QD)-coupled detection antibodies. The emitted fluorescence light from these captured QDs was then imaged using the cellphone camera. The fluorescence micro-images were analysed using a custom-developed Android application running on the same cellphone. With this setup, the cellphone microsphere-based fluorescence immunoassay was successfully applied to milk sample extracts from rbST-treated cows. Next, the cellphone immunoassay was benchmarked against a newly developed planar imaging array alternative. Using cellphone-based on-site analysis in future residue monitoring can limit the number of samples for laboratory analysis already at an early stage. Therewith, the entire monitoring process can become much more efficient and economical.

1 Introduction

Current contaminant and residue monitoring throughout the food chain comprises several steps that are standardized and require extensive administration. First, samples are taken either at the farm, in the food industry, in retail or even at the consumer's home. Samples are then transported to specialized control laboratories, where they are registered and stored. Thereafter, depending on the residue to be monitored, all samples are subject to an initial screening procedure, which identifies suspicious samples in a high-throughput manner. Any identified suspicious sample undergoes the subsequent confirmation procedure, in which the residue is unequivocally identified and, if necessary, quantified [1]. Throughout the entire residue monitoring process, the number of transported, analysed and administrated samples is enormous, since only after screening at a specialized laboratory, they become narrowed down to the actual suspicious ones (**Figure 1**). This process is very inefficient and costly since typically more than 99 % of the samples are found to be compliant.

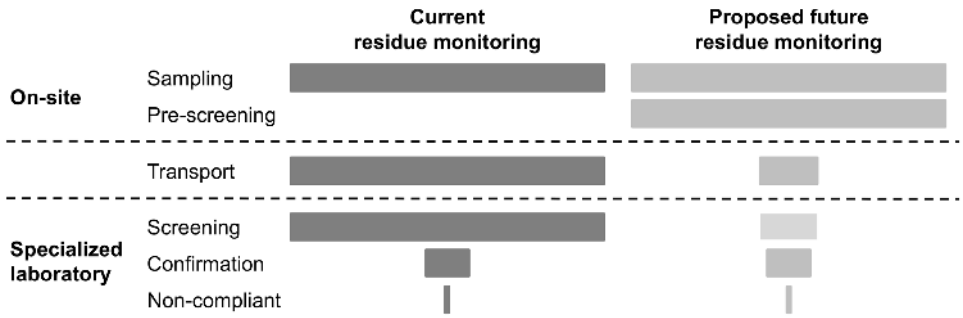

Figure 1: Comparison of current and proposed future residue monitoring in the food chain. Bar widths represent the relative number of samples processed during the respective monitoring step.

For future contaminant and residue monitoring, we propose a slightly different approach: the introduction of a simplified on-site pre-screening step that limits the number of samples for the following steps already at a very early stage (**Figure 1**). Then, only the suspicious samples will be transported, administrated and further analysed in specialized control laboratories. Furthermore, the screening procedure in the specialized laboratory would only remain optional. This proposed approach is much more efficient in terms of transportation, administration and use of equipment in highly specialized laboratories. For the proposed on-site pre-screening procedure, a small attachment on a cellphone may be

used and the administrative data together with the results can be transmitted wireless to a food quality and safety officer.

As a first step on the road towards future cellphone-based food analysis we adopted a cellphone attachment, originally designed for cell analysis [2-5], and turned it into a microsphere fluorescence immunoassay using the analysis of recombinant bovine somatotropin (rbST) biomarker in milk extracts as a showcase. RbST is a proteohormone and increases milk production in dairy cows up to 10-20 % [6]. While rbST use is approved by the Food and Drug Administration in the United States, it is banned in the European Union [7]. To implement European regulations and have accurate 'rbST-free' labelling of milk in for example the US, field monitoring of rbST use and abuse is necessary and would be greatly facilitated by the use of cellphone-assisted rapid screening assays even at farm settings. To screen for rbST, rbST-dependent protein biomarkers can be measured [8]. Protein biomarkers include antibodies, which are endogenously produced by the cow upon treatment with rbST. These anti-rbST antibodies are not only present in serum but also in milk [9]. Previously, a microsphere-based flow cytometric immunoassay (FCIA) method for the detection of anti-rbST antibody (rbST biomarker) in milk was developed [9]. In this previous method, rbST is covalently coupled to microspheres. After incubating the microspheres with a milk sample from an rbST-treated cow, the biomarker binds to the rbST on the surface of the microspheres. The presence of the rbST biomarker can then be detected by a fluorescently labeled anti-bovine-IgG detection antibody. Finally, the fluorescence on the microspheres is measured using a flow cytometer (used as a reference method in this paper) [9]. In the present work, we redesigned that rbST biomarker detection assay and combined it with a cellphone-based analysis by imaging the total fluorescence on a number of microspheres. To this end, a low-cost opto-mechanical cellphone attachment was designed, which uses ultraviolet (UV) light emitting diodes (LEDs) to excite fluorescent Quantum Dot (QD)-labeled anti-bovine-IgG detection antibodies. An optical filter and an external lens in this attachment were used to image the emitted light onto the cellphone camera. A custom-developed Android application, which we term as 'GotMilk', enabled image analysis to be performed on the same cellphone to obtain immediate results.

The developed cellphone immunoassay platform was benchmarked against a newly developed transportable planar imaging array version of the original FCIA approach. The results of this comparison revealed that our cellphone-based approach could detect milk extracts from rbST-treated cows equally well. We believe that the cost-effective and field-portable design of our detection platform provides a good match for field testing of

milk samples even in farm conditions and permits remote reporting and analysis of the acquired test results.

2 Materials and Methods

2.1 Chemicals and instruments

Monsanto rbST standard was obtained from the National Hormone & Peptide Program of Dr. Parlow (Torrance, CA, USA). Posilac 500 mg single-dose syringes and syringes with only the slow-release formula were purchased from Monsanto company (St Louis, MO, USA) and Ely Lilly and Company (Indianapolis, IN, USA). Sodium chloride (NaCl), monosodium phosphate monohydrate ($NaH_2PO_4xH_2O$), potassium dihydrogen phosphate (KH_2PO_4), Tween 20, sodium azide (NaN_3) and glass microscope cover slides (rectangular: 24 x 32 mm, thickness 1; round: Ø 10 mm, thickness 1) were obtained from VWR International (Amsterdam, The Netherlands) and off-the-shelf transparent nail polish was from Herome Cosmetics B.V. (Almere, The Netherlands). Sodium hydroxide (NaOH), disodium hydrogen phosphate dihydrate (Na_2HPO_4 x 2 H_2O) and hydrochloric acid (HCl) were purchased from Merck (Darmstadt, Germany). Sodium dodecyl sulfate (SDS) was obtained from Serva (Heidelberg, Germany) and sulfo-N-Hydroxysuccinimide (sulfo-NHS) from Fluka (Buchs, Switzerland). N-(3-dimethylaminopropyl)-N´-ethylcarbodiimide hydrochloride (EDC), 2-(N-morpholino)ethanesulfonic acid (MES hydrate) and bovine serum albumin (BSA) were purchased at Sigma-Aldrich (St. Louis, MO, USA). MagPlexTM microspheres (set 064) and the MagPixTM planar imaging array platform were from Luminex (Austin, TX, USA). Carboxylated paramagnetic polystyrene microspheres (diameter 8-9.9 µm) were obtained from Microspheres-Nanospheres (Cold Spring, NY, USA) and R-phycoerythrin (PE)-coupled and biotinylated goat anti-bovine immunoglobulins were both from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Protein LoBind Tubes and 1.5 mL reaction tubes were from Eppendorf (Hamburg, Germany). Streptavidin-coupled Quantum Dots (QD; semiconductor CdSe crystal core coated with a semiconductor ZnS shell, a polymer coating and streptavidin protein; total size 15-20 nm; emission at 625 nm) were from Life Technologies (Grand Island, NY, USA) and MultiScreen HTS filter plates were purchased from Millipore (Billerica, MA, USA). The 96-well plates were from Greiner Bio-One B.V. (Alphen aan de Rijn, The Netherlands), the magnetic separator was from Dexter Magnetic Technologies, Inc. (Elk Grove Village, IL, USA) and the orbital shaker was obtained from Salm en Kipp B.V. (Breukelen, The Netherlands). White light emitting diodes (3 mm) were from Conrad Electronic Benelux BV (Oldenzaal, The Netherlands) and the Samsung Galaxy SII was obtained from Amazon.com, Inc. (Seattle, WA, USA). Ultra-bright ultraviolet (380

nm) 5 mm LEDs were bought from Parts Express (Springboro, OH, USA) and 610 nm long pass filter (25 mm diameter) and aspherical lens (focal length 8 mm) were obtained from Thorlabs (Newton, NJ, USA). The battery compartment was obtained from DigiKey (Thief River Falls, MN, USA) and the 3-D printer model Dimension Elite was from Stratasys (Eden Prairie, MN, USA).

2.2 Sample material

Milk samples from two *Bos taurus* animal experiments were used, which had been analysed previously with the FCIA method [9]. The following milk sampling time points were used in the present study: one week before the rbST or placebo treatment and 36 and 58 days after the start of the treatment. Milk samples from untreated animals were randomly selected to determine a decision limit, which was calculated by averaging the test results and adding two-times their standard deviation. Thereafter, 20 milk samples from rbST-treated cows with known rbST antibody responses were tested and a sample was considered as being suspicious for rbST treatment, when its test result was higher than the calculated decision limit. All tested methods, *viz.* cellphone, planar imaging array, and original FCIA, were evaluated for their capability of truly detecting rbST treatment samples.

2.3 Buffers and solutions

Buffers and solutions used were as follows: phosphate-buffered saline (PBS; 154 mM NaCl, 5.39 mM Na_2HPO_4, 1.29 mM KH_2PO_4, pH 7.4), PBST (PBS, 0.05 % v/v Tween 20), activation buffer (100 mM NaH_2PO_4, pH 6.2), MES buffer (50 mM, pH 5), blocking buffer (PBS, 0.1 % w/v BSA, 0.02 % v/v Tween 20, 0.05 % w/v NaN_3), sample diluent (PBST, 0.1 % w/v BSA, 0.008 % w/v SDS).

2.4 Microsphere preparation

RbST was covalently coupled to carboxylated non-coloured magnetic polystyrene microspheres for the cellphone platform using the two-step carbodiimide reaction as described previously [10]. All protocol steps were done in ProteinLobind Tubes to avoid protein loss. Briefly, 200 µl microsphere suspension was used and microspheres were washed in deionized water, activated with 10 µL 50 mg mL^{-1} sulfo-NHS in dH_2O, 10 µL 50 mg mL^{-1} EDC in dH_2O and 80 µL activation buffer for 18 minutes, washed twice in 500 µL MES buffer, covalently coupled with 0.1 mg mL^{-1} rbST in 500 µL MES buffer for 2

hours, blocked in 500 µl blocking buffer for 30 minutes, washed in 500 µL blocking buffer twice and stored in the dark in 500 µL blocking buffer until further use at 2-8 °C. The same protocol was followed for coupling rbST to colour-encoded MagPlex microspheres (microsphere set number: 064) for the planar imaging array platform.

2.5 Preparation of milk extracts

Before conducting the assay protocol, milk samples must be extracted to lower non-specific binding to the microspheres. The extraction procedure is summarized in **Figure S1** of the Supplementary Information. Note that the development of a simplified on-site extraction method was not the objective of the present study yet. Therefore, filtration for removal of fat micelles was simply done by centrifugation at 3000 g for 5 minutes using 1.2 µm pore size 96-well filter bottom plates.

2.6 Cellphone-based detection platform

2.6.1 Opto-mechanical attachment design

The cellphone attachment was designed specifically for an Android-based Samsung Galaxy SII cellphone and based on the model of the previously described fluorescence microscopy cellphone platform [2-5]. Similar attachments can also be created for other smart-phones. This cellphone attachment module (overall dimensions: 88 x 73 x 31.25 mm) consisted of several parts (**Figure 2A and 2B**):

- a cellphone holder to align all optical parts with the camera,
- a sample tray to position the cover slides, having the microsphere suspension sandwiched in between,
- twelve excitation light emitting diodes (wavelength 380 nm) for exciting the QDs for fluorescence imaging. These LEDs were arranged on three of the four sides of the sample tray perpendicular to the glass cover slides, so that the glass slides could serve as planar waveguides for the excitation light,
- two white light LEDs for darkfield imaging,
- an optical filter (long pass 610 nm, 25 mm diameter) was placed in the sample tray for filtering the scattered excitation light,
- an aspherical lens that provides 2X demagnification of the microspheres and an increase of the imaging field of view,
- a battery compartment and
- a mechanical lid to protect fluorescence measurements from ambient light.

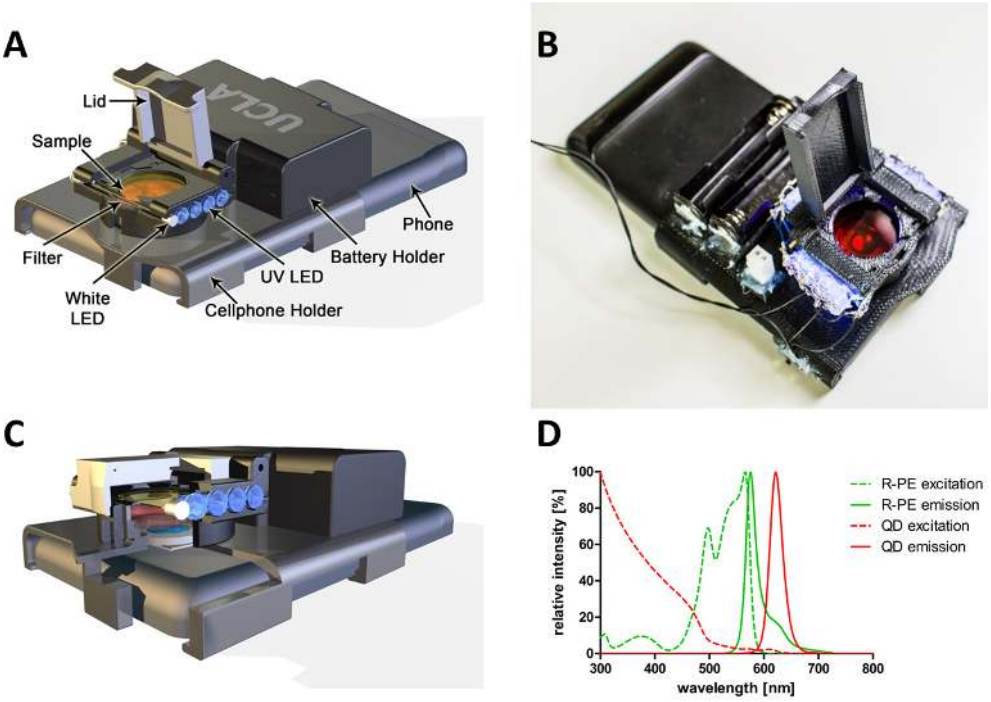

Figure 2: Schematic overview (A, C) and a picture (B) of the cellphone attachment for the detection of rbST biomarker in milk extracts. (D) Excitation (dotted lines) and emission (solid lines) spectra of R-phycoerythrin (R-PE, green) and 625 nm emitting Quantum Dots (QD, red).

2.6.2 Cellphone-based assay procedures

The cellphone-based assay procedures utilize the specific binding of the cows' endogenous rbST biomarker to rbST-coupled magnetic microspheres. After a washing step, a QD-labeled anti-bovine antibody is used to detect the presence of the rbST biomarker (**Figure 3A**).

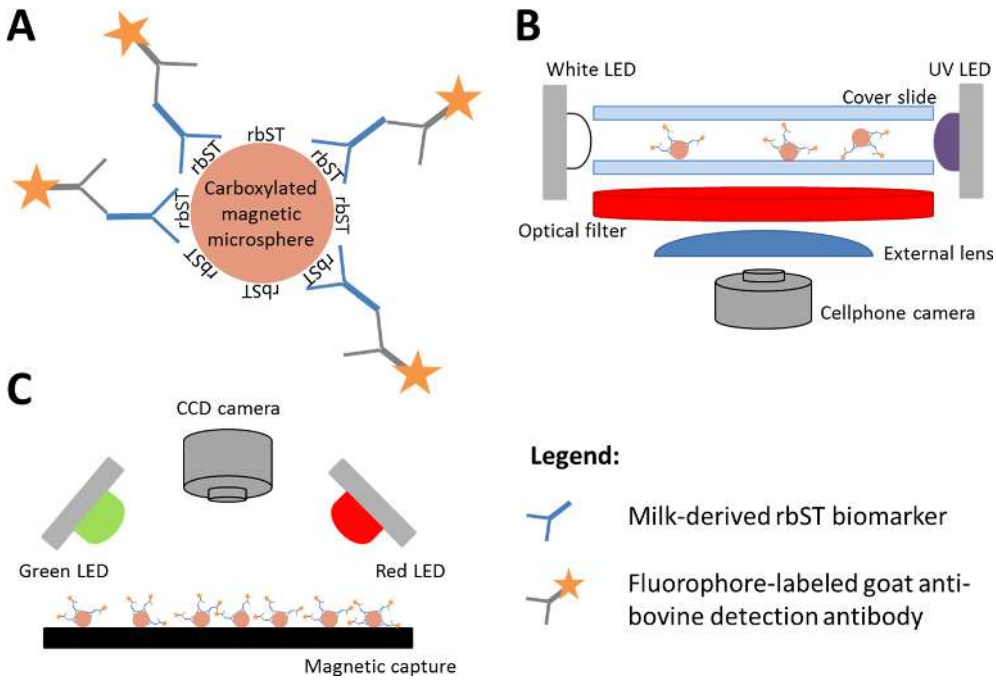

Figure 3: (A) Assay principle for rbST biomarker detection, (B) detection setup for the cellphone-based platform, and (C) detection setup of the planar imaging array platform (C).

After the milk extraction, the immunoassay (**Figure S1**) could be performed in transparent 96-well plates. For all dilutions and washing steps, PBST was used and the procedure was performed at room temperature. While the FCIA reference method used R-PE as a fluorescence label, 625 nm emitting QDs were used for the cellphone-based detection because their excitation and emission spectra allow the use of a standard long pass filter (**Figure 2D**) and QDs do not show photo-bleaching effects. The prepared sandwiched glass slides were slid into the cellphone attachment and a darkfield image was taken by using the white LEDs (**Figure 3B**) and the internal camera of the cellphone operated in the 'night mode' for increased sensitivity. Thereafter, the white LEDs were switched off and UV LEDs were switched on (**Figure 3B**) for taking a fluorescence image with the same settings on the cellphone. The acquired fluorescence images were analysed by using a custom-designed Android application, termed 'GotMilk'. Using this smart application, it is possible to analyse images located at the internal memory of the cellphone or to capture images with the camera and analyse them immediately. After a region of interest was selected

(unless otherwise specified, by default the centre region of the image was analysed), total microsphere count, average fluorescence intensity and its standard deviation were given as the result of the measurement (**Figure 4**). This analysis procedure was possible for both fluorescence images and darkfield images; in the latter the number of total microspheres was counted, which was used for normalization in the fluorescence image analysis.

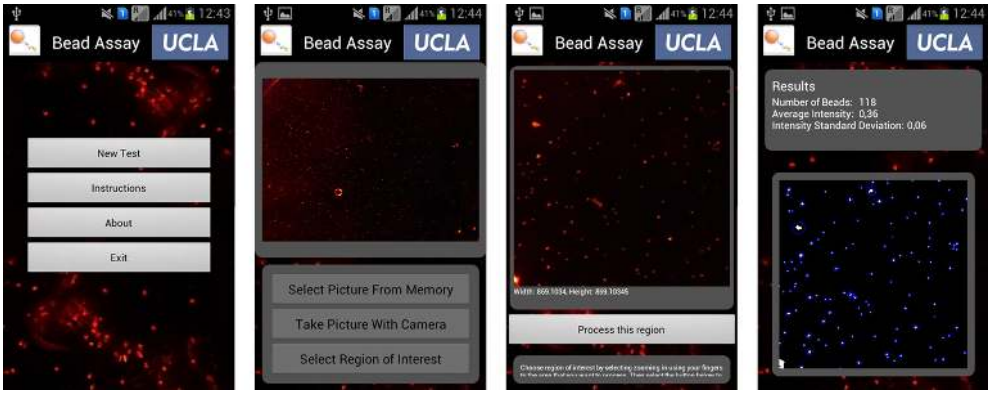

Figure 4: Overview of the GotMilk Android application user interface for analysis of cellphone camera images (fluorescence and darkfield).

2.7 Planar imaging array detection platform

The FCIA reference method described by Ludwig *et al.* [9] was slightly modified in a way that for all tested platforms in this work, the same sample extraction procedure could be applied (**Figure S1**). Next, the assay principle of the FCIA reference method was transferred to a newly developed transportable planar imaging array. For this, colour-encoded MagPlex microspheres (microsphere set number: 064) were coupled with rbST as described in **Section 2.4**, milk samples were extracted as detailed in **Section 2.5** and the samples were prepared following the procedures shown in **Figure S1**. Since colour-encoded microspheres and the photo-labile fluorophore R-PE were used for this platform, all the assay procedures were performed in the dark. The readily prepared microspheres in the 96-well plate were put into the planar imaging array instrument for detection (**Figure 3C**). The colour code of the microspheres was identified after excitation with a red LED (621 nm) and the signal was detected with a CCD camera and two optical filters. The amount of fluorescence (*i.e.*, the signal) for each microsphere was quantified after excitation with a green LED (511 nm) and detected with the CCD camera and an optical filter (590 nm). The quantified fluorescence signal was reported as the median

fluorescence intensity (MFI) of the particles. A decision limit was calculated as described in **Section 2.2**.

3 Results

3.1 Cellphone-based detection

In this work, a cellphone-based analysis method for the detection of anti-rbST antibodies (rbST biomarker) was developed, following the capture of rbST biomarker by rbST-coupled magnetic microspheres. The binding event was detected using a Quantum Dot (QD)-labeled detection antibody together with the specific imaging design of the cellphone attachment.

3.1.1 Cellphone-based fluorescence and darkfield imaging

The developed cellphone attachment for visualizing the presence of rbST biomarker is a light-weight and low-cost device, which can easily be attached to and detached from the cellphone. Its compactness, light weight and low-power consumption makes it a versatile tool, suitable for laboratory and field use. It can be adapted to any available cellphone that has a camera module by simply modifying the dimensions of the cellphone holder and 3D-printing another one accordingly. Our specific attachment was designed for the excitation and emission light spectrum of the 625 nm-emitting QDs (**Figure 2D**), which were used as a label in the rbST biomarker assay. Therefore, UV LEDs (at 380 nm) were used for excitation of these QDs and a 610 nm long pass filter was used for filtering the emission light. Furthermore, an external aspherical lens was used for demagnification of the microspheres such that a large field of view of 80 mm^2 was imaged by the cellphone camera. White light LEDs were also used in the same design for capturing darkfield images of the microspheres such that the total microsphere count could be determined. Since the long pass filter was not removed for darkfield imaging, the microspheres in darkfield images appeared also in red colour. The cellphone holder and the sample tray positioned all the optical parts correctly and aligned the external and camera lenses for imaging. Using this cellphone attachment setup, darkfield (**Figure 5A.1 and 5B.1**) and fluorescence (**Figure 5A.2 and 5B.2**) images were captured, where a custom-designed application (GotMilk) was used to process these images to count the number of microspheres and determine their mean fluorescence intensity.

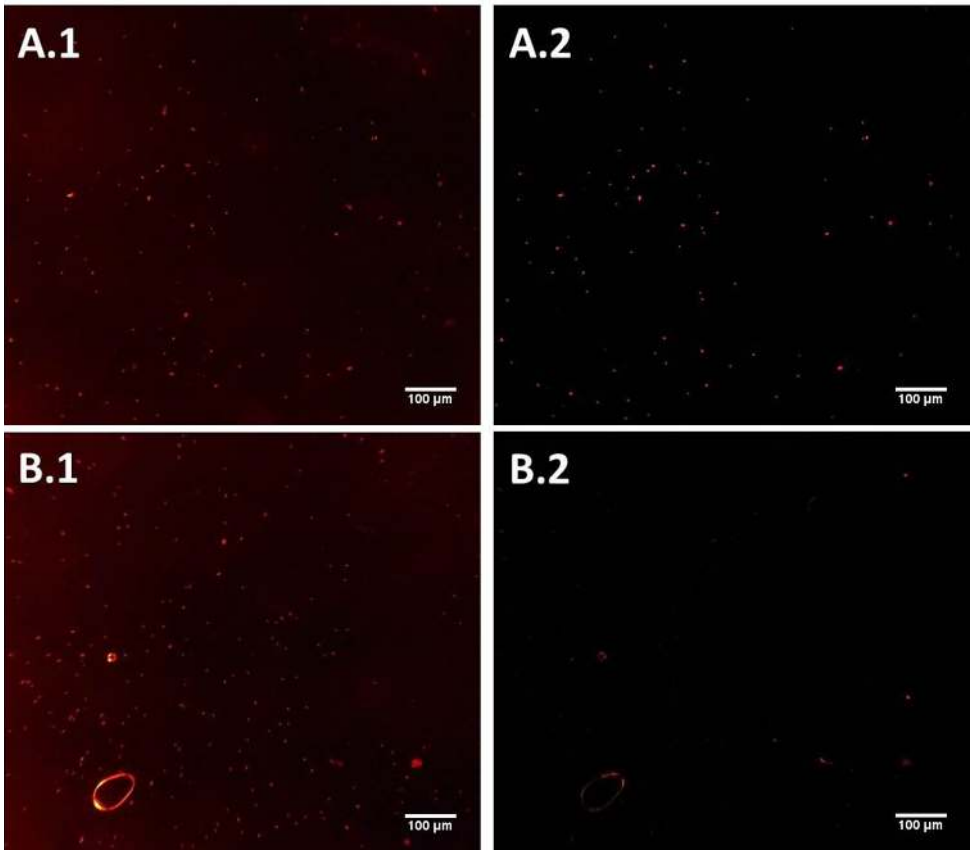

Figure 5: Sample images obtained using the presented cellphone-based fluorescence immunoassay platform. Darkfield (1) and fluorescence images (2) are shown for a milk extract from (A) an rbST-treated animal and (B) a milk extract from an untreated animal. Note that the darkfield images also appear in red colour due to the long pass filter present in the optical path.

3.1.2 Cellphone image data analysis

To be able to discriminate samples deriving from rbST-treated and untreated animals, two different image analysis approaches, namely the intensity analysis approach and the microsphere count-based approach were tested for reproducibility and linearity. For reproducibility, the same milk extract was analysed repeatedly and for linearity, samples with no, low and high rbST biomarker levels were correlated to the results of the FCIA reference method. Note that it is not possible to measure absolute rbST biomarker

concentrations due to a lack of a suitable standard protein. In the intensity analysis approach, the average intensity values of the detected microspheres and the standard deviations calculated by the GotMilk application were used. This approach was expected to deliver similar results as the planar imaging array platform (**Section 2.7 and 3.2**), in which also median fluorescence intensities (MFIs) are obtained. However, this approach was found less useful in our cellphone platform: first, the dynamic range of the obtained intensities varied between 0.09 and 0.22 on a scale from 0 to 1.00 (**Figure 6A.1**). Second, the standard deviation of the microspheres from the same sample was approximately 25 % of the total intensity, which is high and makes the discrimination between milk samples from rbST-treated and untreated animals difficult. Third, there was no obvious biomarker-dependent increase in signal observed (see for example **Figure 6A.1 and 6A.2**).

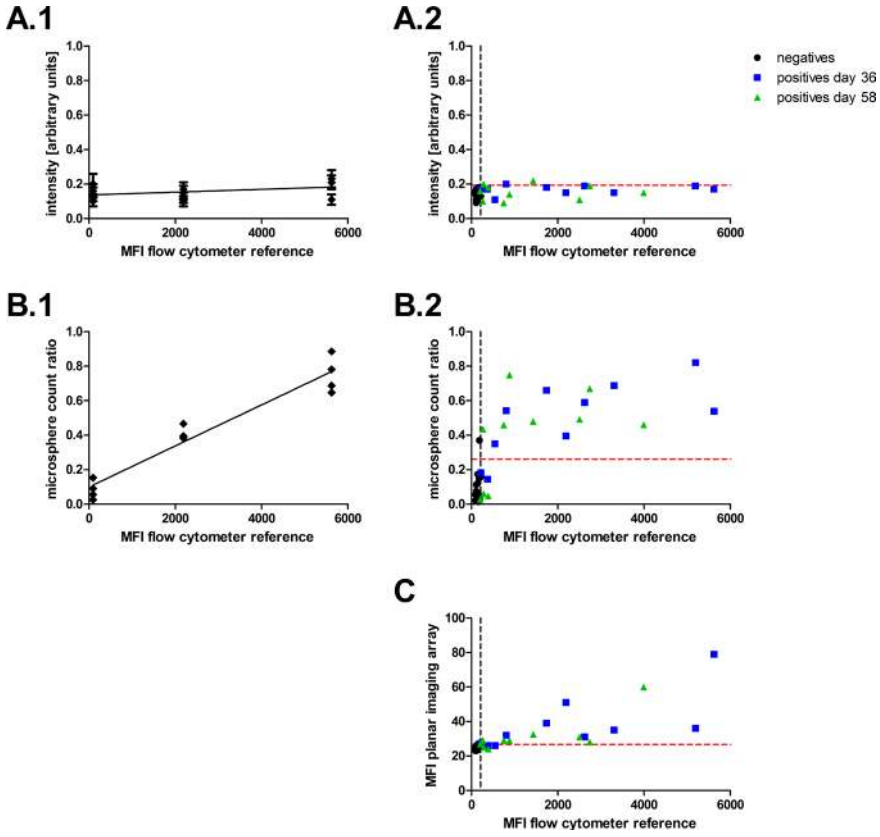

Figure 6: The signal intensity obtained using the cellphone platform with (A) the intensity analysis approach, and (B) the microsphere count ratio obtained with the microsphere count-based approach. Reproducibility and linearity (A.1 and B.1) and individual results

for milk sample extracts from rbST-treated (squares and triangles) and untreated (circles) animals (A.2 and B.2). (C) Correlation of MFI results obtained using the planar imaging array detection approach compared to the reference FCIA method. The decision limit, above which a sample was considered as 'suspicious for rbST treatment' is marked as red dotted line. The decision limit of the flow cytometer reference method is marked with a black dotted line. MFI: median fluorescence intensity.

As an alternative method, a microsphere count-based approach was tested, in which the total microsphere count of the fluorescence image was normalized against the total microsphere count of the darkfield image and this ratio was used for analysis. Using this approach, an increased dynamic range (0.01 to 0.88 on a scale from 0 to 1.00) and an improved reproducibility could be obtained as illustrated in **Figure 6B.1**. Furthermore, this microsphere count ratio correlated quite well with the biomarker presence in the milk extracts. Therefore, this approach was selected to analyse 20 milk sample extracts deriving from rbST-treated cows.

3.1.3 Results of milk sample extracts analysed with the cellphone-based immunoassay platform

For evaluation of the developed cellphone-based rbST biomarker detection platform, 20 milk sample extracts from untreated cows were tested for decision limit calculation and thereafter, 20 further milk extracts from rbST-treated cows, having anti-rbST antibody response according to FCIA, In both cases the microsphere count-based approach as detailed in the previous sub-section was applied. Examples of darkfield and fluorescence images of milk samples taken from rbST-treated and untreated cows can be seen in **Figure 5**. In the darkfield images (**Figure 5A.1 and 5B.1**), microspheres are well visible as red dots. In the fluorescence image of the sample from an rbST-treated animal (**Figure 5A.2**), the majority of microspheres shows a fluorescence signal, whereas in the sample from an untreated animal, only a few fluorescence signals are visible (**Figure 5B.2**). The decision limit was determined by averaging the microsphere count ratios of 20 milk extracts from untreated cows and adding two-times their standard deviation (**Section 2.2**) and was found to be at a microsphere count ratio of 0.261. Based on this decision limit, our tests revealed that fifteen out of the 20 tested milk extracts (75 %) from rbST-treated animals were found suspicious for rbST treatment. Of course, the 75 % truly positive screening rate of the cellphone is not good enough for official testing yet; on the other hand, it is quite remarkable that such a simple low-cost device demonstrates such a rate already now.

The laboratory-based FCIA reference method was used to test the same 20 milk samples from untreated animals for decision limit calculation (211.9 MFI; calculated according to **Section 2.2**); followed by the 20 milk samples from rbST-treated animals, whereof 19 were found to exceed the decision limit, meaning that 95 % of the samples were found suspicious for rbST treatment using the lab-based FCIA method.

3.2 Planar imaging array assay

3.2.1 Development of the planar imaging array assay

For the planar imaging array-based detection method, the same sample preparation protocol of the cellphone-based approach (with a single step of sample dilution) was used. The assay procedure were adopted from the FCIA reference method (**Figure 3C, S1 and Section 2.7**).

3.2.2 Results of milk sample extracts analysed with the planar imaging array instrument

When the 20 milk samples from untreated animals were analysed using the planar imaging array instrument, a decision limit of 26.72 MFI was calculated as described in **Section 2.2**. Furthermore, the variability in between the negative samples was quite low (4.3 % CV). Of the 20 analysed milk sample extracts from rbST-treated animals, sixteen (80 %) were correctly identified as being suspicious for rbST treatment with the planar imaging array platform. Note that this achievement is hardly better than the cellphone results. The individual results of each milk sample are depicted in **Figure 6C** in correlation to the results of the reference flow cytometer method.

4 Discussion

We presented the development and initial real-life applicability testing of a field-portable cellphone-based analysis platform for the detection of rbST biomarker in milk extracts. Other cellphone-based detection platforms were developed before for several different applications [2-5, 11-23]. The here presented platform, however, combines for the first time immunofluorescence detection of single molecules from a sample extract using a cellphone for fluorescence and dark field imaging.

When testing 20 milk extracts derived from rbST-treated cows, our cellphone-based

detection platform performed similar to the planar imaging array (**Table 1**). Currently, the assay time is substantial and therefore, future studies should focus on simplified on-site sample preparation and shorter incubation times. For example, as a first step, in-field sample extraction could be performed by simply diluting the milk sample and remove fat micelles by quick syringe-filtration. Second, the QD detection antibody may be prepared as a single reagent thereby omitting the 30 minutes incubation step for streptavidin-QD in **Figure S1**. Third, co-incubating all assay reagents simultaneously may reduce the several individual incubation steps down to only one. Fourth, the employment of a microfluidic chip comprising all assay reagents prepared would facilitate the entire assay procedure. One should be aware that with the cellphone-based approach the results can be obtained at the site where the sample was taken, *i.e.*, transporting the samples to a specialized laboratory is no longer required. When a suspicious sample is identified during the on-site screening process, further samples, for instance blood samples, can also be taken for subsequent laboratory-based analysis (**Figure 1**). In that case, the multiple serum protein biomarker screening test previously developed [8] and direct confirmatory analysis of rbST itself using LC-MS/MS [24] can be applied.

When benchmarking the anti-rbST platforms available so far, the cellphone-based detection device is the only option for on-farm analysis of tank milk by inspection services (or truck milk at the dairy gate). The planar imaging array platform is, compared to the flow cytometer instrument, transportable, but its dimensions (16.5 x 60 x 43 cm) and its weight of 17.5 kg (the necessary operating computer not included) do not favour in-field use. Note that, as highly desired, the cellphone immunoassay platform combines not only a camera and image analysis tool, but it also allows data storage and wireless transmission via a mobile network to food quality and safety officers, central inspection agencies, control laboratories or industrial QA/QC decision makers.

Multiplexing can be easily achieved by the planar imaging array platform and the flow cytometer reference method, which are designed especially for colour-encoded microsphere ligand binding assays. For the cellphone-based detection device, multiplexing may be achieved easily by using either different fluorescent labels for additional analytes or different colour-encoded or size-encoded microspheres in the microsphere counting process. In both cases, the cellphone attachment needs LEDs of different wavelengths and exchangeable optical filters as demonstrated recently by Zhu *et al.* [5]. Alternatively, using microspheres of a different size would only require adjustments in the GotMilk application in order to discriminate bigger and smaller particles in the fluorescence and darkfield images.

Table 1: Performance of the cellphone assay for rbST biomarker detection in cows' milk versus alternative approaches

	Cellphone detection	Planar imaging array platform	Flow cytometer reference method
Amount of samples found suspicious for rbST treatment	75 %	80 %	95 %
Reading time	5 minutes per sample	1 minute per sample	1 minute per sample
Portability	Portable	Transportable	Not transportable
In-field applicability	No external power supply needed	Operation requires external power supply	Not applicable in field
Multiplexing capability	Requires inter-changeable filters	Multiplex ready	Multiplex ready
Wireless Connectivity	Yes	No	No

In conclusion, we demonstrated the development and real-life applicability of a novel cellphone-based assay platform for the analysis of rbST biomarker in milk as a pre-screening method for the detection of rbST abuse in dairy cattle. Applying this cellphone-based on-site pre-screening in future contaminant and residue monitoring can limit the number of samples to be processed already at an early stage. Therewith, the administration and transport of an extensive number of compliant samples can be avoided and the entire monitoring process can become much more efficient and economical (**Figure 1**). The cellphone platform's small dimensions, light weight and cost-effectiveness make it highly desirable for field testing even in farm settings.

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Supplementary information

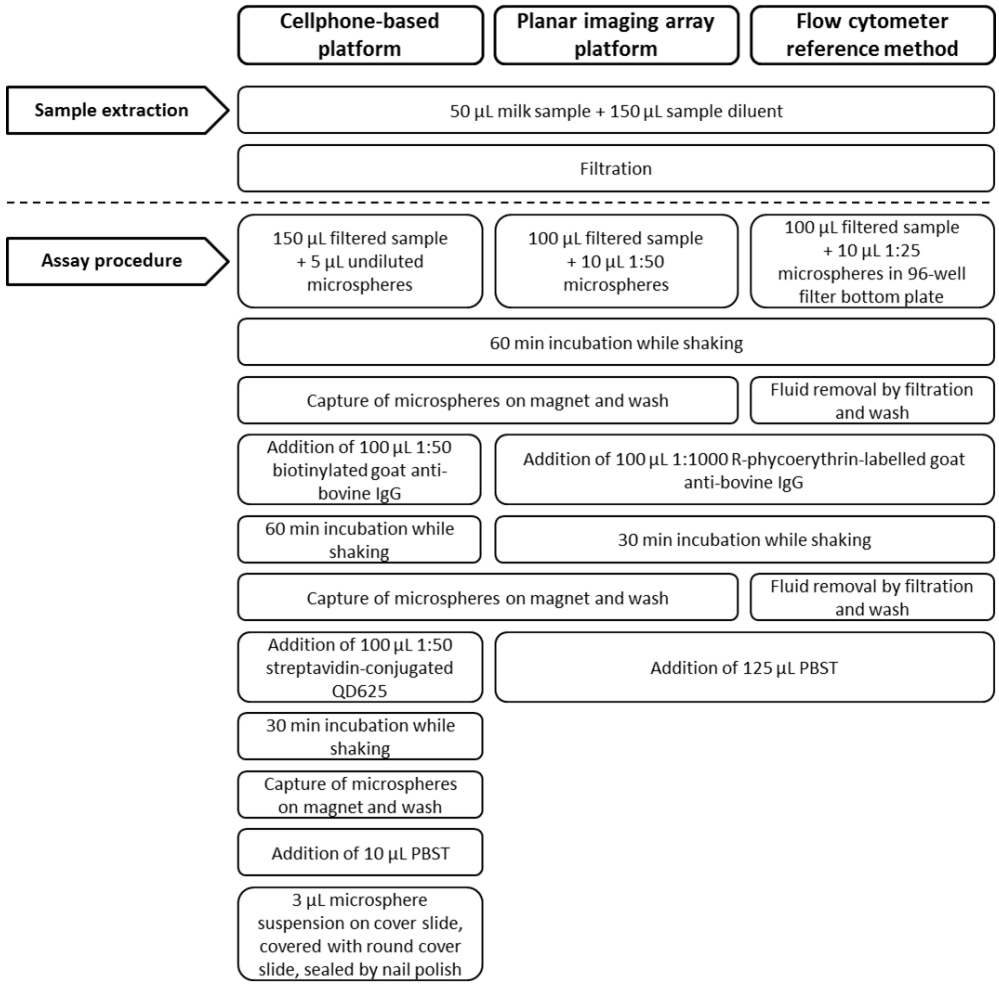

Figure S1: Sample preparation procedures for the three platforms that are used in this work and the flow cytometer reference method.

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8

Calling biomarkers using a protein microarray on your cellphone

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Nielen, M.W.F. (2014). Submitted manuscript.

Abstract

On-site testing has a broad significance for food safety, environment and health monitoring and simultaneous analysis of multiple analytes (contaminants or biomarkers) saves analysis time, money and sample volume. Here we present the scientific concept of a protein microarray-based platform for multiple biomarker detection on a cellphone. We selected the detection of recombinant bovine somatotropin (rbST) abuse based on altered biomarker profiles of IGF-1 and anti-rbST antibodies in milk extracts as a showcase. A multiplex immunoassay having built-in positive and negative controls was designed on a microarray chip. The 48 microspots were labelled with Quantum Dots depending on the biomarker levels in the sample. Fluorescence was detected by the cellphone camera under UV light excitation from LEDs embedded in a simple 3D-printed opto-mechanical attachment on the cellphone and images were analysed by custom software. RbST-treated and untreated cows clearly showed a treatment-dependent biomarker profile in milk. Broad application and adoption of this concept is envisaged.

On-site testing platforms have a broad significance for food safety, environment and health monitoring as they allow the analysis of samples on the spot and the initiation of immediate measures depending on the outcome of the test. In food safety controls, on-site testing platforms can be used to detect the presence of antibiotics, contaminants or veterinary drugs for instance in milk directly on farm. In environmental monitoring, toxins, heavy metals or endocrine disrupting chemicals can be analysed directly at the sampling site. And in health care, disease biomarkers can be monitored using an on-site testing platform as a point-of-care (POC) device. In recent years, several on-site testing platforms have been developed, for instance for food allergen testing [1], monitoring of polycyclic aromatic hydrocarbon (PAH) presence in river water [2], detection of mercury contamination in water [3] and red and white blood cell analysis [4, 5]. Topol summarized that "the 2010s will probably be known as the era of digital medical devices" [6], but these recent developments demonstrate a broader significance of on-site testing platforms beyond disease diagnosis. Another trend within the area of on-site monitoring is the use of standard cellphones, which are equipped with simple attachments for analysis [4, 5, 7]. These attachments usually house simple optical components to enable imaging and analysis of the sample using the built-in camera module of the cellphone. For all applications, the simultaneous analysis of multiple analytes, such as contaminants, chemicals or biomarkers, is advantageous, because it saves analysis time, money and sample volume. To meet the demands of multiple analyte detection using an on-site platform, we present here a protein microarray for biomarker detection on a standard cellphone as a scientific concept. Design features are 48 array spots thus allowing replicate measurements of multiple biomarkers and built-in positive and negative quality controls.

We chose the biomarker-based detection of recombinant bovine somatotropin (rbST) abuse in milk sample extracts from dairy cows as a challenge. RbST is a proteohormone that can be used to increase milk production but its administration is illegal in EU [8] and of consumer's concern in the USA. To detect rbST abuse, it has been shown that rbST-dependent protein biomarkers in serum and milk of dairy cows can be monitored, but levels are typically low (10 ng mL^{-1} range) [9-11]. Here, we analyse the biomarkers anti-rbST antibodies and insulin-like growth factor-1 (IGF-1) in milk sample extracts by a fluorescence immunoassay microarray. The array was conveniently imaged using a cellphone equipped with a 3D-printed opto-mechanical attachment originally designed as a fluorescent microscope for *e.g.*, particle analysis (**Figure 1A**) [12].

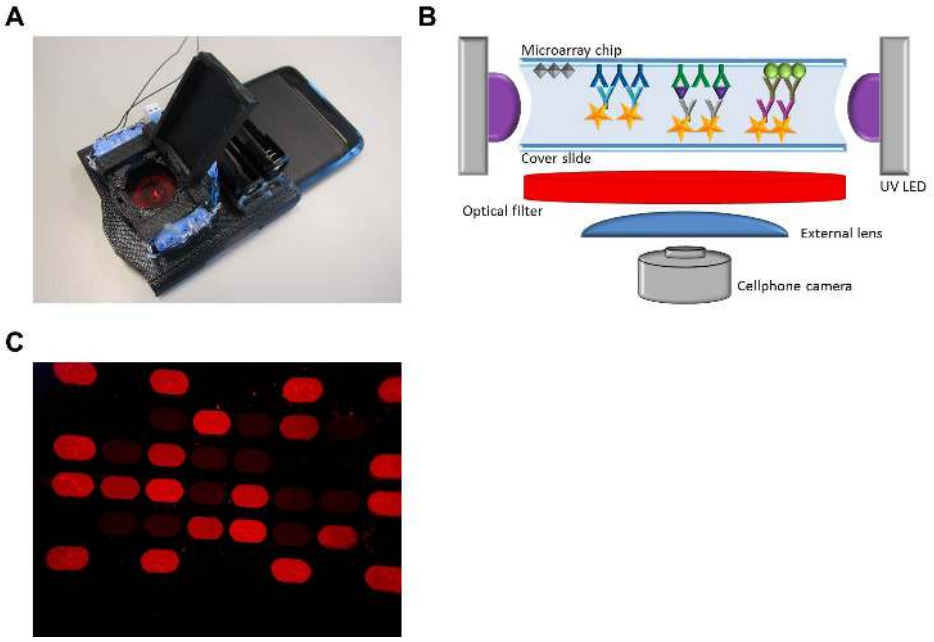

Figure 1: Cellphone with fluorescent microscope attachment, detection setup and microarray image. (A) Photograph of the 3D-printed microscopic imaging attachment on the cellphone, which was used for analysis. (B) Setup of the cellphone biomarker detection platform. A detailed description of the microarray immunoassay setup can be found in the Supplementary Material. (C) Exemplary microarray image obtained using the cellphone fluorescent microscope.

The microarray for rbST biomarkers comprised four different proteins spotted on a single functionalized glass slide: rbST for anti-rbST antibody analysis, anti-IGF-1 antibody for IGF-1 analysis, ovalbumin as negative control and sheep IgG as positive control. The positive control was used to monitor whether the assay was performed properly and provided reference points for image analysis and for normalization of all the other protein spots (further details can be found in the **Materials and Methods section**, **Figure S1** and **Figure S2A** in the Supplementary Material). The microarray chip was successively incubated with milk sample extract, secondary antibodies and Quantum Dots, whereupon the microarray spots were fluorescently labelled depending on the amount of biomarker present in the sample extract. The microarray chip was subsequently imaged using the low-cost cellphone attachment housing UV LEDs for fluorescence excitation, an external lens for an

increased field of view and an optical filter (**Figure 1A** and **Figure 1B**) [12]. An exemplary microarray image as it was taken by the cellphone camera is shown in **Figure 1C**. The luminance of each single protein spot in the microarray images was automatically analysed using a custom-written MATLAB software code, which detected all protein microspot locations (**Figure S2C**) and reported median luminance values per protein spot. The luminance data of respective protein spots were averaged, the background signal (negative control) was subtracted and the background-corrected luminance was normalized against the positive control on the same microarray chip.

As a first step for evaluating the applicability of the developed protein microarray, an IGF-1 standard addition curve was measured, which was prepared in milk extracts from an untreated cow. The best results were obtained with 100 µg mL^{-1} anti-IGF-1 antibody spotted onto the microarray chip. Despite the relatively small increase in fluorescence signal, a concentration-dependent trend was clearly obtained (**Figure 2**), demonstrating that the low IGF-1 levels in milk extracts can be analysed in the relevant physiological range using a cellphone-based fluorescent microarray platform. Note that no standard curve can be obtained for the anti-rbST antibodies because of the lack of a pure standard of that biomarker. Then, milk sample extracts from rbST-treated and untreated cows were tested to investigate whether these can be discriminated on the basis of their biomarker responses. In milk sample extracts from rbST-treated cows increased IGF-1 levels and anti-rbST antibodies were detected (**Figure 3A** and **Figure 3B**). These results are in agreement with previously found increased IGF-1 levels and anti-rbST antibody presence in serum and milk after rbST treatment [9-11]. Furthermore, similar as with more sophisticated lab-based methods, in this protein microarray approach anti-rbST antibodies also appear to be more discriminative than IGF-1 [9, 10]. Anti-rbST antibodies form a very specific biomarker and are only induced after rbST treatment and not present in serum or milk otherwise [9, 10], whereas IGF-1 is always present in serum and milk at certain background levels.

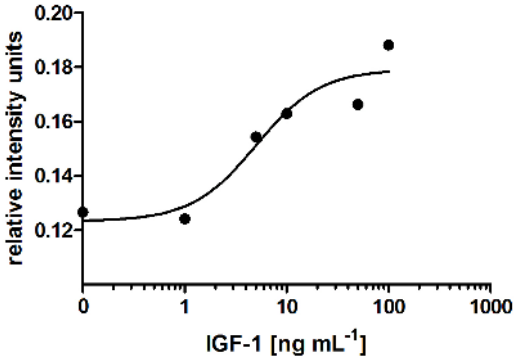

Figure 2: IGF-1 standard addition curve. The curve was prepared in milk from an untreated cow.

Compared to the particle-based cellphone concepts [12, 13], the presented microarray format shows several advantages. First, no white LED is required in the microarray format. Previously, a white LED was used to locate and count the total number of particles in the darkfield image. In the multiplexed microarray format presented in this work, the positions of the positive control spots determine the location of the other protein spots, which can then be easily analysed using the automatic image analysis. Second, in our previous work, a particle count ratio between the fluorescence and darkfield images was calculated, which correlated with the biomarker level in the sample. Therefore, two individual images had to be taken and analysed in our previous work. In the current format, however, the luminance of each protein spot is determined from a single image in the fluorescence mode. And third, using the multiplexed microarray format, the simultaneous detection of multiple replicates of several different biomarkers is possible and even positive and negative controls can be incorporated. The fixed location of each individual spot in the array allows the discrimination of multiple biomarkers by using the same Quantum Dot label for all markers analysed.

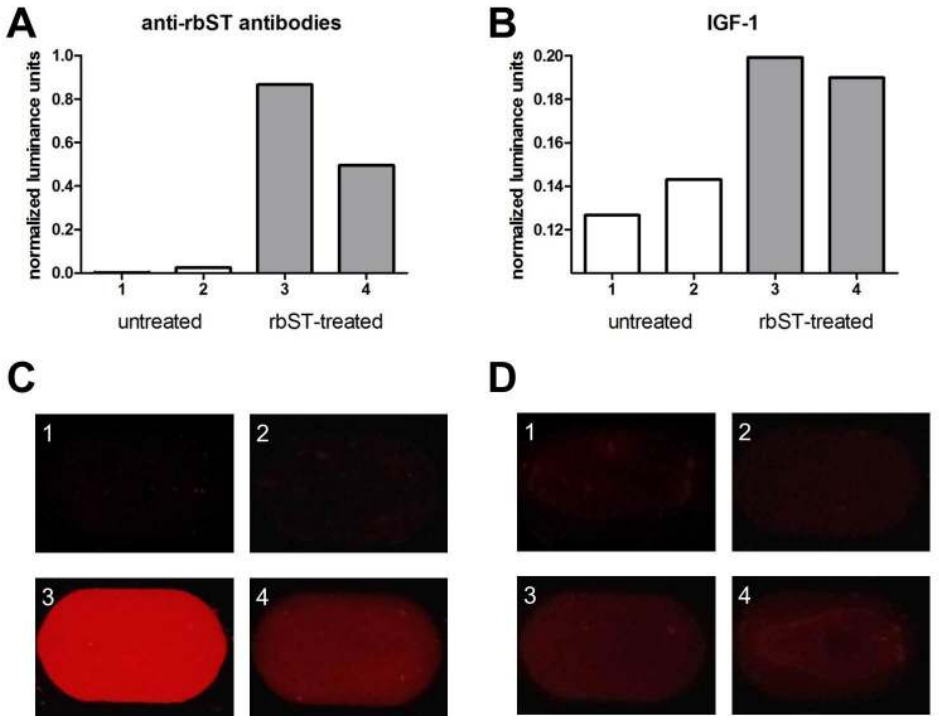

Figure 3: Biomarker responses of rbST-treated and untreated cows. (A) Normalized luminance units of the biomarker anti-rbST antibodies in milk extracts from untreated (1, 2; white bars) and rbST-treated (3, 4; grey bars) cows. (B) Normalized luminance units for the biomarker IGF-1 in the same milk extracts from the same untreated (1, 2; white bars) and rbST-treated (3, 4; grey bars) cows. Corresponding selected individual microspot images obtained with the cellphone-based fluorescent microscope for the biomarkers (C) anti-rbST antibodies and (D) IGF-1.

In order to validate the developed cellphone-based biomarker microarray approach for rbST abuse detection during official on-farm dairy controls, higher numbers of milk samples from rbST-treated and untreated cows need to be tested. The same is necessary to account for physiological fluctuations in biomarker levels, which are expected to occur in milk as well as in serum [10]. The continuous flow microspotter used for production of the microarray chips allows spotting 48 different protein spots at the same time (**Figure S2**), therewith providing the opportunity to detect more distinct biomarkers on the same chip and/or to perform replicate analysis. Note that the number of spots may be easily

increased using a microcontact printing technique. For applicability of the developed cellphone-based microarray platform in resource-limited environments, the assay protocol and its incubation times need to be improved. For rbST abuse detection, such an improvement could include a co-incubation step of the different assay components. For a general microarray approach using a cellphone, the incorporation of a microfluidic device having all assay components included [14] could minimize the required manual handling steps. Moreover, the use of a membrane filter microarray format, in which the sample and the bioreagents are flowing through a membrane filter, would overcome diffusion-limited binding assay kinetics and thereby reduce the required incubation time to a minimum [15].

This cellphone-based protein microarray approach clearly demonstrates that it is possible to monitor several biomarkers simultaneously for food safety monitoring. Since the microarray format is broadly applicable, multiple contaminants and/or biomarkers may be analysed in a similar manner for environmental monitoring and health care. The latter includes POC applications, home-diagnostics, mobile-health, or telemedicine analysis. The showcase assay presented here only required a few drops of milk and therefore this platform is very well suited for assaying small volumes, such as blood, saliva and urine. The hardware costs for such a cellphone attachment are significantly reduced compared to conventional fluorescent microscopes [13, 16]; the housing dimensions of the attachment can be easily adapted to the rapidly changing cellphone market by simply 3D-printing a new housing; the microarray assay kit may be eventually purchased through the internet. A cellphone-based device offers the opportunity to instantly analyse the images using the internal computing power of the phone and transmit the results to food safety authorities, environmental monitoring centres or medical specialist doctors via wireless network connection. Therewith, immediate measures can be initiated on the spot depending on the outcome of the test. We envisage that in all these application areas, cloud-based cellphone analysis networks may be established to create patio-temporal maps of contaminant or disease occurrence and spreading, also providing an important tool for *e.g.*, epidemiology.

Acknowledgments

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Supplementary Material

Materials and Methods

Reagents

Monsanto rbST standard was obtained from the National Hormone & Peptide Program of Dr. Parlow (Torrance, CA, USA). Posilac 500 mg single-dose syringes and syringes with only the slow-release formula were purchased from Monsanto Company (St Louis, MO, USA) and Ely Lilly and Company (Indianapolis, IN, USA). Rabbit anti-IGF-1 antibody (H-70), biotinylated donkey anti-sheep antibody and biotinylated goat anti-bovine antibody were bought from Santa Cruz Biotechnologies (Santa Cruz, CA, USA). Sheep IgG was obtained from Jackson Immunoresearch Europe Ltd (Suffolk, UK) and albumin from chicken egg white (ovalbumin), N-hydroxysuccinimide (NHS), 2-(N-morpholino) ethane sulfonic acid (MES), (w/v) 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimid (EDC), ethanolamine hydrochloride and tris(hydroxymethyl)-aminomethan (Tris) were bought from Sigma-Aldrich (St. Louis, MO, USA). MultiScreen HTS filter plates (pore size 1.2 µm) and Amicon Ultra 0.5 mL 30 K centrifugal filter units were purchased from Millipore (Billerica, MA, USA). Polycarboxylate hydrogel-coated borosilicate glass chips (24.9 x 24 x 0.13 - 0.16 mm) were from XanTec bioanalytics GmbH (Düsseldorf, Germany). Biotinylated rabbit anti-IGF-1 antibody was obtained from R&D Systems (Minneapolis, MN, USA) and Streptavidin-conjugated Quantum Dots (QD) were from Life Technologies (Grand Island, NY, USA). Prot G HP SpinTrap columns and 30000 MWCO VivaSpin500 units were obtained from GE Healthcare Europe GmbH (Diegem, Belgium). Sodium chloride (NaCl), monosodium phosphate monohydrate ($NaH_2PO_4 \times H_2O$), potassium dihydrogen phosphate (KH_2PO_4), Tween 20 and glass microscope cover slides (rectangular: 24 x 32 mm, thickness 1) were obtained from VWR International (Amsterdam, The Netherlands) and glycine was from Duchefa (Haarlem, The Netherlands). Sodium hydroxide (NaOH), sodium hydrogen carbonate ($NaHCO_3$), disodium hydrogen phosphate dihydrate ($Na_2HPO_4 \times 2H_2O$), acetic acid and hydrochloric acid (HCl) were purchased from Merck (Darmstadt, Germany). Sodium dodecyl sulfate (SDS) was obtained from Serva (Heidelberg, Germany).

Buffers and solutions

Antibody purification: binding buffer (20 mM NaH_2PO_4, pH 7.0), elution buffer (0.1 M glycine-HCl, pH 2.7), neutralizing buffer (1 M Tris-HCl, pH 9.0); microarray spotting: elution buffer (1 M NaCl, 0.1 M $NaHCO_3$, pH 10.0), activation buffer (0.1 M (NHS), 0.1 M MES,

0.5 % (w/v) EDC) washing buffer (5 mM acetic acid), coupling buffer (50 mM MES, pH 5.0), quenching buffer (0.5 M ethanolamine hydrochloride, pH 8.5); sample preparation: glycine solution I (GS I; 27.5 mM glycine, pH 0.5 adjusted with HCl); glycine solution II mixture (GS II; 230 mM glycine, 250 ng mL^{-1} IGF-2, 0.015 % SDS (w/v), pH 10 adjusted with NaOH); microarray procedure: phosphate-buffered saline with Tween (PBST; 154 mM NaCl, 5.39 mM Na_2HPO_4, 1.29 mM KH_2PO_4, 0.05 % v/v Tween20, pH 7.4)

Instruments

The NanoDrop instrument was obtained from Isogen LifeScience (De Meern, The Netherlands).The continuous flow microspotter was from Wasatch Microfluidics (Salt Lake City, UT, USA) and the Samsung Galaxy SII cellphone was obtained from Amazon.com, Inc. (Seattle, WA, USA). The cellphone fluorescent microscope attachment was constructed as described in [12]. In short, the mechanical components, such as the cellphone holder, the sample tray and the lid were made from thermoplastic using a 3D printer from Stratasys (Eden Prairie, MN, USA). For fluorescence excitation, twelve 380 nm UV LEDs, obtained from Parts Express (Springboro, OH, USA), were positioned around the sample tray. A 610 nm long pass filter (25 mm diameter) and an aspherical lens (focal length 8 mm), both were obtained from Thorlabs (Newton, NJ, USA), were used to filter the fluorescence light and improve the imaging field of view, respectively.

Sample material

Milk samples were collected from *Bos taurus* controlled animal treatment experiments as described before [9]. For both studies, permissions were obtained from the respective local ethical committees.

Antibody purification

Rabbit anti-IGF-1 antibodies (H-70) had to be purified from their storage solution to avoid interference with stabilizing proteins during protein spotting. For purification, Prot G HP SpinTrap columns were used according to the protocol of the manufacturer followed by membrane ultrafiltration using 30kDa MWCO centrifugal filter units. Protein concentration was determined with the NanoDrop.

Microarray chip spotting

Before protein spotting, polycarboxylate hydrogel-coated borosilicate glass chips were activated. Therefore, they were kept in elution buffer for 5 minutes, were washed three times in Milli Q water, activated in activation buffer for 15 minutes, washed three times in washing buffer and dried in a sharp stream of nitrogen gas. Then, they were mounted in the continuous flow microspotter and spotted with the protein solutions. Protein concentrations were 100 µg mL^{-1} rbST, ovalbumin, IGF-1 antibody and sheep IgG and 250 µg mL^{-1} for IGF-1 antibody in coupling buffer. RbST and each of the IGF-1 antibody concentrations were spotted in replicates of 6 each. The remaining spots were allocated with 15 replicates each of positive and negative control. The spotting instrument settings were as follows: location 3, air gap 5 µL, flow time 30 minutes and rinse 2 minutes. After spotting, the remaining binding sites were quenched for 30 minutes in quenching buffer and washed three times in Milli Q water. Readily spotted chips were kept in PBST at 4 °C until use.

Milk extraction

For the protein microarray procedure, milk samples had to be extracted. Therefore, 250 µL milk sample (just a few drops) was mixed with 250 µL glycine solution I while vortexting and kept at room temperature for 60 minutes. Thereafter, 500 µL glycine solution II mixture was added while vortexing and samples were filtered via a 1.2 µm pore size filter.

Microarray procedure

The spotted microarray chip was washed with PBST and 1 mL milk extract was pipetted onto the chip and incubated for 60 minutes (**Figure S1**). The chip was washed again and 500 µL antibody mixture was pipetted onto the chip surface (1:10,000 biotinylated donkey anti-sheep antibody, 1:10,000 biotinylated goat anti-bovine antibody, 1:125 biotinylated anti-IGF-1 antibody in PBST) and incubated for 60 minutes. After washing the microarray chip, 500 µL 20 nM QD solution was pipetted onto the chip and incubated for 30 minutes. After a final washing step, the microarray chip was covered with a cover slide to keep the fluorescence label in an aqueous environment and for easier handling. The microarray chip was then positioned into the cellphone microscope attachment and was imaged using the ultraviolet LED-based excitation in the cellphone attachment. The phone was set to ‘night mode’ for increased sensitivity.

Image analysis

In order to analyse the spots automatically, the MATLAB® Image Processing ToolboxTM was used (MATLAB 8.1, The MathWorks Inc., Natick, MA, 2013). The aim of the image analysis is to determine the luminance in every spot systematically and calculate luminance values of the spot area in a standardized way. Since all images showed spatial aberrations (see *e.g.*, **Figure S2B**), it was necessary to transform each image to a standardized image from which the luminance values could be calculated. In a first step, the positive control spots were detected automatically by finding connected illuminating areas of a certain minimum size. Since the positive control spots were always at the same location, their centroid was used to perform a first transformation (*i.e.*, cubic polynomial transformation) to distinct locations (**Figure S2C to S2D**). Therewith, shearing was removed and curving was reduced. Next, the image was cut to the area containing only the spots and omitting the remaining surrounding area. In a second cubic polynomial transformation, the slightly curved image was uniformly sized and therewith, the spots were positioned on specific locations (**Figure S2D to S2E**). After these image transformation steps, a standardized grid of centroids was used to determine luminance of each spot by taking all pixels within a defined ellipse into account (**Figure S2E**). The median luminance of each spot was reported. Then, the luminance data of respective protein spots were averaged, the background signal (negative control) was subtracted and background-corrected luminance was normalized against the positive control on the same microarray chip.

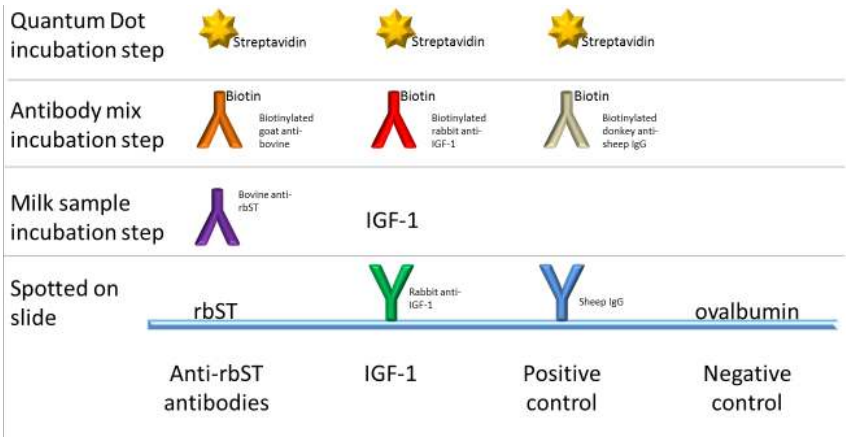

Figure S1: Protein microarray immunoassay principle. Recombinant bovine somatotropin (rbST), rabbit anti-insulin-like growth factor-1 (IGF-1), sheep IgG and ovalbumin were covalently coupled to polycarboxylate hydrogel-coated borosilicate glass chips (spot size: 400 µm x 600 µm). During the milk incubation step, anti-rbST antibodies and IGF-1

bound to their respective binding partners and during the antibody mix incubation step, biotinylated goat anti-bovine, rabbit anti-IGF-1 and donkey anti-sheep bound to their respective binding partners. In the final Quantum Dot incubation step, streptavidin-coupled Quantum Dots labelled the biotinylated antibodies present on the microarray chip.

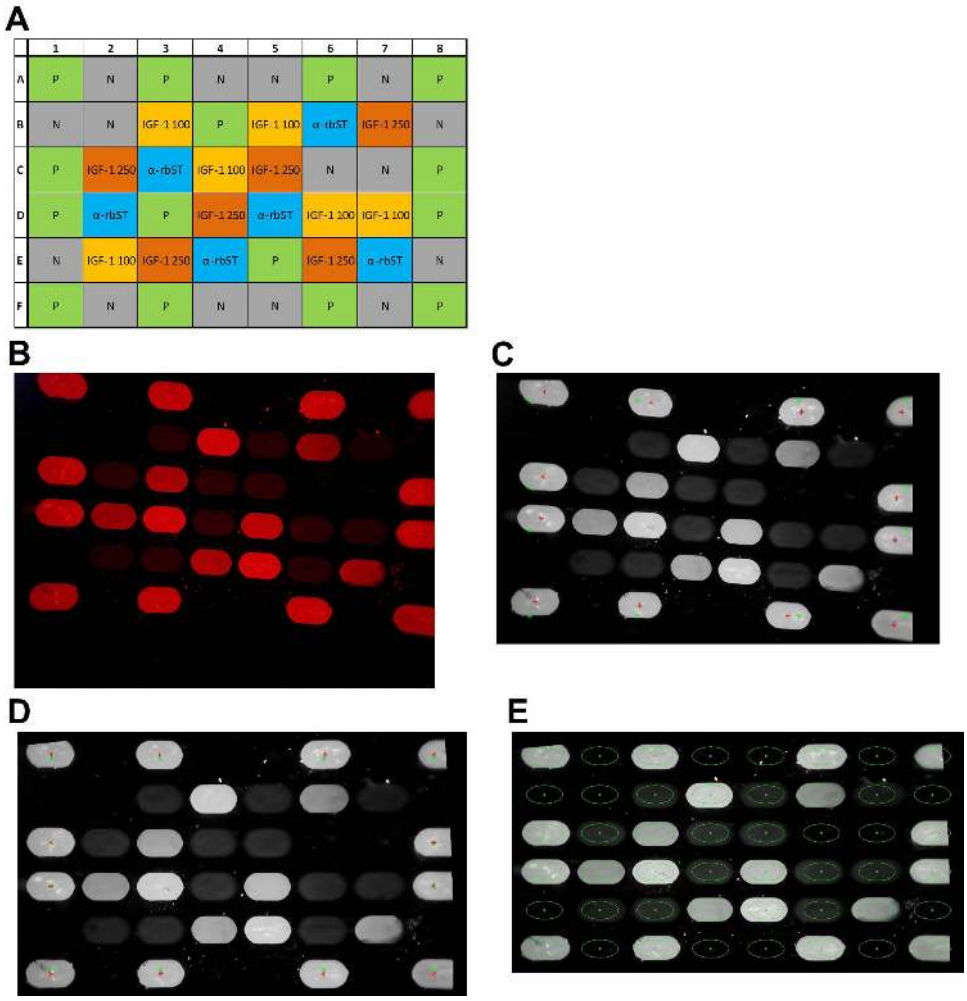

Figure S2: Microarray images. (A) Microarray layout for spotting of the four different proteins recombinant bovine somatotropin (rbST) for detection of the biomarker anti-rbST antibody (α-rbST, blue), anti-insulin-like growth factor-1 (IGF-1) antibody (spotted in two concentrations: 100 µg mL^{-1} and 250 µg mL^{-1}) for detection of the biomarker IGF-1 (IGF-1

100, yellow; IGF-1 250, orange), ovalbumin as a negative control (N, grey) and sheep IgG as positive control (P, green). (B)-(E) Image analysis workflow showing the original cellphone camera image (B), transformation points (red to green) for (C) correcting aberrations in a first transformation step and (D) size correction in a second transformation step. (E) Final image with elliptic spots, from which the median luminance is obtained. Luminance data of respective protein spots were averaged, the background signal (negative control) was subtracted, background-corrected luminance was normalized against the positive control on the same microarray chip and normalized values were plotted in Figure 2A and Figure 2B.

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9

General discussion and future outlook

1 A protein biomarker-based screening tool for rbST abuse detection

As reported by the Spanish newspaper La Voz de Galicia in April 2012, an illegal storage of hundreds of rbST syringes was uncovered and it turned out that rbST had been used on more than 300 Spanish farms [1]. This shows that even after rbST has been banned since 2000, the topic of illegal rbST use in milk production remains ongoing. Most likely, milk producers continuously use the hormone, because of its economic benefits and so far, there is no analytical method implemented EU-wide to control for this abuse. The development of proper methodology to directly detect the hormone itself in the cow's body fluids is facing several challenges: First, the molecular structure of the recombinant proteohormone is more than 99 % identical with its endogenous form; the 22 kDa proteins differ by only 60 Da, which is due to an N-terminal amino acid exchange from alanine to methionine [2]. Second, there are strong natural fluctuations in somatotropin concentrations in blood circulation. And third, expected rbST concentrations are very low: in serum they are less than 30 ng mL^{-1} and in milk less than 1 ng mL^{-1} [3, 4].

The approach followed in this thesis therefore focussed on the detection of rbST-dependent biomarkers. This is an indirect approach, meaning that the rbST molecule itself is not measured. Instead, rbST-dependent proteins, which are specifically up- or downregulated by physiological rbST actions, give an indication whether the hormone was used. This approach is advantageous since it is not affected by low rbST or fluctuating bST concentrations and molecular similarities of the recombinant and endogenous hormone. Furthermore, biomarkers remain altered for a prolonged period of time and offer therewith an extended time window for detection of rbST abuse.

1.1 Biomarker screening in serum

Analysed serum protein biomarkers were IGF-1 and IGFBP2, which are members of the GH-IGF-axis, and anti-rbST antibodies, which are produced by the cow as an immunological response towards rbST (**Chapter 3**) [5]. This triplex assay was complemented with the biomarker osteocalcin, a marker of bone turnover (**Chapter 4**) [6]. All four biomarkers were simultaneously analysed in serum of rbST-treated and untreated cows using the FCIA technology. The individual biomarker results were combined using the statistical model *k*-nearest neighbours (kNN) and true-positive rates were calculated and plotted over time (**Figure 3 in Chapter 3** and **Figure 5 in Chapter 4**). According to European legislation (Commission Decision 2002/657/EC), screening methods should be capable of at least

detecting 95 % of the samples from treated animals as being non-compliant [7]. Both, the triplex and the fourplex FCIA formats did not differ too much in their performances: both detected 4 out of 11 time points during rbST-treatment above the 95 % target (**Table 1**). And also during the wash-out phase for both FCIA formats, 3 out of 5 time points were above the 95 % target. The false-positive rates for the entire animal study period were only 2.3 % for the triplex FCIA and 5.5 % for the fourplex FCIA. Even if both FCIA formats seem to show similar performances in correctly identifying rbST-treated and untreated cows, there is a major difference: For the statistical model of the triplex FCIA, the very same data were used as training and test set data. Therefore, this model and data outcome is over-optimistic. In the fourplex FCIA model, however, two different data sets were used for model building and testing: data from one group of cows were used to build the model (training set) and data from an independent group of cows were used to test the model (test set). This means that the outcome is more realistic than in the triplex format and that the performance results are statistically spoken more robust than the triplex results.

Table 1: Time points above the 95 % true-positive target during treatment and wash-out phases and overall false-positive rates of triplex, fourplex and reduced duplex FCIAs for rbST abuse detection in rbST-treated and untreated dairy cows.

		Time points above the 95 % target		False-positive rate
Assay	**Chapter**	**Treatment phase**	**Wash-out phase**	**Overall**
Triplex FCIA	3	4/11	3/5	2.3 %
Fourplex FCIA	4	4/11	3/5	5.5 %
Reduced duplex FCIA	4	7/11	3/5	11.8 %

A next step was to assess and evaluate which of the 11 possible biomarker combinations was most indicative for rbST abuse and it appeared that the combination osteocalcin and anti-rbST antibodies showed the most time points above the 95 % target during the treatment phase (**Chapter 4** and **Table 1**). The biomarkers IGF-1 and IGFBP2 did not remain in the final reduced duplex FICA format. IGF-1 is a biomarker, which rapidly reacts upon rbST presence with a transient increase in serum concentration. Already before the next rbST injection, IGF-1 concentrations were back to baseline levels (**Figure 2A.1 Chapter 4**). These fluctuating IGF-1 levels are difficult to use as robust and reliable predictors for rbST abuse detection. IGFBP2 levels also showed a fluctuating pattern and furthermore, inter-individual differences were high (**Figure 2B.1 Chapter 4**). Hence, kNN models including IGFBP2 as a biomarker, performed less than others (**Figure 5 Chapter 4**).

For all presented formats, there were time points for which the 95 % true-positive target

was not reached. The most difficult time points for rbST abuse detection are short after beginning of the treatment, when there is a delayed biomarker response, and during the wash-out phase, when altered biomarker levels return to baseline. If rbST is administered according to manufacturer's suggestions, the wash-out phase is at the same time as the dry period, which usually lasts for 50 days (approximately 7 weeks). In the animal studies performed for this thesis, the wash-out phase lasted only 4 weeks, meaning that not the entire dry period was covered. Moreover, the triplex, fourplex and reduced duplex formats were all capable of only detecting rbST administration up to 2 weeks after the last administration. This means that the biomarker-based approach is so far not good enough for all time points and needs to be improved to cover the entire treatment phase and a large period of the wash-out phase.

The animal studies performed for obtaining the data of this thesis comprised of four or six rbST injection time points respectively. In reality, cows are treated for 25-30 weeks, which results in 13-15 rbST injections per lactation period. No data are available how well the here presented FCIA formats perform in long-term treated cows. Especially when looking at the declining immunological response after a few rbST administrations (**Figure 2C.1 in Chapter 4**), the performance of the anti-rbST antibodies as a biomarker needs to be assessed in long-term rbST-treated cows. Also Zwickl *et al.* [8] saw a declining immunological response in long-term treated cows after 3 months of rbST treatment. Furthermore, they did not see an immunological memory effect with boosted antibody titres in cows, which were treated in two subsequent years. In both years however, they found that approximately 83 % of the rbST-treated cows produced anti-rbST antibodies. For long-term and repeatedly treated cows, the fourplex FCIA should be tested and all biomarkers should be evaluated individually and in combination for their capability of detecting rbST abuse.

To improve the detection capabilities of the FCIA, additional protein biomarkers can be included. Potential biomarkers are for instance myostatin, N-terminal propeptide of procollagen III (PIIINP) and C-terminal cross-linked telopeptide of collagen I (CTx). For all these potential biomarkers, commercially available immunoassays were obtained to test the feasibility of the biomarkers in serum samples from rbST-treated and untreated cows. Even though these commercial assays were initially developed for analysis of human samples, amino acid sequence homology of the target proteins has been verified or these assay kits have been shown to be applicable to bovine samples in other studies [9-11]. Myostatin levels measured with an enzyme-linked immunosorbent assay (ELISA) kit remained unaffected by rbST treatment (**Figure 1A**).

An increase in PIIINP levels was observed in rbST-treated cows using a radio immunoassay (**Figure 1B**). PIIINP concentrations of untreated cows were similar except one cow showed a very high PIIINP concentration already before treatment (cow E). Note that cows A-D were all approximately 4.5 years old whereas cow E was only two years old and PIIINP levels are known to be age-dependent [12].

CTx concentrations were found to be very different between individual cows (**Figure 1C**). Baseline values before rbST treatment differed a lot and these differences were not age-dependent (data not shown), even though an age-dependent decline in CTx levels was observed in children and young adults [13]. CTx levels have been shown to vary during the day and these circadian fluctuations can be lowered by using samples that were taken after a 24 hour fasting period [14], which is not realistic for the analysis of samples from dairy cows. Also the responses upon rbST treatment were different: some cows (*e.g.*, cows J and K) did not show any response, some showed increasing CTx concentrations over time (*e.g.,* cows B, E and I) and some showed the highest CTx concentration after 3 weeks of rbST treatment (*e.g.*, cows C, D and F).

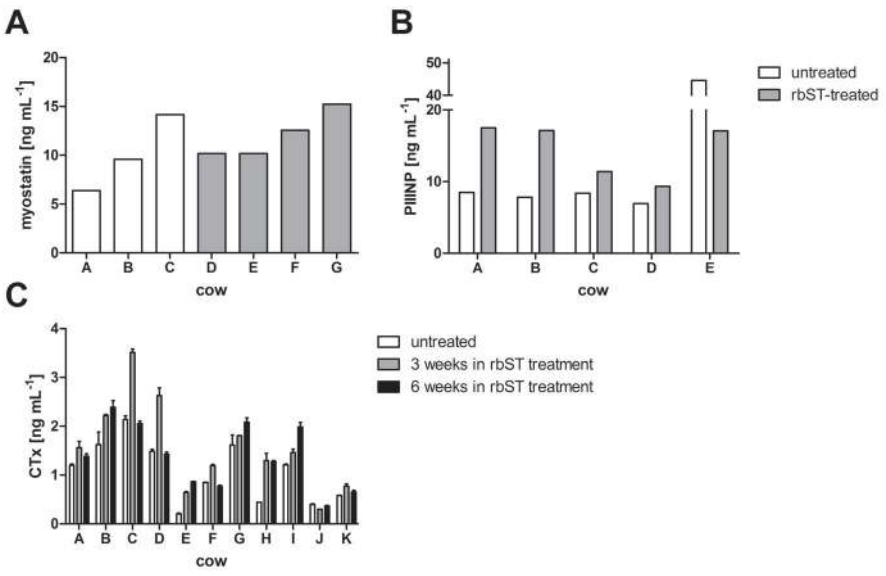

Figure 1: (A) Myostatin, (B) PIIINP and (C) CTx levels in serum of rbST-treated and untreated animals. Cow labels do not correspond between individual graphs.

In conclusion, based on these preliminary results, PIIINP can be considered a candidate biomarker but needs to be further investigated and validated on serum samples from more rbST-treated and untreated dairy cows. Successful FCIA assay development for PIIINP and inclusion in the existing fourplex FCIA was hindered by the lack of a suitable PIIINP standard protein.For additional serum biomarkers, biomarker discovery studies as outlined in **Chapter 2** can be performed. Similar studies have been performed for equine somatotropin (eST) detection in horses and hGH abuse detection in humans and revealed a high number of candidate biomarkers. Also in cattle and race horses for detection of steroid and other anabolic agent treatments, biomarker discovery studies have been performed and candidate biomarkers, such as reticuloalbin, apolipoprotein A1 and clusterin, were identified (**Chapter 2**) [15-17].

1.2 Biomarker screening in milk

Another important part of this thesis is the targeted analysis of protein biomarkers in milk, such as anti-rbST antibodies, which can be found in more than 62 % of the milk samples from the rbST treatment animal studies. They were detectable starting from the second rbST administration and showed the maximum response around 20 days in treatment. Thereafter, the response declined, but remained above the decision limit for two to three more weeks during the wash-out phase (**Figure 2 in Chapter 6**). For the untreated cows, a false-positive rate of 7 % was found. Obviously, the true-positive rate of 62 % (equivalent to false-compliant rate of 38 %) is not sufficient according to Commission Decision 2002/657/EC for screening methods [7]. But note that in real practice, tank milk will be analysed and therefore, milk from high-, low- and no-responders will be mixed, leading to a detectable antibody response as shown in **Chapter 6** [18].

Similarly to the FCIA formats for multiple biomarker analysis in serum, several different rbST-dependent biomarkers can be added to the existing milk FCIA. For preparing the cellphone-based protein microarray analysis of milk samples (**Chapter 8**), a duplex FCIA was tested for the simultaneous detection of anti-rbST antibodies and IGF-1. In this duplex FCIA, increased IGF-1 levels and increased anti-rbST response were detectable after rbST treatment (**Figure 2A and 2B**). The expected levels of IGF-1 in milk typically range from 1 ng mL^{-1} to 50 ng mL^{-1} and can further increase after rbST treatment [19, 20]. The standard addition curve of the duplex FCIA (**Figure 2C**) has its linear range from approximately 10 ng mL^{-1} to 50 ng mL^{-1}. Taking into account that there is IGF-1 present in the milk used for the standard addition curve, this method is not accurate for milk samples with a very low IGF-1 concentration.

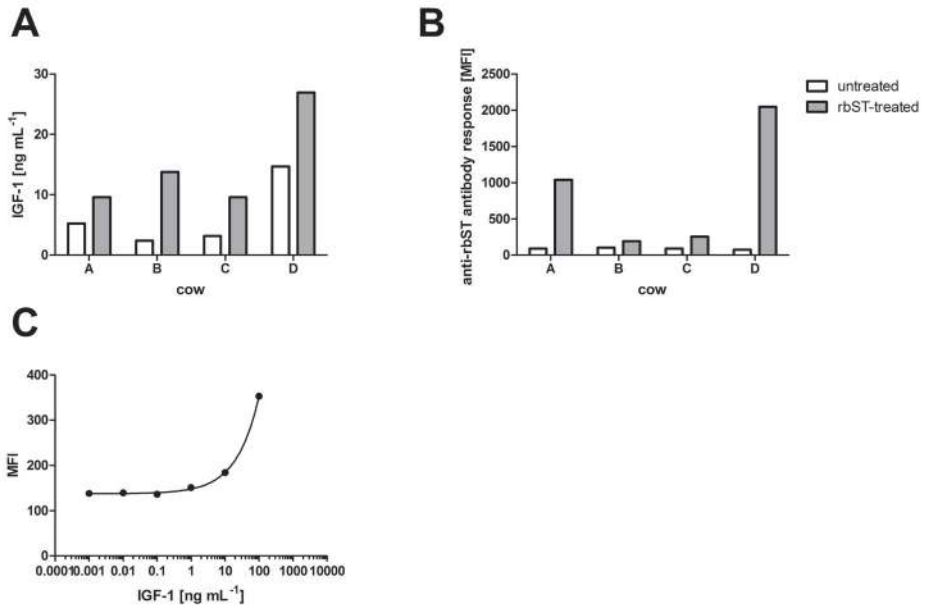

Figure 2: (A) IGF-1 concentrations and (B) anti-rbST antibody response in milk samples from untreated and rbST-treated cows. (C) IGF-1 standard addition curve in milk from an untreated cow (MFI – median fluorescence intensity).

According to serum biomarker discovery studies (**Chapter 2**), milk biomarkers for detecting rbST abuse can also be discovered in an untargeted manner using mass spectrometry techniques. Biomarkers for rbST treatment in milk could be growth factors, similarly to IGF-1 (insulin-like growth factor-1). Growth factors belong to the low-abundant proteins in milk and discovery of low-abundant proteins poses challenges as already described in **Chapter 2**. One possibility to facilitate the detection of low-abundant proteins is the use of suitable depletion methods for removal of high-abundant proteins. Unfortunately, the majority of existing depletion methods is optimized for serum or plasma use. Caseins and β-lactoglobulin, the two major bovine milk protein fractions, are both not present in serum or plasma [21, 22]. Therefore, they are not sufficiently removed by serum-specific depletion methods. Another possibility for the detection of low-abundant proteins is the use of selective enrichment strategies to enrich the low-abundant protein fraction from the complex milk matrix. Boehmer *et al.*, however found serum-optimized selective enrichment tools not suitable for bovine milk samples because of their limited capability in removing high-abundant proteins, such as caseins and β-lactoglobulin [23]. Milk proteins

can also be analysed after removing a major fraction of the caseins by ultracentrifugation of the raw milk sample. Using this approach, Hettinga *et al.* detected 269 milk proteins in bovine milk using a qualitative setup; only a limited amount of these proteins was subsequently also quantified [24]. For untargeted milk biomarker discovery studies, there is a lot of future research needed on suitable sample preparation strategies.

1.3 Biomarker screening in other matrices

For the detection of certain substances in sports doping and veterinary control, urine is used for analysis. The detected substances are for example steroids, which are also excreted via the renal route. In the recent years, the urinary proteome was studied and it was also used for protein biomarker discovery in clinical studies [25, 26]. In a protein biomarker discovery study for hGH-dependent biomarkers in serum, α1-antitrypsin has been identified as a candidate biomarker (**Chapter 2**) and this protein has also been found in the human urinary proteome [25, 27]. Similarly to many serum proteins, urinary protein concentrations underlie inter- and intra-individual differences and therefore, characterization of the normal fluctuations is required [25]. In conclusion, it might be possible to detect protein biomarkers for rbST abuse in urine of cows.

2 Biomarker panels for discrimination of different substance classes

In **Chapter 5** in this thesis it is shown that a biomarker panel originally developed for detection of rbST abuse can also be used to indicate steroid hormone administration. Likewise, in **Chapter 2** it is described that similar protein biomarkers, such as IGF-1, leucine-rich α-2-glycoprotein or PIIINP, were indicative for more than one specific treatment. The reason for this is that partly the same physiological machinery is used as a response upon different growth promoter treatments. This finding highlights the significance of biomarker-based approaches. It is conceivable that in the future, multiplex assays comprising a full range of different biomarkers are used to indicate a class of substances, where the abused substance belongs to. With this knowledge, subsequent targeted instrumental analysis can be performed for confirmation of the abused substance.

Even though biomarker profiling for an entire range of different substances offers great advantages, the issue of confounding factors has to be carefully considered. Confounding factors are for instance age, gender, nutrition, exercise, injuries, medication or co-

administration of other drugs. In general, the more a biomarker is influenced by other factors or treatments, the more extensive validation procedures are required. This extensive validation is particularly essential for biomarkers, which are indicative for more than one administered substance.

3 Multiple-analyte assays: extensive development procedure

When multiple biomarkers are measured, multiple-analyte, also called multiplex assays, have major advantages over single-analyte assays. First, the required sample material is kept at a minimum volume, because all biomarkers are simultaneously analysed in one sample aliquot. Second, sample preparation and assay time is minimized, because all steps are performed only once. And third, material, bioreagents and chemicals are saved for the same reasons [28].

Although the conduction of a multiplex assay is time-saving, because several assays are combined in one, the development of such a multiplex assay is more laborious compared to the development of a single-analyte assay. Several additional experiments are required to ensure that no unwanted cross-reactions occur during an assay for combined detection of several analytes. Moreover, these experiments have to be performed again if an existing multiplex analyte panel is extended by additional analytes. In general, the higher the number of different antibodies and standard analytes in one well, the higher the chance of unwanted cross-reactions of the antibodies with each other, of antibodies with non-specific analytes and analytes with each other [28, 29].

By decreasing the total number of different assay components (antibodies and protein standards), possible unwanted cross-reactions can be avoided. **Figure 3** shows three exemplary immunoassay formats with decreasing number of assay components. Whereas in the classical sandwich format, typically four assay components are required, in the inhibition format only three and in the direct inhibition format only two are required. Despite the fact that less assay components show a lower chance of unwanted cross-reactivity, additional detection antibodies can amplify the final signal and can therefore lead to more sensitive assays.

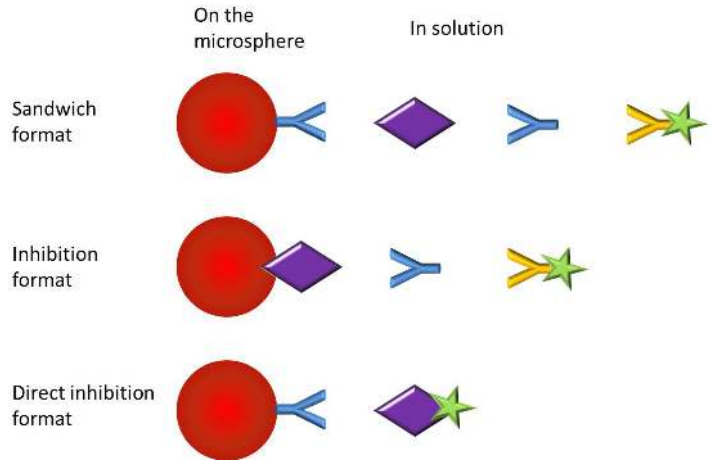

Figure 3: Three exemplary microsphere-based immunoassay formats suitable for multiplexing. In the sandwich format and direct inhibition format, capture antibodies are immobilized on the surface of colour-encoded microspheres, whereas in the inhibition format, a protein standard is immobilized. Depending on the assay format, different bioreagents, such as analytes and detection antibodies (either labelled or unlabelled) are present in solution.

Another important item in the development of a multiplex immunoassay is the sample that shall be measured. To account for sample matrix effects, standard curves should be performed in the final sample matrix. Moreover, all standard curves are to be measured with all assay components simultaneously to keep the assay conditions as similar as possible between the standards and samples. When exogenous analytes are analysed, which are normally not present in a sample, the analyte can be simply spiked in blank samples to create a standard curve. However, when endogenous analytes are analysed, which are always present in a sample at a certain concentration, the choice of matrix for a standard curve is more difficult. On one hand, blank samples can be created by depleting the sample from the target analyte. But when multiple analytes are intended to be measured and therefore, multiple analytes are depleted, the character of the standard sample matrix becomes more and more different from the real sample matrix. On the other hand, matrix-matched buffers can be prepared as an alternative for a blank sample. For the development of the serum biomarker FCIA in this thesis, 80 mg mL^{-1} bovine serum albumin in phosphate-buffered saline was used as a serum-matched buffer, which resembled the total protein concentration in a serum sample. Then, according to the analysis of exogenous analytes, the analytes were spiked into the serum-matched buffer

for the standard curve. One has to be aware that both, analyte depletion and the use of matrix-matched buffers do not necessarily completely reflect the character of the final sample, but might be fit-for-purpose.

When combining several single-analyte assays into one multiple analyte method, the different target analyte concentrations in the sample and the sensitivities achieved using the different assays have to be considered. If a developed multiplex assay requires different sample preparations, this method might detect several analytes at the same time but not in the very same sample [30, 31]. One can only make use of all advantages of a multiplex method if the target concentrations of all the analytes in the sample fit into their respective standard curve at the same sample dilution.

4 On the search for treatment-specific biomarkers: Anti-rbST antibodies

Even if protein biomarkers are usually endogenously produced and are therewith influenced by many different factors, some can be specific for one treatment and only present after that treatment. One example is the biomarker anti-rbST antibodies, which are endogenously produced by the cow after administration of rbST (**Chapters 3-8**). There can be several different reasons for the occurrence of anti-rbST antibodies after rbST administration: First, the body might detect the administered rbST as an exogenous protein because of its altered N-terminus and respond via the T-cell-dependent immunological pathway [32]. During this pathway, high titres of IgG are produced and memory cells are generated for rapid and high antibody production in case of a second administration. Considering that there were generally no high IgG titres and no memory effect observed upon a second administration period in the following year [8, 33], the activation of the T-cell-dependent pathway upon rbST administration seems unlikely [34]. Second, antibody production against biopharmaceuticals is a known phenomenon in the clinical environment and is attributed to many factors, such as product-related factors, patient-related factors and treatment-related factors [32]. Product-related factors are for instance protein aggregation, which often occurs in recombinantly produced biopharmaceutical formulas, and impurities from the recombinant protein production process, which both can trigger immunological reactions [35]. RbST is known to aggregate through hydrophobic interactions and these aggregates might therefore be responsible for the observed immunological response [36]. Moreover, since rbST is produced using *E.coli*, there might be impurities deriving from the host, which further influence the immunogenicity of the administered drug [32].

Since antibody production is a frequent phenomenon after treatment with protein biopharmaceuticals, the presence of these antibodies can be used as an indicator for protein hormone abuse, such as human growth hormone or erythropoietin, in human and animal sports doping analysis as well [37]. Compared to the other protein biomarkers used to indicate drug abuse, anti-drug antibodies are very specific for the particular treatment and therefore offer a great potential to complement current biomarker-based detection strategies (**Chapter 2**) [37-39].

For screening, anti-rbST antibodies can be incorporated into a biomarker panel and be measured using a multiplex FCIA for instance, but they can also be monitored using a quick on-site test platform, such as a lateral flow device or dip stick. A general consideration for analysing antibodies in cows' serum or milk is that there are many other different bovine antibodies present, which might affect the sensitivity of the test for the specific antibodies. Two different dipstick formats for the detection of anti-rbST antibodies were developed and tested during this thesis. In the first format (format A), anti-rbST antibodies are sandwiched in between rbST on the nitrocellulose membrane and rbST on coloured latex particles (**Figure 4A**). In this format, no secondary anti-bovine IgG detection antibody is required and therewith, unwanted cross-interactions with the other present milk antibodies can be avoided. In a second format (format B), the anti-rbST antibodies bound to rbST on the nitrocellulose membrane and were detected by a colloid carbon-labelled anti-bovine antibody (**Figure 4B**). Here, the result might be affected by other bovine antibodies present in the same sample, which may be bound by the anti-bovine detection antibodies. For both tests, the presence of anti-rbST antibodies resulted in appearance of a test line (**Figures 5A.1 and 5A.2**).

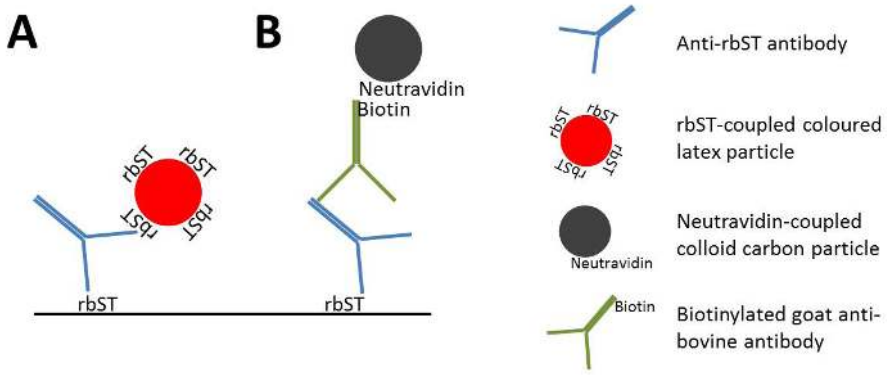

Figure 4: Dip stick formats tested for this thesis. In format A, the anti-rbST antibody interconnects rbST on the nitrocellulose membrane and rbST on coloured latex particles using its two antigenic binding sites. In format B, a labelled secondary antibody detects the presence of the anti-rbST antibody bound to rbST on the nitrocellulose membrane.

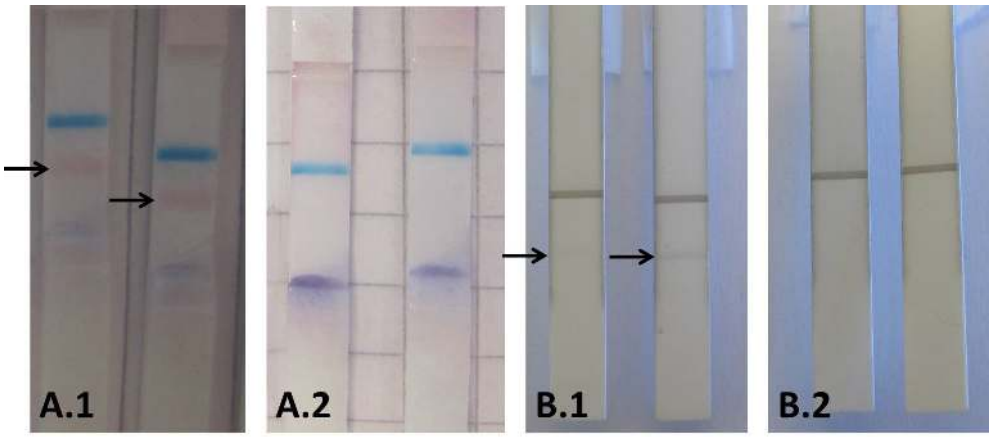

Figure 5: Examples of dipstick results in using (A) format A dipsticks and (B) format B dipsticks with milk samples from (1) rbST-treated and (2) control cows. Arrows indicate the presence of the test line.

To assess the performance of the developed dipsticks, 20 milk samples from rbST-treated and 20 milk samples from untreated cows were tested. These samples were selected based on their responses shown in FCIA experiments. Samples from rbST-treated cows previously found positive and samples from untreated cows previously found negative were selected. For both formats, different assay protocols were used since the biotinylated

antibody and neutravidin-carbon-conjugate required a pre-incubation step. Using the format A dipstick, 50 % of the milk samples from rbST-treated animals were detected (examples in **Figure 5A.1**), whereas with format B, 65 % of the milk samples from rbST-treated animals were detected (examples in **Figure 5B.1**). The false-positive rate obtained by analysis of milk samples from untreated animals was comparable between the two formats (examples see **Figure 5A.2** and **Figure 5B.2**; **Table 2**).

Table 2: True-positive results obtained by testing 20 milk samples from rbST-treated cows and false-positive results from 20 milk samples from control animals by using format A and format B dipsticks.

Cows	Format A	Format B
rbST-treated	50 % (10/20)	65 % (13/20)
untreated	5 % (1/20)	10 % (2/20)

These results indicate that both dipsticks yield anti-rbST antibody-specific signals, but are not sensitive enough to detect sufficient rbST-positive samples according to Commission Decision 2002/657/EC, which requires that 95 % of the treated samples are detected as non-compliant [7]. To improve the sensitivity of the dipstick, additional secondary labelled antibodies can be used to amplify the signal on the test line.

5 Effect-based screening methods in the view of current European legislation

5.1 Validation requirements for effect-based screening methods

Classically, screening methods are defined by Commission Decision 2002/657/EC as "methods that are used to detect the presence of a substance or class of substances at the level of interest.", whereby substance is defined as "matter of particular or definite chemical constitution and its metabolites." [7]. Thus, these definitions and the specified validation requirements for the detection of substances in this legal paper relate to the detection of a substance itself or its metabolites. But effect-based screening strategies, which only indirectly indicate that a substance is or was present, are not covered by the Commission Decision. Effect-based strategies comprise for instance bioassays, steroid profiling and biomarker methods. Bioassays monitor the effect of a bioactive substance, *e.g.,* the specific binding to a receptor using a transcription activation assay [40]. Steroid profiling can be used to monitor the effect of different administered substances on

steroidogenesis [41]. And protein biomarker levels can also be changed as an effect upon substance administration (**Chapters 2-5**). Even though in certain bioassays, a dose-response relation can be made and therewith the classical validation procedures according to Commission Decision 2002/657/EC can be followed, the other effect-based screening methods may not show a dose-response relation because of the complex physiological machinery behind the response. It is therefore advisable that these effect-based methods follow a different validation procedure before they can be used as a screening method. The aim of validating an effect-based screening method is the definition of a background response, background level or a reference profile in an untreated population and the determination of a threshold beyond which the sample is considered suspect. This can be achieved by the assessment of variation in an untreated population and statistical determination of the threshold value, *e.g.*, addition of 2.33-times the standard deviation of the population data to its mean. In this case, only 1 % of the untreated population will be classified as false-positive using the assessed screening method. Additionally, the response of a treated population and the variation therein has to be assessed to determine the false-negative rate, which should be less than 5 % for a screening method according to Commission Decision 2002/657/EC [7]. Since the effect-based methods are expected to identify suspect samples in a time-dependent manner after treatment, time course studies can be performed to determine in which time window the assessed effect can be observed with the required confidence (**Chapter 4**). This procedure is obviously more extensive and laborious compared to the classical validation described in Commission Decision 2002/657/EC and "Guidelines for the validation of screening methods for residues of veterinary medicines" [7, 42] and the extent is dependent on the variation observed in the treated and untreated populations. This means, the more confounding factors are exerting effects on the background response or response upon treatment, the higher the number of samples that are required for validation. Method validation by assessing untreated and treated populations including numerous confounding factors has been performed for sports doping analysis [39].

Despite successful effect-based method development, method validation can be hindered by strong inter-individual differences, which cannot be linked to general confounding factors. Then, the monitoring of individual profiles over time as done with the athlete's biological passport may be performed to see changes in protein biomarker or steroid profiles compared to previous profiles of the same athlete (**Chapter 2**). Abnormal intra-individual changes can then be related to substance administration. Animal passports are unfortunately not feasible due to the high number of individual animals in food production. But farm passports, combining samples from one farm, are conceivable if it is

proven that intra-farm variations are lower than inter-farm variations and that a combined biomarker or steroid profile can be indicative for substance administration.

5.2 Screening and subsequent confirmatory process

Traditionally when screening for a specific substance, a suspicious result can be obtained. The time window for finding a suspicious result is determined by the half-life time of the substance in the sample. Subsequent confirmatory analysis can then either confirm the previous finding, the sample is then considered non-compliant, or it can deliver a negative result, which in turn means that the screening result was a false-positive. The reasons for a false-positive screening result can be a specific (*e.g.*, a related compound was detected by a similar epitope with the antibody used in screening) or an unspecific interference (*e.g.*, an unexpected matrix effect) during screening or a substance level in the sample below the limit of detection of the instrument used for confirmatory analysis (**Table 3**).

Table 3: Screening and subsequent confirmatory process overview for the classical substance detection approach and the effect-based detection approaches.

Screening	Confirmatory positive (true-positive screening result)	Confirmatory negative (false-positive screening result)
Substance screening	Substance identity confirmed Substance level above threshold	Substance level below LOD Screening result was specific or non-specific interference
Effect-based screening	Substance identity confirmed Substance level above threshold Substance-effect relation	Substance level below LOD Screening result was specific (unknown active substance) or non-specific interference

If effect-based methods, such as bioassays, steroid profiles or biomarkers, are used for screening, the presumed time windows for detecting a suspicious sample are diverging. Since a bioassay is detecting the effect of a substance present, the time window for observing a specific signal is depending on the substance presence. Steroid profiles, which are the result of a specific endogenous enzyme activity, however, are presumed to be altered for a longer period of time. And biomarkers or biomarker profiles, which are usually the result of a changed protein synthesis in the animal's body, are presumed to be changed for the longest period and offer therewith the longest time window for detecting a suspicious sample.

If a suspicious sample has been identified using one of the abovementioned effect-based screening methods, the result has to be confirmed by a subsequent confirmatory method.

The confirmatory method can then confirm the presence of the substance, which caused the observed effect during screening, and therewith establish a direct substance-effect relation. But the confirmatory analysis can also give a negative result, thus a false-positive screening result. In the case of effect- or biomarker-based screening, false-positive screening results can also be caused by different reasons. First, the substance level in the sample can be below the limit of detection of the method used for confirmatory analysis. This can be because the effect of the substance can be detected longer than the substance itself or the substance level is in general too low for detection. Second, an unknown other substance caused the same effect during screening, which resulted in a specific interference. And third, an unspecific interference caused the false-positive result.

Note that there is one fundamental difference in reasons for false-positive screening results between the two approaches, the classical substance screening and the effect-based screening. During effect-based screening, a treatment-specific effect might be observed, which cannot be confirmed, because the substance itself is below the limit of detection in the sample. By the strict definition of EU regulation, this would mean a false-positive screening result. In view of the advantages of biomarker-based strategies, namely a prolonged time window for detection, this strict definition cuts back this valuable advantage. In this case, it might be a possibility to adopt the regulations used in sports and horse racing doping control where solely a specific biomarker profile can indicate the hormone abuse (**Chapter 2**). Then, the confirmatory method would not play such an important role anymore.

Another important point to discuss is the example of IGF-1, which is being used as a biomarker in different assays [39] (**Chapters 3, 4 and 8**), but can also be the abused substance itself [43, 44]. In this case it is advisable to follow both approaches, the classical approach detecting IGF-1 itself in blood, as IGF-1 levels are strongly increased after IGF-1 treatment [43], and the biomarker-based approach detecting IGF-1-dependent biomarkers [44]. Both approaches can then be compared in respect of false-negative and false-positive results during an entire treatment and washout period in an IGF-1 treatment study. Depending on which of the approaches gives the better outcome, either a single approach can be chosen for the following thorough validation, or both approaches can be used complimentary to increase the chances of a positive screening result. In the veterinary field, subsequent confirmatory analysis will focus on the direct detection of IGF-1 in the sample by instrumental analysis.

5.3 Towards a new veterinary drug monitoring approach

As described in the general introduction (**Chapter 1**), the Netherlands developed a multi-level surveillance system for food safety control. Within this system, the different levels are usually followed in a certain order depending on the extent of the occurring food safety problem. After a general suspicion is raised by for instance the discovery of injection preparations during general inspections, a surveillance program is run to estimate the extent of the use of the veterinary drug or contaminant. During the surveillance program, up to a few hundred of samples are measured using a simple screening method. In case of the rbST abuse detection, the surveillance step is reached, which means that screening methods have been developed (**Chapters 4 and 6**) and samples are being analysed on small-scale in a reference or specific laboratory. If suspicious samples are identified during screening, then a confirmatory method is required to detect the rbST molecule itself. An immunoaffinity enrichment on monolithic micro-column approach for rbST purification from serum prior to instrumental analysis is currently being developed and will be adopted for milk analysis as well [45]. Using this approach, the general problem of a very limited window of detection can be solved, because of its superior sensitivity performance compared to previously described confirmatory analysis attempts [2, 3, 46]. If screening and confirmation reveal that rbST is widely used in the dairy sector, rbST detection will be incorporated in the yearly national residue monitoring plan using the developed methods. Then, rbST use will be systematically analysed in official laboratories and the results are reported to the EU. At this stage, it is advisable to limit the amount of samples, which need to be transported to and analysed in specialized laboratories, for cost reasons. As discussed in **Chapter 7**, a quick on-farm pre-screening method can identify suspicious samples. Compliant samples, which are usually the majority of the tested samples, do not need to be administrated, transported and analysed using expensive and time-consuming methodology. As an effect of this, money and time can be saved, which in turn can be reinvested in further rbST pre-screening or sample analysis for other high priority substances.

6 Future outlook on biomarker profiling

The benefits of protein biomarker profiling have extensively been discussed in **Chapter 2**. They are not only widespread used in disease diagnosis but are also applied in sports doping control and biomarker-based methods are ready to be used in future veterinary control. For the latter, there are still a number of fields where biomarker-based screening methods can be beneficial especially in view of the expanded time window for detection.

Protein biomarkers can then serve as indicators even when the abused substance itself is not present anymore. Therefore, biomarker methods are imaginable for general hormone abuse detection (**Chapter 5**) [10, 30, 31, 47-49], contaminant pollution assessment [50] or heavy metal exposure analysis [51]. Also in environmental analysis, contaminant pollution and heavy metal exposure are important issues. Therefore, the same biomarkers as in veterinary control for food production can be indicative for the presence of trace levels of these substances.

When biomarker profiles or biomarkers for several different substance exposures are to be measured, the use of a multiplex method, such as the FCIA (**Chapters 3, 4 and 5**) is advisable. But, as discussed earlier, such a multiplex method is only beneficial if there are no cross-interactions between the single assays and if the same sample preparation can be applied for all the single assays. For instance, it can be assessed whether the multiple protein biomarker methods used in sports doping control, which are currently performed with single analyte methods (ELISAs), can also be performed using a multiplex method.

In addition to protein biomarkers, other biomarkers can be monitored as well, such as transcriptomic or metabolomic biomarkers [41, 52-54]. Using proteomic, transcriptomic and metabolomic technologies, combined biomarker profiles can be measured with enhanced statistical significance. Biomarker profiles that are altered after drug treatment can be indicative for a specific abused substance or group of substances. Therefore, expanded biomarker profiling can be used as a general drug abuse monitoring tool and point to samples, which need further targeted investigation.

7 Future outlook on technical developments

As described in **Chapter 8**, the use of an on-site test for multiplex analysis of several substances, which can be analysed with the help of the ligand-binding principle, enables the simultaneous detection of several food safety-related substances. Therewith, the opportunity arises to measure all targets of interest by only using one test. As an example, a single milk sample could be analysed for hormones, allergens, antibiotics, contaminants and other substances, all at the same time, provided they can be detected through the ligand-binding principle and no occurrence of unwanted cross interactions. A cellphone-based multiplex analysis platform can even automatically analyse all the data obtained, show a final report about the sample and send it to central food quality and safety databases. Such a device would not only be beneficial for the food quality and safety sectors but also for any situation, where an on-site test is desired, such as medical

diagnosis and environmental monitoring.

In general for on-site analysis, miniaturisation and automation of the applied assays is envisaged in order to minimize manual handling steps. Therefore, for cellphone-based microarray or microsphere analyses, the fabrication of a microfluidic device including all required assay reagents is advisable. Then, only the sample needs to be added, which will subsequently be diluted and extracted and which follows the assay procedure in a single-step or by passing through consecutive microchambers [55]. The readout and analysis of the result can then be easily performed by the cellphone equipped with an attachment in which the microfluidic device fits exactly.

Using a microarray format for the detection of abused substances in veterinary control, also offers the opportunity to couple the screening step directly to a confirmatory step. A dual read-out microarray approach combining fluorescence detection and subsequent matrix-assisted laser desorption ionization time-of-flight mass spectrometer (MALDI-TOF) has been published [56]. Using this approach, several substances such as residues of veterinary drugs, antibiotics and contaminants present in food samples can be captured on the surface of a microarray chip covered with specific capture antibodies. Then, a quick fluorescence read-out can be performed as screening and in case of a suspicious result, the very same microarray chip can be subsequently used to truly identify the captured substances by MALDI-TOF as confirmatory analysis. Following this approach only one sample aliquot is needed for the entire analysis and time needed for sample preparation is limited because of the combined direct consecutive analysis.

8 Societal impact of research in this thesis

The developed methods for rbST abuse detection, which are presented in this thesis, are envisaged to be used in surveillance and national residue plan analysis in the EU. Moreover, independent control organizations (*e.g.,* consumer associations) that are concerned about hormone use in food production can easily apply the testing. This is not only important for the EU, but also for the countries where rbST use is legal and so far, dairies, vendors, retailers and consumers can only trust the 'rbST-free' labelling. With the use of rbST detection methods, this labelling can be controlled and therewith, the value of the label and the trust in the label can be increased.

The consequences of the possibility to monitor rbST abuse are manifold. First, the EU regulation concerning the ban of rbST administration [57] can be enforced, which has

not been possible so far. Second, animal welfare will improve when rbST administration is stopped, because rbST administration increased incidences of mastitis, foot and leg disorders. Third, the use of veterinary drugs, such as antibiotics, which are used to restore animal health in case of an infection, will therewith decrease as well. Therefore, the overall use of antibiotics can decrease, which is highly appreciated since antibiotics use is a wide problem in food production. Therewith, also the occurrence of antibiotic-resistant bacteria will decrease. Fourth, consumer trust can be maintained that milk is produced without the use of administered hormones. This would permit fair consumer choices on what they want to buy, eat and drink. And fifth, border inspection controls would be enabled to control whether the imported dairy products have been produced according to EU regulations.

In conclusion, this thesis presents the development of protein biomarker-based screening methods for rbST abuse detection in serum and milk of dairy cows. Using the different developed testing platforms, on-site testing and laboratory-based analyses are possible. The future implementation of these testing platforms for rbST abuse detection is a major leap forward concerning the enforcement of the rbST ban in the EU and concerning the value of protein biomarker-based approaches in veterinary control.

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Summary

Recombinant bovine somatotropin (rbST) is a 22 kDa proteohormone, which can be used to increase milk production in dairy cows. It has been marketed since 1994 and while its use in food production is approved in several countries, such as the US, it is banned in the EU since 2000. To enforce the ban on rbST in the EU and to control for 'rbST-free' –labelling in the US, detection methods are required that identify whether rbST has been used. Existing rbST detection methods focus on the detection of rbST itself in bovine serum. The recombinant form of the hormone has one amino acid exchanged at the N-terminus of the protein. RbST can therefore be potentially discriminated from the endogenous bST by mass spectrometric methods. Other methods employ sandwich enzyme-linked immunosorbent assays (ELISAs) with antibodies having a higher affinity to rbST than bST. These methods, however mainly lack sensitivity, reproducibility or selectivity for rbST and are therefore not widely applied. Hence, no method has been implemented so far to monitor rbST abuse in dairy farming. Screening methods developed for veterinary drug residue control in the EU have to perform according to Commission Decision 2002/657/EC and have to identify at least 95 % of the treated animals.

An alternative approach for rbST abuse detection is the analysis of rbST-dependent biomarkers. A biomarker is defined as an indicator of normal physiological processes, pathogenic processes, or pharmacological responses to a therapeutic intervention. Therefore, the levels of rbST-dependent protein biomarkers are either up- or down-regulated after administration of rbST and rbST-specific biomarker profiles can be used to detect its abuse. RbST exerts similar physiological actions in the cow's body as the endogenous bST. Therefore, proteins involved in the regulatory circuit of bST have been chosen as candidate biomarkers, such as insulin-like growth factor-1 (IGF-1), IGF binding protein 2 (IGFBP2) and osteocalcin. Additionally to that, the administration of rbST induces anti-rbST antibodies in the cow's body, which can be detected as biomarkers. This approach is according to the growth hormone (GH) abuse detection in sports doping control, where solely protein biomarker profiles are used to identify the abuse.

Chapter 2 introduces protein biomarkers and how biomarkers can be used in sports doping and veterinary control to detect the abuse of illegal substances. The advantages of using biomarkers are that the biological effect of a substance usually lasts longer than the substance itself can be detected and therewith, the window of detection is expanded. Moreover, since different substances exert similar effects on physiological machineries for growth or production enhancement, biomarker-based-detection methods have the potential to detect a whole class of substances. Furthermore, low-dose mixtures of different banned substances, which might escape from direct detection of each individual

substance used, could be still detected by the combined effect they exert. In this chapter, protein biomarker-based detection strategies are discussed against generic challenges in biomarker discovery and method development.

Part I of the thesis concerns biomarker analysis in serum and plasma samples from cattle, which are analysed using laboratory-based equipment.A triplex flow cytometric immunoassay (FCIA), which combines the detection of three rbST-dependent biomarkers, *viz.* IGF-1, IGFBP2 and anti-rbST antibodies is demonstrated in **Chapter 3**. Serum samples from treated and untreated dairy cows from a single animal study were analysed using this triplex FCIA. Characteristic treatment-dependent responses for all three individual biomarkers were shown. These results were combined using the statistical model *k*-nearest neighbours (kNN). This model discriminated rbST-treated from untreated cows with a true-positive rate of 89.1 % and a true-negative rate of 97.7 %.
This triplex FCIA was further extended with the biomarker osteocalcin and the resulting fourplex FCIA was used for biomarker profiling in serum samples from rbST-treated and untreated cows from two independent rbST treatment studies. In **Chapter 4**, different data analysis approaches were tested with the aim to detect the highest possible number of true-positive samples. The statistical model kNN was used on all 11 possible biomarker combinations and the combination of the biomarkers osteocalcin and endogenously produced antibodies against rbST proved to be very reliable and correctly predicted 95 % of the samples of treated cows starting from the second rbST injection until the end of the treatment period and even thereafter. With the same biomarker combination, only 12 % of the samples of untreated animals appeared false-positive. This reliability meets the requirements of Commission Decision 2002/657/EC for screening methods in veterinary control.
It can be expected that rbST-dependent biomarkers also show a response upon other treatments. Therefore in **Chapter 5**, the fourplex FCIA for rbST abuse detection was applied to bovines treated with steroids, such as estradiol, dexamethasone and prednisolone. Each treatment resulted in a specific plasma biomarker profile for IGF-1, IGFBP2, osteocalcin and anti-rbST antibodies, which could be distinguished from the profile of untreated animals. Therefore, the fourplex biomarker FCIA is, apart from rbST, also capable of detecting treatment with other growth-promoting agents and clearly shows the potential of biomarker profiling as a screening method in veterinary control.

Part II of the thesis focusses on protein biomarker analysis in milk samples and the change from laboratory-based to on-site analysis.
In **Chapter 6**, the detection of anti-rbST antibodies in raw milk samples was demonstrated,

which discriminated rbST-treated from untreated cows with a 67 % true-positive and 94 % true-negative rate. The laboratory-based assay was also applied to simulated tank milk and pasteurized milk samples. Using milk as a sample matrix for detection has the advantages of non-invasive sampling, and for tank milk analysis at the farm only one milk sample is needed to screen the whole farm for rbST (ab)use.
As a next step in **Chapter 7**, this assay was translated to an on-site pre-screening platform including a cellphone. Using this on-site platform, samples can be tested at the point where they were taken. Only samples that are suspect are transported to an laboratory for further analysis. To this end, a cellphone-based fluorescence imaging platform for the detection of anti-rbST antibodies in milk extracts was developed, which is based on a microsphere fluorescence immunoassay. After performing the assay, the fluorescence is excited by UV LEDs embedded in a dedicated cellphone attachment and the emitted fluorescence light is imaged by the cellphone camera. The fluorescence micro-images were analysed using a custom-developed Android application running on the same cellphone and milk samples from rbST-treated and untreated cows were discriminated.
Also in milk samples, the simultaneous detection of several biomarkers is advantageous as they can increase the confidence of a positive finding. Therefore in **Chapter 8**, a protein microarray-based platform for multiple rbST biomarker detection on a cellphone is presented, which detects anti-rbST antibodies and IGF-1 in milk samples. The 48 microspots on the microarray were labelled with Quantum Dots depending on the biomarker levels in the sample. Quantum Dot fluorescence was detected by the cellphone camera and the same opto-mechanical attachment as in **Chapter 7** and images were analysed by custom software. RbST-treated clearly showed a treatment-dependent biomarker profile in milk that could be discriminated from the profile of untreated cows.

Future research should focus on the simultaneous detection of different targets of interest in milk samples, such as hormones, allergens, antibiotics, contaminants and other substances, all at the same time using the microarray platform on the cellphone. Moreover, sample handling can be facilitated by the use of pre-fabricated microfluidic devices including all required assay reagents.
With the work presented in this thesis, screening for rbST abuse in serum and milk becomes possible: in the laboratory and on-site. The future implementation of these testing platforms for rbST abuse detection is a major leap forward concerning the enforcement of the rbST ban in the EU and concerning the value of protein biomarker-based approaches in veterinary control.

Samenvatting

Recombinant bovien somatotropine (rbST) is een 22 kDa peptide hormoon, dat in koeien melkproductie verhogend werkt. In 1994 is het middel op de markt gebracht en sindsdien is het gebruik in veel landen goedgekeurd. In de EU is het gebruik van rbST echter sinds 2000 verboden. Om het verbod in de EU te handhaven, maar ook om etikettering met 'rbST-vrij' in de VS te kunnen controleren, zijn detectiemethoden die rbST-gebruik aantonen nodig. Bestaande detectiemethoden focussen op het identificeren van rbST in het serum van de koe. Zo is het mogelijk om met massaspectrometrie onderscheidt te maken tussen rbST en endogeen geproduceerd bST doordat deze 1 aminozuur verschillen aan de N-terminus. Andere methoden, zoals sandwich enzyme-linked immunosorbent assays (ELISAs), maken gebruik van antilichamen die een hogere affiniteit voor rbST dan voor bST hebben. Deze methoden worden echter niet breed toegepast doordat ze niet gevoelig genoeg zijn en het ontbreekt aan herhaalbaarheid en specificiteit voor rbST. Screeningsmethoden ontwikkeld voor controle op residuen van diergeneesmiddelen moeten voldoen aan de eisen van richtlijn 2002/657/EG van de Europeese Commissie. Dit houdt in dat behandeling bij minimaal 95 % van de behandelde dieren aangetoond moet kunnen worden. Tot nu toe is er echter nog geen methode beschikbaar die gebruikt kan worden voor controle op rbST-misbruik in de zuivelproductie, en voldoet aan richtlijn 2002/657/EG.

Een alternatieve aanpak om misbruik van rbST aan te tonen is de detectie van rbST-afhankelijke biomarkers. Een biomarker is gedefinieerd als een stof die wordt gebruikt als indicator van een bepaalde biologische toestand. Na rbST toediening zijn de concentraties van rbST-afhankelijke biomarkers verhoogd of verlaagd. Dit levert rbST-specifieke biomarkerprofielen op, die vervolgens gebruikt kunnen worden voor de identificatie van rbST-misbruik. De effecten van rbST en het endogeen geproduceerde bST op het lichaam van een koe zijn gelijk. De eiwitten, die door bST gereguleerd worden zoals insulin-like growth factor-1 (IGF-1), IGF binding protein 2 (IGFBP2) en osteocalcine zijn daarom als kandidaat-biomarker gekozen. Daarnaast vindt na toediening van rbST, antilichaam productie tegen rbST plaats, welke als biomarker gebruikt kunnen worden. Deze aanpak is grotendeels gelijk aan de detectiemethode die in de sport gebruikt wordt voor het aantonen van groeihormoon-misbruik.

In **hoofdstuk 2** wordt de toepassing van eiwit-biomarkers voor sport-doping controles en bij veterinaire inspecties toegelicht. Een voordeel van het gebruik van biomarkers is dat het biologische effect, veroorzaakt door de gebruikte stof, langer aantoonbaar is dan de gebruikte stof zelf. Een ander voordeel is dat verschillende stoffen voor zowel groei-, als productieverhoging hetzelfde fysiologische mechanisme gebruiken. Detectiemethoden

gebaseerd op biomarkers hebben hierdoor het potentieel gehele stofklassen aan te tonen. Daarnaast kunnen mengsels van laag gedoseerde illegale stoffen, die afzonderlijk niet opgespoord kunnen worden, op basis van hun gecombineerde effect op het biomarkerprofiel aangetoond worden. In dit hoofdstuk worden op biomarker gebaseerde detectiemethoden, de bijbehorende algemene uitdagingen van biomarker-identificatie en methode ontwikkeling beschreven.

Deel I van dit proefschrift beschrijft op laboratorium apparatuur gebaseerde biomarker analyses in koeienserum en plasma.
In **hoofdstuk 3** wordt een drievoudige flowcytometrische immunoassay beschreven, die de detectie van drie rbST-afhankelijke biomarkers, namelijk IGF-1, IGFBP2 en anti-rbST antilichamen combineert. Serummonsters van behandelde en onbehandelde melkkoeien zijn met deze drievoudige flowcytometrische immunoassay gemeten. De drie biomarkers hebben allen specifiek op de behandeling gereageerd. De resultaten zijn vervolgens met behulp van het statistische *k*-nearest neighbours (kNN) model gecombineerd. Dit model maakt het mogelijk een onderscheid te maken tussen rbST-behandelde en onbehandelde koeien met een juist-positief percentage van 89,1 % en een juist-negatief percentage van 97,7 %.
Deze drievoudige flowcytometrische immunoassay is uitgebreid met de biomarker osteocalcine, resulterend in een viervoudige flowcytometrische immunoassay. Deze immunoassay is gebruikt voor de analyse van serummonsters van behandelde en onbehandelde koeien. In **hoofdstuk 4** worden, met de verkregen resultaten, verschillende data analyse methoden getest om te bepalen hoe het maximaal aantal juist-positieve resultaten behaald kan worden. Met 4 biomarkers is het mogelijk 11 verschillende combinaties te maken. Het statistische kNN model is vervolgens gebruikt om alle 11 mogelijke combinaties van biomarkers te testen. Bij de biomarker combinatie, osteocalcine en anti-rbST antilichamen worden, vanaf de tweede injectie tot het na het einde van de behandelingsperiode, 95 % van de serummonsters van rbST-behandelde koeien juist geïdentificeerd. Daarnaast worden met deze combinatie van biomarkers maar 12 % van de monsters afkomstig van onbehandelde koeien foutief geïdentificeerd. Deze resultaten zijn in overeenstemming met de eisen voor screeningsmethoden voor veterinaire inspectie, zoals beschreven in richtlijn 2002/657/EG.
Het vermoeden bestaat dat biomarkers geselecteerd om rbST-misbruik aan te tonen ook na behandelingen met andere stoffen een reactie laten zien. Daarom wordt in **hoofdstuk 5** een onderzoek gepresenteerd waarbij de viervoudige flowcytometrische immunoassay voor detectie van rbST-misbruik is gebruikt om serum van koeien behandeld met estradiol, dexamethason en prednisolon te testen. Iedere behandeling leverde

behandelings-specifieke biomarkerprofielen op die afwijkend waren van het profiel van onbehandelde koeien. De viervoudige biomarker immunoassay kan daarom naast rbST ook behandelingen met andere groeibevorderaars aantonen en demonstreert daarmee duidelijk het potentieel van biomarkerprofilering als een screeningsmethode in de veterinaire inspectie.

Deel II van dit proefschrift beschrijft de transitie van lab-gebaseerde methoden voor eiwit-biomarker analyses in melkmonsters naar veldtesten.
In **hoofdstuk 6** wordt de detectie van anti-rbST antilichamen in rauwe melkmonsters besproken, waarmee 67 % van rbST behandelde koeien en 94 % van onbehandelde koeien juist geïdentificeerd worden. Deze lab-gebaseerde methode is gebruikt om anti-rbST antilichamen in een op het lab nagebootste tankmelk en gepasteuriseerde melk te meten. Melk als monstermateriaal heeft als voordeel dat de monsters niet-invasief afgenomen hoeven worden. Daarnaast geeft het analyseren van tankmelk de mogelijkheid om met één enkel monster een heel melkveebedrijf op rbST-misbruik te controleren.
Deze lab-gebaseerde assay is vervolgens omgezet naar een methode die ter plaatse uitgevoerd kan worden; een pre-screening gebaseerd op een mobiele telefoon (**hoofdstuk 7**). Hiervoor is een houder voor op de mobiele telefoon ontwikkeld, waarmee de aanwezigheid van anti-rbST antilichamen in melk door middel van fluorescentie aangetoond kan worden. Hiervoor zijn UV LEDs op de mobiele telefoonhouder geplaatst. Deze zorgen voor excitatie van de fluorescente Quantum Dots gekoppeld aan de anti-rbST antilichamen. Door met de camera van de mobiele telefoon een foto te maken is deze fluorescentie als afbeelding vast te leggen. Met behulp van een speciaal ontwikkelde Android-app kunnen de fluorescentie afbeeldingen aanwezig op de telefoon geanalyseerd worden. Melkmonsters van rbST-behandelde en onbehandelde koeien kunnen op deze manier onderscheiden worden, en kunnen monsters direct na afname gemeten worden. Uitsluitend verdachte monsters worden vervolgens naar een laboratorium getransporteerd voor aanvullende analyses.
Ook voor melkmonsters geldt dat de betrouwbaarheid van een positieve vondst verhoogd wordt wanneer meerdere biomarkers tegelijkertijd gemeten worden. In **+** wordt een eiwit-microarray geïntroduceerd welke compatibel is met de mobiele telefoon. Deze microarray kan meerdere rbST-afhankelijke biomarkers in melkmonsters meten, namelijk anti-rbST antilichamen en IGF-1. Aan de 48 spots op de microarray worden, afhankelijk van de aanwezige concentratie van de biomarker in het monster, Quantum Dots gebonden. De fluorescentie van de Quantum Dots wordt vervolgens op dezelfde manier, en met dezelfde mobiele telefoonhouder als gebruikt in **hoofdstuk 7**, gemeten. De verkregen afbeeldingen worden vervolgens met speciaal ontwikkelde software geanalyseerd. De

biomarkerprofielen in melkmonsters van rbST-behandelde koeien zijn specifiek, en duidelijk te onderscheiden van het profiel in melk van onbehandelde koeien.

In toekomstige studies kan gelijktijdige detectie van verschillende stofklassen, zoals hormonen, allergenen, antibiotica en verontreinigingen, in melk onderzocht worden met de microarray methode op de mobiele telefoon. De monstervoorbewerking kan vereenvoudigd worden door geprefabriceerde microfluidische modules te gebruiken die alle nodige assay-reagentia bevatten. Met de resultaten van dit proefschrift wordt aangetoond dat het mogelijk is serum en melkmonsters in het laboratorium of in het veld op rbST-misbruik te testen. De toekomstige introductie van deze testmethoden voor het aantonen van rbST-misbruik is een grote stap vooruit om het verbod op rbST-gebruik binnen de EU te kunnen handhaven. Daarnaast verduidelijken de verkregen resultaten de waarde van biomarker-gebaseerde methoden voor de veterinaire inspectie.

Zusammenfassung

Rekombinantes bovines Somatotropin (rbST) ist ein 22 kDa großes Proteinhormon, das zur Steigerung der Milchproduktion in Milchkühen eingesetzt werden kann. Seit 1994 wird es kommerziell vermarktet und seine Verwendung ist in vielen nicht-europäischen Ländern zugelassen. In der Europäischen Union (EU) hingegen ist es aus tiergesundheitlichen Gründen seit dem Jahr 2000 verboten. Um das Verbot in der EU durchzusetzen und die „rbST-frei"-Kennzeichnung von Lebensmitteln in den USA zu kontrollieren, sind Nachweismethoden erforderlich, die die rbST-Anwendung aufzeigen. Bisher bestehende Nachweismethoden konzentrieren sich auf die Identifizierung des rbST-Proteins im Blutserum von Kühen. Die rekombinante Form des Hormons hat eine andere N-terminale Aminosäure als das endogen produzierten bST. Deswegen kann rbST potenziell mittels massenspektrometrischen Methoden identifiziert werden. Andere Methoden, wie der sandwich enzyme-linked immunosorbent assay (ELISA), nutzen Antikörper mit einer größeren Affinität zu rbST als bST. Allerdings sind diese Methoden oftmals nicht sensitiv genug, die Ergenisse sind nicht reproduzierbar oder die Antikörper sind nicht selektiv gegen rbST gerichtet. Deshalb sind diese Methoden nicht weit verbreitet. Demzufolge gibt es bisher keine Methode, die offiziell zur Überwachung von möglichem rbST-Missbrauch in der Milchwirtschaft eingeführt ist. Allgemein sollte beachtet werden, dass Screeningmethoden, die in der EU zur Überwachung von Tierarzneimittelrückständen entwickelt werden, die Richtlinie 2002/657/EG der Kommission der Europäischen Gemeinschaften erfüllen müssen und demnach mindestens 95 % der behandelten Tiere als solche identifizieren müssen.

Eine Alternative zum Nachweis von rbST-Missbrauch ist die Analyse von rbST-abhängigen Biomarkern. Per Definition ist ein Biomarker ein Indikator von physiologischen oder krankheitserregenden Prozessen oder pharmakologischen Reaktionen auf einen therapeutischen Eingriff. Demzufolge ist es zu erwarten, dass die Spiegel von rbST-abhängigen Biomarkern nach rbST-Behandlung erhöht oder abgesenkt sind. Dadurch können rbST-spezifische Biomarkerprofile zur Identifizierung von rbST-Missbrauch herangezogen werden. RbST hat ähnliche Effekte auf den Körper des Rindes wie das endogen produzierte bST. Deswegen wurden Proteine, die im hormonellen Regelkreis von bST involviert sind, als Biomarker-Kandidaten ausgewählt. Dazu zählen Insulin-like growth factor-1 (IGF-1), IGF binding protein 2 (IGFBP2) und Osteocalcin. Außerdem induziert die rbST-Behandlung die Antikörperproduktion gegen rbST in der Kuh, welche auch als Biomarker nachgewiesen werden können. Diese Herangehensweise ist analog zur Nachweismethode von Wachstumshormonmissbrauch, die in der Dopingkontrolle im Sport angewendet wird. Hierbei werden auch ausschließlich Biomarkerprofile zum Nachweis von Missbrauch genutzt.

Kapitel 2 befasst sich mit Protein-Biomarkern und deren Potenzial, illegal verwendete Substanzen nachweisen zu können und somit bei Dopingkontrollen und Veterinärkontrollen zum Einsatz zu kommen. Die Vorteile der Verwendung von Biomarkern liegen in der verlängerten Nachweisbarkeit der zugeführten Substanz. Der biologische Effekt der angewendeten Substanz hält meist länger als die Substanz selbst nachgewiesen werden kann. Desweiteren besitzen biomarkerbasierte Nachweismethoden das Potenzial ganze Substanzklassen aufzuspüren, da ähnliche physiologische Mechanismen zur Wachstums- und Produktionssteigerung von unterschiedlichen Stoffen angesprochen werden. Weiterhin können niedrigdosierte Mixturen mehrerer verbotener Substanzen, die einzeln durch ihre niedrige Konzentration nicht nachweisbar sind, auf Basis ihres kombinierten Effekts auf das Biomarkerprofil nachgewiesen werden. In diesem Kapitel werden biomarkerbasierte Nachweisstrategien vor dem Hintergrund allgemeiner Herausforderungen der Biomarkeridentifizierung und Methodenentwicklung diskutiert.

Teil I dieser Doktorarbeit beschäftigt sich mit Biomarkeranalyse in Rinderserum und –plasma, die mit Hilfe von Laborgeräten untersucht wurden.
In **Kapitel 3** wird ein dreifacher durchflusszytometrischer Immunassay dargestellt, der den Nachweis von drei rbST-abhängigen Biomarkern, IGF-1, IGFBP2 und Anti-rbST-Antikörpern, kombiniert. Mit diesem dreifach durchflusszytometrischen Immunassay wurden Serumproben von behandelten und unbehandelten Milchkühen eines Tierexperiments analysiert. Alle drei Biomarker zeigten charakteristische behandlungsabhängige Reaktionen. Die Ergebnisse wurden mittels des statistischen Modells *k*-nearest neighbours kombiniert. Dieses Modell konnte zwischen rbST-behandelten und unbehandelten Kühen mit einer Echt-Positiv-Rate von 89,1 % und einer Echt-Negativ-Rate von 97,7 % unterscheiden.
Dieser dreifache Immunassay wurde mit dem Biomarker Osteocalcin erweitert und der resultierende vierfache Immunassay zur Analyse von Serumproben rbST-behandelter und unbehandelter Kühe aus zwei unabhängigen Tierexperimenten genutzt. In **Kapitel 4** wurden verschiedene Datenanalyseverfahren getestet um die höchstmögliche Anzahl echt-positiver Proben zu erhalten. Das statistische *k*-nearest neighbours-Modell wurde auf alle 11 möglichen Biomarkerkombinationen angewendet. Die statistische Kombination der Biomarker Osteocalcin und Anti-rbST-Antikörper identifizierte zuverlässig 95 % der Proben von rbST-behandelten Kühen im Zeitraum beginnend von der zweiten rbST-Anwendung bis zum Ende des Behandlungszeitraumes sowie darüber hinaus. Mit derselben Biomarkerkombination traten nur 12 % der Proben von unbehandelten Kühen als Falsch-Positive auf. Diese Ergebnisse sind im Einklang mit den von der oben genannten Richtlinie 2002/657/EG geforderten Bedingungen für Screeningmethoden in der Veterinärkontrolle.

Es wird angenommen, dass rbST-abhängige Biomarker auch nach anderen Behandlungen Reaktionen zeigen. Deswegen wird in **Kapitel 5** eine Studie vorgestellt, in der der vierfach durchflusszytometrische Immunassay für den rbST-Missbrauchsnachweis verwendet wurde. Ziel der Analyse war die Untersuchung von Kühen, die mit den Steroiden Östradiol, Dexamethason und Prednisolon behandelt wurden. Das Resultat jeder einzelnen Behandlung war ein behandlungsspezifisches Biomarkerprofil für die vier Biomarker IGF-1, IGFBP2, Osteocalcin und Anti-rbST-Antikörper, die jeweils vom Profil unbehandelter Tiere unterschieden werden konnten. Deswegen kann der vierfache Biomarker-Immunassay, neben rbST, auch Behandlungen mit anderen wachstumsfördernden Substanzen nachweisen und zeigt damit deutlich das Potenzial von Biomarkerprofiling als eine Screeningmethode in der Veterinärkontrolle.

Teil II dieser Doktorarbeit behandelt die Protein-Biomarkeranalyse in Milchproben und den Übergang von Labor- zu Vor-Ort-Analysen.
In **Kapitel 6** wird der Nachweis von Anti-rbST-Antikörpern in Rohmilchproben dargelegt, mit Hilfe derer 67 % der Proben von rbST-behandelten Kühen und 94 % der Proben von unbehandelten Kühen identifiziert werden konnten. Diese laborbasierte Methode wurde auch bei simulierten Kühltankmilchproben und pasteurisierten Milchproben angewendet. Der Vorteil ist hier, dass Milchproben im Gegensatz zu Serumproben nichtinvasiv genommen werden und bei der Untersuchung von Kühltankmilchproben ist eine einzige Probe ausreichend um einen gesamten Milchviehbetrieb auf möglichen rbST-Missbrauch zu kontrollieren.
In einem nächsten Schritt wurde dieser Assay in eine Handy-basierte Vor-Ort-Vorscreeningmethode umgewandelt, die in **Kapitel 7** näher beschrieben ist. Mittels dieser Vor-Ort-Methode können die Proben direkt dort untersucht werden, wo sie genommen wurden. Ausschließlich die auffälligen Proben würden anschließend zu einem Labor für weitere Analysen transportiert werden. Zu diesem Zweck wurde ein Handy-basiertes Fluoreszenz-bildgebendes System zum Nachweis von Anti-rbST-Antikörpern in Milchextrakten entwickelt, dem ein Fluoreszenzimmunassay auf der Oberfläche von Mikrokugeln zugrunde liegt. Nach der Assaydurchführung wurde die Fluoreszenz durch ultraviolette Leuchtdioden, die in einer zugehörigen Handyhalterung eingebettet waren, angeregt. Das abgegebene Fluoreszenzlicht wurde mittels der Handykamera abgebildet. Die Auswertung der Fluoreszenzbilder erfolgte mit einer speziell entwickelten Android-App, die auf demselben Handy ausgeführt wurde.Damit konnten Milchproben von rbST-behandelten und unbehandelten Kühen voneinander unterschieden werden.
Auch bei der Untersuchung von Milchproben hat es Vorteile mehrere Biomarker gleichzeitig zu analysieren, da dies die Zuverlässigkeit eines positiven Ergebnisses erhöht.

In **Kapitel 8** wird ein Proteinmikroarray vorgestellt, mit dem mehrere rbST-abhängige Biomarker, Anti-rbST-Antikörper und IGF-1, in Milchproben mittels eines Handys nachgewiesen werden können. Die 48 Protein-Mikropunkte auf dem Mikroarray wurden in Abhängigkeit von den Biomarkerspiegeln in der Probe mit fluoreszenten Quantum Dots markiert. Die Fluoreszenz der Quantum Dots konnte mittels der Handykamera und derselben opto-mechanischen Handyhalterung wie in **Kapitel 7** detektiert werden. Die Bildasuwertung erfolgte mit speziell entwickelter Software. Behandlungs-spezifische Biomarkerprofile wurden in Milchproben von rbST-behandelten Kühen nachgewiesen, das deutlich von dem unbehandelter Kühe zu unterscheiden war.

Zukünftige Forschungstätigkeiten können sich auf den gleichzeitigen Nachweis verschiedener Stoffgruppen in Milch (zum Beispiel Hormone, Allergene, Antibiotika und Schadstoffe) ausrichten und dabei die Proteinmikroarraymethode auf dem Handy anwenden. Desweiteren kann die Probenbearbeitung durch den Gebrauch von vorgefertigten mikrofluidischen Elementen vereinfacht werden. Letzere beinhalten bereits alle nötigen Assayreagenzien. Die in dieser Doktorarbeit dargelegten Ergebnisse ermöglichen das Screening von Serum- und Milchproben auf möglichen rbST-Missbrauch sowohl im Labor und vor Ort. Die Einführung dieser Testmethoden wäre ein großer Meilenstein für die Durchsetzung des rbST-Verbots in der EU . Des Weiteren unterstreichen die Ergebnisse die Relevanz von biomarkerbasierten Methoden in der Veterinärkontrolle.

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Susann

About the author

Curriculum vitae

Susann Ludwig was born on the 17th of June 1985 in Jena (Germany). In 2003, she graduated from the grammar school "Carl Zeiss" in Jena, which offers an intensified education in mathematics, science and technical subjects. Thereafter, she started her biology studies at the Free University in Berlin (Germany) and changed to the Dresden University of Technology (Germany) in 2005. During her studies she did several internships. These included the EAWAG in Zürich (Switzerland), where she performed field research at the Tagliamento, the German Sports University in Cologne and the Hong Kong University of Science and Technology. At the German Sports University, she was involved in a project in which several androgenic compounds were tested for their anabolic actions. At the Hong Kong University of Science and Technology, she learnt several *in vitro* assays to assess amyloid β aggregation and toxicity that are important parameters in Alzheimer's disease research. With this expertise, she returned to Dresden and worked on her thesis about neuroprotective effects of certain phytoestrogens and she graduated from university in 2009. In the beginning of 2010, she started her PhD project at RIKILT in Wageningen (Netherlands). One part of her PhD research she conducted in Prof. Ozcan's lab at the University of California in Los Angeles (USA). Then, she started a cooperation project with the Cambridge Centre for Proteomics with the support of a PRIME-XS grant. For the entire period of her PhD she was board member of the annual Summer School on Endocrinology in Bregenz. The results of her PhD project are described in this thesis.

List of publications

Ludwig, S.K.J. , Smits, N.G.E. , Bremer, M.G.E.G. , Nielen, M.W.F. (2012) Monitoring milk for antibodies against recombinant bovine somatotropin using a microsphere immunoassay-based biomarker approach, *Food Control*, 26(1), 68-72.

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Ludwig, S.K.J., Tokarski, C., van Ginkel, L. A., Zhu, H., Ozcan, A., Nielen, M.W.F. (2014) Calling biomarkers using a protein microarray on your cellphone, submitted manuscript.

Overview of completed training activities

Discipline specific activities

Summer School on Endocrinology, German Society of Endocrinology (2010-2013)
Chemometrics, ANAC (2010)
YAR meetings, German Society of Endocrinology (2010, 2011)
BioPhotonics 2011, Università di Parma (2011)
10th workshop on biosensors and bioanalytical microtechniques in environmental and clinical analysis, University of Applied Sciences Jena (2011)
NWO symposia Analytical Chemistry, Lunteren (2010-2013)
Symposium "Dierbehandelingsmiddelen: samenwerking, trends en innovatie"
Adviescommissie van het WOT-Voedselveiligheid (2011)
EuroResidue 2012, Egmond aan Zee (2012)
RAFA 2013, Prague (2013)
Advanced proteomics, VLAG (2013)
Summer School of Advanced Proteomics, Max Planck Institute for Biophysical Chemistry (2013)

General courses

VLAG PhD week (2010)
Statistics for the Life Sciences, WIAS (2010)
Techniques for writing and presenting a scientific paper, WGS (2010)
Competence Assessment, WGS (2010)
Adobe InDesign, WUR Library (2010)
Scientific Publishing, WGS (2011)
Project and Time Management, WGS (2011)
Minisymposium: How to write a world class paper?, WUR Library (2011)
Oictures, Tables and infographics in your research, VLAG & AFSG (2011)
Teaching and Supervising Thesis Students, DO at WUR (2011)
Philosophy and Ethics of Food Science and Technology, VLAG (2011)
Safe handling with radioactive materials and sources, Van Hall Larenstein (2012)
Career Orientation, WGS (2013)
Voice Matters - Voice and Presentation Skills Training (V&PT), WGS (2013)
Writing Grant Proposals, WGS (2013)

Optionals

Cluster Meetings, RIKILT (2010-2014)
Colloquia Organic chemistry, ORC (2010-2014)
Preparing PhD research proposal (2010)
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