Inaugural-Dissertation zur Erlangung der Doktorwürde

der Tierärztlichen Fakultät

der Ludwig-Maximilians-Universität München

Investigations on selected aspects involved in the aetiology of bovine neonatal pancytopenia (BNP)

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München 2015

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der Ludwig-Maximilians-Universität München

Lehrstuhl für Molekulare Tierzucht und Biotechnologie

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Gedruckt mit der Genehmigung der Tierärztlichen Fakultät

der Ludwig-Maximilians-Universität München

Dekan:	Univ.-Prof. Dr. Joachim Braun
Berichterstatter:	Univ.-Prof. Dr. Eckhard Wolf
Korreferenten:	Univ.-Prof. Dr. Holm Zerbe Univ.-Prof. Dr. Walter Hermanns Priv.-Doz. Dr. Valeri Zakhartchenko Univ.-Prof. Dr. Dušan Palić

Tag der Promotion: 31. Januar 2015

Publications included in this thesis:

Demasius, W., Weikard, R., Kromik, A., Wolf, C., Müller, K., and Kühn, C. (2014) Bovine neonatal pancytopenia (BNP): novel insights into the incidence, vaccination-associated epidemiological factors and a potential genetic predisposition for clinical and subclinical cases. *Research in Veterinary Science* **96**(3): 537-542.

Demasius, W., Weikard, R., Hadlich, F., Müller, K., and Kühn, C. (2013) Monitoring the immune response to vaccination with an inactivated vaccine associated to bovine neonatal pancytopenia by deep sequencing transcriptome analysis in cattle. *Veterinary Research* **44**(1): 93.

Publications, talks and poster presentations not included in this thesis:

Weikard, R., Demasius, W., Hadlich, F., and Kühn, C. Different blood cell-derived transcriptome signatures in cows exposed to vaccination pre- or postpartum. *Manuscript submitted*

Demasius, W., Weikard, R., Hadlich, F., Rümenapf, T., Buitkamp, J., Kühn, C. Identification of previously unpublished classical and non-classical MHC class I sequences in cattle with a new method for immunotyping MHC class I applying RNA-Seq. First Next-Generation-Sequencing (NGS) Workshop in Mecklenburg-Vorpommern. Leibniz-Institut für Nutztierbiologie (FBN), Dummerstorf. April 4th, 2014.

Kühn, C., Demasius, W., Hadlich, F., and Weikard, R. (2014) Monitoring Novel Functional Elements in the Bovine Genome Applying RNAseq. International Plant and Animal Genome XXII. San Diego, CA. Poster Presentation. January 11 – 15, 2014.

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To my parents

Table of Contents

1 Abbreviations

AMIR	Antibody-mediated immune response
APC	Antigen-presenting cell
BoLA	Bovine leukocyte antigen
BNP	Bovine neonatal pancytopenia
BNP-dam	Dam that has given birth to a BNP-calf
BTV	Bluetongue virus
BVDV	Bovine Viral Diarrhoea Virus
CD	Cluster of differentiation
CMIR	Cell-mediated immune response
DCVC	S-(1,2-Dichlorovinyl)-L-cysteine
DNA	Deoxyribonucleic acid
dsRNA	Double-stranded RNA
EHDV	Epizootic haemorrhagic disease virus
FAIT	Foetal alloimmune thrombocytopenia
hCG	Human chorionic gonadotropin
IFN	Interferon
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL	Interleukin
IPA	Ingenuity Pathway Analysis
ISCOM	Immune stimulating complex
i.v.	Intravenous
kDa	Kilodalton
KEGG	Kyoto Encyclopedia of Genes and Genomes
MDBK cells	Madin-Darby bovine kidney cells
MHC	Major histocompatibility complex

mRNA	Messenger RNA
NAIT	Neonatal alloimmune thrombocytopenia
ncp	Noncytopathic
NGS	Next-generation sequencing
NI	Neonatal Isoerythrolysis
Non-BNP-dam	Dam that has not given birth to a BNP-calf
PBMC	Peripheral blood mononuclear cell
p.c.i.	Post colostrum intake
PCV-2	Porcine circovirus type 2
PDNS	Porcine dermatitis and nephropathy syndrome
PMWS	Postweaning multisystemic wasting syndrome
Poly(I:C)	Polyriboinosinic acid-polyribocytidylic acid
RBC	Red blood cells
RIG-1	Retinoic-acid-inducible gene 1
RNA	Ribonucleic acid
RNA-Seq	RNA sequencing
RT-PCR	Reverse transcriptase polymerase chain reaction
SNP	Single nucleotide polymorphism
Spp.	Subspecies
ssRNA	Single-stranded RNA
Th cell	T-helper cell
TLR	Toll-like receptor

2 Introduction

Bovine neonatal pancytopenia (BNP) is an alloimmune disease, which has recently emerged in newborn calves. This disease has been registered in several European countries as well as in New Zealand during the last few years (Bell *et al.*, 2010b; Corbière *et al.*, 2009; Ellis-Iversen and Colloff, 2009; Friedrich *et al.*, 2009c; Gentile *et al.*, 2009; Ministry of Agriculture and Forestry - MAF - New Zealand, 2011; Pardon *et al.*, 2010; Sánchez-Miguel *et al.*, 2010; Smolenaars and Mars, 2009). In all of these countries a specific inactivated vaccine (PregSure® BVD) against the Bovine Viral Diarrhoea Virus (BVDV) had been applied, which is strongly associated with BNP (Lambton *et al.*, 2012; Sauter-Louis *et al.*, 2012). This vaccine comprises a unique adjuvant and was shown to induce very high antibody titres against BVDV in vaccinated animals (Bastian *et al.*, 2011; Raue *et al.*, 2011). However, during aetiological investigations on BNP it was revealed that vaccination with PregSure® BVD additionally induces alloreactive antibodies in some vaccinated animals, which are directed against calf leucocytes (Bridger *et al.*, 2011; Pardon *et al.*, 2011). These alloantibodies are transferred to susceptible calves by ingestion of colostrum from these specific dams (Friedrich *et al.*, 2011; Schröter *et al.*, 2011). As a result of this BNP-colostrum intake, affected calves suffer from external and internal haemorrhages, marked thrombocytopenia and leucopenia and a depletion of the bone marrow (panmyelophthisis) (Friedrich *et al.*, 2009c; Pardon *et al.*, 2010). Although the number of observed clinical BNP-cases is low compared to the number of PregSure® BVD vaccinated dams or vaccination doses sold (Kasonta *et al.*, 2012), case fatality is very high (Pardon *et al.*, 2010). Besides these clinical cases of BNP, subclinical BNP-cases have been described. These cases show a transient thrombocytopenia and leucopenia, but no clinical signs characteristic of BNP (Pardon *et al.*, 2010; Witt *et al.*, 2011). The incidence of subclinical cases has not been investigated thoroughly up to now, and therefore it is possible that clinical cases of BNP only represent the tip of the iceberg.

Only a subset of PregSure® BVD-vaccinated dams are known to produce colostrum which can induce BNP in related, but also in unrelated calves after ingestion of the respective colostrum (Friedrich *et al.*, 2011; Schröter *et al.*, 2011). A possible genetic predisposition of the dam involved in clinical BNP has been revealed in a study by Krappmann et al. (Krappmann *et al.*, 2011). This study investigated a well-characterised cattle resource population with occurrence of clinical BNP-cases and pointed out that all dams, which had clinical BNP-cases, were confined exclusively to one specific sire line of this population.

Investigations on the target antigen of BNP-associated alloantibodies showed that alloantibodies bind to the surface of the Madin-Darby Bovine Kidney (MDBK) cell line, which had been used for

the production of PregSure® BVD (Bastian *et al.*, 2011). The nature of the targeted antigen was revealed as MHC class I (Deutskens *et al.*, 2011; Foucras *et al.*, 2011). Hence, it was proposed that the vaccine was contaminated with MHC class I antigens from the MDBK cell line, which elicit the production of alloantibodies in BNP-dams. However, not only BNP-dams possess alloantibodies directed against MHC class I, but also some non-BNP-dams (Deutskens *et al.*, 2011) and also a large proportion of animals experimentally vaccinated (Kasonta *et al.*, 2012).

So far, several questions regarding the aetiopathogenesis of BNP still remain unresolved. The study presented here addresses some of these questions. Firstly, a well-characterised cattle resource population was monitored for the incidence and for a possible genetic predisposition also involved in subclinical BNP-cases. Secondly, the immune response after vaccination with PregSure® BVD was characterised on trancriptome level with an RNA-Seq approach in order to gain an overview of the immune response to this viral inactivated vaccine and novel insights into potential characteristic immune reactions evoked by this vaccine.

3 Literature Review

3.1 Bovine Neonatal Pancytopenia (BNP)

3.1.1 Definition

Since 2007, an increase in the number of neonatal calves showing clinical signs of a haemorrhagic diathesis has been documented in many European countries (Bell *et al.*, 2010b; Ellis-Iversen and Colloff, 2009; Friedrich *et al.*, 2009c; Gentile *et al.*, 2009; Pardon *et al.*, 2010; Sánchez-Miguel *et al.*, 2010; Smolenaars and Mars, 2009). This syndrome, initially denoted as "bleeding calf syndrome", "blood sweating" or "haemorrhagic diathesis", was officially termed "Bovine Neonatal Pancytopenia" (BNP) at the Satellite Symposium of the European Buiatric Congress in Marseille, France, in 2009. Several criteria were defined in order to assign calves with a bleeding disorder as a BNP-case (Friedrich *et al.*, 2009a):

1) Bleeding disorder (typical haematological alterations may exist some days before clinical signs or even without clinical signs)
2) Negative test results for Bovine Viral Diarrhoea Virus (Panpestivirus PCR)
3) Thrombocytopenia (< 200 G/L) and leucopenia (< 4.0 G/L)
4) Calves younger than four weeks
5) Calves show no signs of septicaemia
6) *Post mortem:* Panmyelophthisis (Friedrich *et al.*, 2009c)

In addition to clinical and haematological findings indicative of BNP, the proof of a panmyelophthisis in calves up to the age of four weeks is considered to be the gold standard for defining a BNP-case. In case of a missing pathological examination, the combination of clinical and haematological (thrombocytopenia and leucopenia) findings associated with BNP is required for case confirmation (Reichmann, 2012; Sauter-Louis *et al.*, 2012).

3.1.2 Epidemiology

Prior to 2007, sporadic cases of idiopathic haemorrhagic diathesis in neonatal calves had been perceived (Friedrich *et al.*, 2009b). However, since 2007 an accumulation of calves with a bleeding disorder has been observed in Bavaria and thereafter also in other parts of Germany (Friedrich *et al.*, 2009b; Friedrich *et al.*, 2009c). Similar reports from other European countries like Belgium (Pardon *et al.*, 2010), France (Corbière *et al.*, 2009), the Netherlands (Smolenaars and Mars, 2009), Great Britain and Scotland (Bell *et al.*, 2010b; Ellis-Iversen and Colloff, 2009), Italy (Gentile

et al., 2009) and Ireland (Sánchez-Miguel *et al.*, 2010) were recorded. In neighbouring countries like Switzerland, Austria and Denmark, which are free of BVD or where no BVD-vaccinations had been undertaken, no such cases were observed (Friedrich *et al.*, 2011). In August 2011, a first case of BNP was registered in New Zealand (Ministry of Agriculture and Forestry - MAF - New Zealand, 2011).

Both genders are equally affected by BNP (Pardon *et al.*, 2010), and the disease was also observed in a variety of different breeds such as Simmental, Holstein Friesian, Belgian Blue, Aberdeen Angus, Charolais, Blonde d'Aquitaine, Montbéliarde and Limousine (Bell *et al.*, 2009; Corbière *et al.*, 2009; Friedrich *et al.*, 2009c; Pardon *et al.*, 2010). Pardon et al. (Pardon *et al.*, 2010) reported that 40% of BNP-calves were born to heifers, 30% to dams from 2nd parity, 25% to dams from 3rd parity and 5% to dams from 4th parity. Some dams gave birth to more than one BNP-calf, but not necessarily in subsequent years (Friedrich *et al.*, 2011; Pardon *et al.*, 2010).

First observations pointed out that BNP may be linked to vaccinations against the Bovine Viral Diarrhoea Virus (BVDV) (Friedrich *et al.*, 2009c). Two independently conducted epidemiological studies then revealed a strong association between the occurrence of BNP and the use of a specific inactivated vaccine against BVD (PregSure® BVD, Pfizer Animal Health) (Lambton *et al.*, 2012; Sauter-Louis *et al.*, 2012). PregSure® BVD had been launched in 2004 in Germany (Kasonta *et al.*, 2012) and was retracted from the European market in 2010 (Paul-Ehrlich-Institut - PEI, 2010). Following a first case report, the vaccine was also recalled in New Zealand in 2011 (Ministry of Agriculture and Forestry - MAF - New Zealand, 2011). Until 28th February 2011, more than 3.000 case reports had been registered in Germany (Paul-Ehrlich-Institut - PEI, 2011). By the end of August 2012, 6913 suspected BNP-cases had been reported across Europe, with a decreasing tendency of reported cases in 2011 and 2012 compared to previous years (Jones *et al.*, 2013). BNP is a non-notifiable disease. Therefore, the precise number of cases in Germany is likely to have been much higher since not all cases have been reported, as a German study revealed (Reichmann, 2012). Considering the large amount of vaccination doses sold (Doll *et al.*, 2013), the overall reported incidence of clinical cases of BNP is low (Kasonta *et al.*, 2012; Pardon *et al.*, 2010). On individual farm level, the incidence rate was generally not more than 10% (Foucras *et al.*, 2011; Witt *et al.*, 2011), but there were farms with an incidence rate up to 20% (Reichmann, 2012). There are reports of an increase of clinical BNP-cases during summer and autumn, potentially due to insect bites (Friedrich *et al.*, 2009c; Pardon *et al.*, 2010). Furthermore, field observations revealed a high proportion of BNP-affected herds, in which a subclinical case of BNP could be detected (Pardon *et al.*, 2010). A study on a farm with a high incidence rate of BNP demonstrated a remarkable proportion of neonatal calves showing alterations in haematological

parameters without clinical signs of BNP (Witt *et al.*, 2011). Although these studies point to the occurrence of subclinical cases, extensive investigations on the true incidence of subclinical BNP-cases are missing.

Alloreactive antibodies contained in colostrum of certain dams were shown to be the causal agents for inducing BNP in calves (Bridger *et al.*, 2011; Friedrich *et al.*, 2011; Pardon *et al.*, 2011; Schröter *et al.*, 2011). Kasonta et al. (Kasonta *et al.*, 2012) reported that in most cases, repetitive vaccinations with PregSure® BVD, such as a basic immunisation consisting of two doses and a booster vaccination, were required for the induction of alloreactive antibodies. Varying incidences of BNP were observed amongst European countries and also within other European countries (Lambton *et al.*, 2012). For different regions of Germany, different vaccination regimens were found to be a potential causative factor for varying incidences of BNP (Kasonta *et al.*, 2012). In the German federal state of Bavaria, where a much higher incidence rate of BNP was observed in comparison to the federal state of Lower Saxony, PregSure® BVD had been applied according to the manufacturer's instructions. Therefore, dams in Bavaria had received at least two doses of PregSure® BVD. In Lower Saxony, however, a two-step vaccination scheme, which consists of a primary vaccination with an inactivated BVD-vaccine like PregSure® BVD and a booster immunisation with a live attenuated BVD vaccine, had been applied. Thus, in Lower Saxony most dams had received a maximum of one dose of PregSure® BVD (Kasonta *et al.*, 2012). In some cases, one dose of PregSure® BVD was still sufficient for the development of BNP-associated alloantibodies, as demonstrated by the very low incidence rate of BNP-cases in Lower Saxony.

3.1.3 Clinical Signs

The background for the most prominent clinical signs found in BNP-calves is a haemorrhagic diathesis due to thrombocytopenia. Increased bleeding tendencies can be due to defects in primary haemostasis like thrombocytopenia or qualitative disorders like thrombopathy. Underlying mechanisms for disorders in secondary haemostasis involve defects during fibrin formation from coagulation factors, for example caused by vitamin K antagonists or inherited coagulopathies (Grubbs and Olchowy, 1997). A thrombocytopenia can result from a decreased platelet production, increased platelet destruction, consumption of thrombocytes, or sequestration.

Initially, BNP-calves are born healthy and with a normal birth weight (Bell *et al.*, 2010b; Kappe *et al.*, 2010). First abnormalities in affected calves are not necessarily signs of a bleeding disorder, and calves can present typical neonatal diseases such as diarrhoea, umbilical infections and/or

lung infections. Some calves displayed fever of unknown origin and a dull demeanour (Friedrich *et al.*, 2009c). First indications of a bleeding disorder are sometimes prolonged bleeding after ear-tagging, injections or dehorning (Bell *et al.*, 2010b; Friedrich *et al.*, 2009c; Klemt, 2010; Pardon *et al.*, 2010). The typical clinical signs of BNP are sometimes already observed at the age of one week (Bell *et al.*, 2010b; Buck *et al.*, 2011), but mostly show up during the 2nd and 3rd week after birth (Friedrich *et al.*, 2009c; Kappe *et al.*, 2010; Klemt, 2010; Pardon *et al.*, 2010), and up to the age of approximately four weeks *post partum* (Kappe *et al.*, 2010; Pardon *et al.*, 2010; Smolenaars and Mars, 2009). On average, the bleeding disorder becomes noticeable at the age of 12.7 days (Friedrich *et al.*, 2009c) or 14 days (Smolenaars and Mars, 2009). According to Pardon et al. (Pardon *et al.*, 2010), calves on average die at the age of 13 days *post partum*. In experimental studies with challenge calves, first clinical signs of a bleeding disorder were already noticed at the age of 3 – 4 days (small amounts of blood in faeces) (Friedrich *et al.*, 2011). After the onset of obvious clinical symptoms, most affected calves die peracutely or acutely within a few days (Bell *et al.*, 2010b; Friedrich *et al.*, 2009c; Kappe *et al.*, 2010; Klemt, 2010). Irrespective of any medical treatment, lethality in affected calves is high, with reports of a lethality rate ranging between 60% (Buck *et al.*, 2011) up to 90% (Pardon *et al.*, 2010).

One of the most frequent clinical findings in BNP-affected calves is differing amounts of blood in the faeces, which ranges from single bloody smears to single coagula up to a bloody diarrhoea (Friedrich *et al.*, 2009c). In addition, petechiae on mucous membranes are a common observation and can be observed mainly on the oral mucous membranes, especially sublingually, but also on buccal, nasal and vaginal mucosae and on the edges of the eyelids (Friedrich *et al.*, 2009c; Pardon *et al.*, 2010). In the initial phase of the disease, mucous membranes are pink (Pardon *et al.*, 2010), and in the progress of the disease turn anaemic (Klemt, 2010; Pardon *et al.*, 2010). Less common clinical findings are epistaxis, subscleral bleedings, haematomas and blue discolouration at the chin, the opening of the mouth and muzzle (Friedrich *et al.*, 2009c; Pardon *et al.*, 2010). Only 60% of clinically affected calves displayed bleedings of the skin, which resulted in the initial colloquial term “blood sweating” used for the disease. However, this term is misleading, because the bleedings do not originate from large areas, but rather from very small, punctual localisations (Friedrich *et al.*, 2009c). According to Pardon et al. (Pardon *et al.*, 2010), areas around the eyes, ears, the back and distal part of the limbs are mainly affected by transcutaneous bleedings, which might be due to insect bites (Friedrich *et al.*, 2009c; Pardon *et al.*, 2010). These fly bites might also have been the reason for a higher number of reported clinical BNP-cases during the summer and autumn (Friedrich *et al.*, 2009c; Pardon *et al.*, 2010). In first case reports, pyrexia, resistant to medical treatment, was revealed as a common finding in BNP-calves (Bell *et al.*, 2010b; Friedrich *et al.*, 2009c; Klemt, 2010). However, not all animals display fever, and especially in the final stage

of the disease calves may show hypothermia (Pardon *et al.*, 2010). In experimental studies with challenge calves, only a small number of calves showed fever (Friedrich *et al.*, 2011), and challenge calves did not display this symptom more frequently compared to control calves (Bell *et al.*, 2013). The general condition of calves deteriorates during the course of the disease up to a severely depressed state and permanent recumbency (Friedrich *et al.*, 2009c; Pardon *et al.*, 2010). Along with these characteristic findings of BNP, several BNP-calves were affected by other infections, such as diarrhoea, umbilical infections, pneumonia or septicaemia, which did not respond to medical treatment (Friedrich *et al.*, 2009c). Calves that survived clinical BNP showed stunted growth (Witt *et al.*, 2011).

3.1.4 Haematological Findings

Reference ranges for blood parameters in neonatal calves differ from those applied to adults (Knowles *et al.*, 2000). There are few studies only that deal with haematological profiles in neonatal calves (Egli and Blum, 1998; Knowles *et al.*, 2000; Mohri *et al.*, 2007; Tennant *et al.*, 1974). Studies in the context of BNP applied reference ranges of 200 – 800 G/l for thrombocytes (Buck *et al.*, 2011; Friedrich *et al.*, 2011) or 300 G/l (Kappe *et al.*, 2010; Klemt, 2010; Witt *et al.*, 2011) or only 100 G/l (Pardon *et al.*, 2010) for the threshold. For the leucocytes, reference ranges from 4.0 – 10.0 G/l (Friedrich *et al.*, 2011; Pardon *et al.*, 2010) or up to 12.0 G/l for the upper margin (Kappe *et al.*, 2010; Klemt, 2010; Witt *et al.*, 2011) were previously used.

The most distinctive haematological aberration found in clinically affected BNP-calves is a severe thrombocytopenia (Bell *et al.*, 2010b; Buck *et al.*, 2011; Friedrich *et al.*, 2009c; Kappe *et al.*, 2010; Pardon *et al.*, 2010) along with a normal morphology of the platelets (Buck *et al.*, 2011; Friedrich *et al.*, 2009c). Shortly before death, calves can have thrombocyte counts < 10.0 G/l (Buck *et al.*, 2011; Krappmann *et al.*, 2011; Pardon *et al.*, 2010), which is incompatible with life. Depending on the outcome of the disease in clinical BNP-cases, thrombocyte counts increased continually back to reference ranges in calves that survived BNP, whereas lethal BNP-cases showed no recovery from thrombocytopenia (Buck *et al.*, 2011; Friedrich *et al.*, 2009c; Pardon *et al.*, 2010). In challenge studies, which applied a frequent haematological monitoring, platelet numbers already decreased during the first three hours post colostrum intake in challenge calves (Friedrich *et al.*, 2011). This phenomenon of decreasing thrombocyte counts after birth could also be found in control or healthy calves (Egli and Blum, 1998; Knowles *et al.*, 2000; Schröter *et al.*, 2011), however already being significantly less distinctive 8 hours after colostrum intake than in challenge calves (Bell *et al.*, 2013). Thereafter, in healthy or control calves and BNP-affected calves, platelet counts again increased up to day 3 (Bell *et al.*, 2013; Schröter *et al.*, 2011).

However, after this increase, BNP-calves showed a second decline in thrombocytes, with values below the applied reference ranges (Bell *et al.*, 2013; Friedrich *et al.*, 2011; Schröter *et al.*, 2011).

Some animals with clinical BNP display a severe leucopenia besides a marked thrombocytopenia, especially shortly before death (Bridger *et al.*, 2011; Buck *et al.*, 2011; Friedrich *et al.*, 2009c; Kappe *et al.*, 2010; Krappmann *et al.*, 2011; Pardon *et al.*, 2010). However, the leucopenia does not always occur simultaneously with the thrombocytopenia (Schröter *et al.*, 2011) and can show varying courses over time, with leucocyte counts being temporarily back above the threshold of 4.0 G/l (Bridger *et al.*, 2011; Friedrich *et al.*, 2011; Schröter *et al.*, 2011). Immunosuppression as a result of sustained leucopenia is likely to be the reason for attendant bacterial infections and lacking success of medical treatment (Friedrich *et al.*, 2011). Leucocyte differential counts revealed a monocytopenia, lymphopenia (relative lymphocytosis) and neutropenia (Bell *et al.*, 2010b; Kappe *et al.*, 2010; Pardon *et al.*, 2010). After an initial significant decrease of neutrophils after colostrum ingestion in challenge calves, there was no significant difference in neutrophil counts after 8 hours post colostrum intake and thereafter in challenge calves compared to control animals until both groups of calves were euthanised for experimental reasons on day 10 after birth (Bell *et al.*, 2013). At the time points 8 hours and 12 hours after colostral intake, challenge calves showed a significantly higher proportion of band neutrophils than control calves, which suggests myeloid stress (Bell *et al.*, 2013).

Anaemia was found in several calves (Bell *et al.*, 2010b; Kappe *et al.*, 2010; Krappmann *et al.*, 2011; Pardon *et al.*, 2010), especially in the final stage of the disease (Schröter *et al.*, 2011). The anaemia was normocytic and normochromic (Bell *et al.*, 2010b). Initially it was of haemorrhagic nature, but then turned into an aplastic anaemia, since hardly any reticulocytes could be found in affected calves (Bell *et al.*, 2010b; Friedrich *et al.*, 2009c). In accordance with these findings, the packed cell volume was within applied reference ranges or below the threshold (Buck *et al.*, 2011; Friedrich *et al.*, 2011; Kappe *et al.*, 2010; Pardon *et al.*, 2010).

All observed changes in peripheral blood cell counts correspond to the life span of these cells in the blood combined with a destruction of the bone marrow stem cells: the shortest life span can be found in granulocytes (less than one week), followed by thrombocytes (5 – 10 days) and erythrocytes (110 – 150 days) (Baker *et al.*, 1998; Bell *et al.*, 2013; Friedrich *et al.*, 2009c; Mizuno *et al.*, 1959). As indicated in Bell et al. (Bell *et al.*, 2013), lymphocytes in the periphery have a variable lifespan.

There are several reports either in the field or in challenge studies on calves that showed cell counts below the applied reference ranges in at least two haematopoietic cell lineages, but no

clinical symptoms consistent with BNP (Friedrich *et al.*, 2011; Pardon *et al.*, 2010; Schröter *et al.*, 2011; Witt *et al.*, 2011). These calves were classified as subclinical BNP-calves.

Except for a hypoproteinaemia, which might be due to the haemorrhagic diathesis, blood biochemical parameters did not show any abnormalities in clinically affected BNP-calves (Pardon *et al.*, 2010). Bell et al. (Bell *et al.*, 2010b) found no abnormalities in the prothrombin and activated partial thromboplastin times, while Pardon et al. (Pardon *et al.*, 2010) reported of a slightly prolonged prothrombin time and in some calves a slightly prolonged activated partial thromboplastin time. In contrast, Krappmann et al. (Krappmann *et al.*, 2011) revealed an extremely prolonged prothrombin time in a number of BNP-cases. Fibrinogen was either elevated or normal (Pardon *et al.*, 2010).

3.1.5 Pathology and Histology

Gross pathological findings in BNP-calves revealed generalised haemorrhages in varying degrees, ranging from petechiae to ecchymoses or suggilations, which could be found in various organs, tissues and localisations (Buck *et al.*, 2011; Friedrich *et al.*, 2009c; Kappe *et al.*, 2010; Klemt, 2010; Krappmann *et al.*, 2011; Pardon *et al.*, 2010). Haemorrhages were found most frequently on the skin and subcutaneously, especially at bony prominences, external mucosae, on the serosae, subepi- and subendocardially, in the meninges and in the skeletal muscle (Friedrich *et al.*, 2009c; Kappe *et al.*, 2010; Krappmann *et al.*, 2011; Schröter *et al.*, 2011). The gastrointestinal tract can be filled with coagulated blood (Krappmann *et al.*, 2011; Pardon *et al.*, 2010). Less common findings are haemorrhagic fluid in the pleura, the abdomen or intra-articular (Pardon *et al.*, 2010). After venipuncture or injections, severe haemorrhages were found at respective locations (Schröter *et al.*, 2011). Due to the haemorrhagic diathesis, carcasses appear anaemic (Friedrich *et al.*, 2009c; Kappe *et al.*, 2010; Klemt, 2010; Pardon *et al.*, 2010). The bone marrow of sternum and long bones has a pale red appearance (Kappe *et al.*, 2010). In challenge experiments, lymph nodes like prescapular or inguinal lymph nodes were found to be enlarged in some calves (Bell *et al.*, 2013; Schröter *et al.*, 2011), as well as the thymus (Bell *et al.*, 2013). In necropsy of challenge calves, Bell et al. (Bell *et al.*, 2013) showed at 10 days after first colostrum intake a significantly reduced length of the femurs in comparison to control calves, while Kappe et al. (Kappe *et al.*, 2010) reported in BNP-calves a good nutritional condition and bodyweight according to the respective age at necropsy. Besides these findings, other pathological observations in several BNP-calves were bronchopneumonia, inflammations in the oral cavity or catharral enteritis and detection of pathogens associated to these diseases (Friedrich *et al.*, 2009c; Kappe *et al.*, 2010; Klemt, 2010; Krappmann *et al.*, 2011; Pardon *et al.*, 2010).

The most distinctive finding in histopathology is a depletion of the bone marrow haematopoetic cells (panmyelophthisis) (Bell *et al.*, 2010b; Buck *et al.*, 2011; Friedrich *et al.*, 2009c; Friedrich *et al.*, 2011; Kappe *et al.*, 2010; Klemt, 2010; Krappmann *et al.*, 2011; Pardon *et al.*, 2010; Schröter *et al.*, 2011). Depending on the degree of hypoplasia, ranging from hypo- to aplastic bone marrow, various numbers of precursor cells are still present, but all precursor cells of the erythroid, myeloid and lymphoid lineage, including megakaryocytes, are reduced (trilineage hypoplasia) (Bell *et al.*, 2010b; Friedrich *et al.*, 2009c; Kappe *et al.*, 2010; Pardon *et al.*, 2010; Schröter *et al.*, 2011). Clusters of macrophages were found, many of them being activated and in some calves showing haemophagocytosis (Pardon *et al.*, 2010). In some animals, the eosinophilic component was increased (Pardon *et al.*, 2010). A comprehensive study, in which bone marrow biopsies were taken at 24 hours, 6 days and at necropsy 10 days after giving a standardised dose of the same colostrum to all challenge calves, a reduced cellularity, involving all cell lineages, was revealed 6 days post challenge. At 10 days post colostrum ingestion, the reduced cellularity was marked, but small numbers of eosinophil, mature neutrophils and erythroid progenitors could still be detected. Hence, the destruction of the bone marrow does not affect the more mature stages of neutrophil, eosinophil and erythrocyte precursors (Bell *et al.*, 2013). Areas affected by hypocellularity are replaced by fatty stromal tissue or filled with erythrocytes or a proteinaceous fluid (Bell *et al.*, 2010b; Bell, 2011). Regarding the vascular architecture, neither damages relating to a vasculopathy, nor endothelial changes or transmural inflammation were detected (Pardon *et al.*, 2010). An extravasation of red blood cells could be demonstrated, whereby the affected tissue and organ architecture did not show any abnormalities (Pardon *et al.*, 2010). Other histopathological findings include a diffuse lymphoid depletion equally involving T- and B-compartments in spleen and lymph nodes; in some calves the lymphoid depletion was also found in the thymus (Kappe *et al.*, 2010; Pardon *et al.*, 2010). There is an increase of apoptotic lymphocytes in lymphoid follicles (Kappe *et al.*, 2010). Bell et al. (Bell *et al.*, 2013) revealed wider cortices and no well-defined secondary follicle formation compared to control calves 10 days after colostral intake in lymph nodes of challenge calves. Lymphocyte depletion can be observed in the periarteriolar lymphoid sheets and lymphoid follicles in the spleen. Additionally the red pulp can display a hypocellularity (Pardon *et al.*, 2010).

3.1.6 Therapy and Prophylaxis

Blood transfusions were undertaken in some BNP-calves. These proved to be a short term success only, and the lethality rate was not decreased (Buck *et al.*, 2011; Pardon *et al.*, 2010). On a long-term perspective, haematological alterations like thrombocytopenia and leucopenia were not

improved with this approach (Pardon *et al.*, 2010). Moreover, several attempts were made in treating bacterial infections and fever with different antibiotics and antiphlogistics, either without (Friedrich *et al.*, 2009c; Kappe *et al.*, 2010; Pardon *et al.*, 2010) or with limited success (Klemt, 2010). It had been reported that prophylactic treatment of calves with dexamethasone on day 7 after birth resulted in a lower incidence of clinically affected BNP-calves, and haematological parameters also showed less dramatic deviations from reference ranges (Klemt, 2010).

In first experimental studies with challenge calves, colostrum from specific dams was shown to be the key causal agent for the induction of BNP (Friedrich *et al.*, 2011; Schröter *et al.*, 2011). Feeding a group of calves with a colostrum substitute in a herd with previous BNP-incidence proved to prevent BNP-cases, while some calves of the same herd, which were fed colostrum from their own dams, showed haematological alterations or developed clinical signs of BNP (Schröter *et al.*, 2012). Similarly, Bell et al. (Bell *et al.*, 2010a) reported that calves from BNP-dams were muzzled immediately after birth and received a colostrum substitute, which prevented development of BNP in these calves. Thus, feeding of mixed colostrum in herds with previous BNP-cases as well as colostrum from dams that are known to have had BNP-calves should be avoided.

3.2 Alloimmune Diseases

Alloimmunity is defined as an immune response towards antigens of a genetically different member of the same species. The prefix “allo-” means “other”. The term is most often used in connection with graft rejection or blood transfusions. In autoimmune diseases, with the prefix “auto” meaning “self”, an individual’s own tissue is attacked by the immune system. Finally, the prefix “xeno”, originating from “xenos”, the Greek word for “foreign”, describes a graft between two animals of different species (Tizard, 2012a; Tizard, 2012b).

Cell surface molecules of red blood cells (RBC), comprising membrane proteins, glycoproteins or glycolipids, differ between individuals and can act as antigens (Urbaniak, 2002). In humans, more than 20 blood group systems have been recognised, and well-known antigens are the polysaccharide antigens AB0 or protein antigens such as Rhesus. Alloimmunisation against RBC antigens can occur during blood transfusions, pregnancy or tissue/organ transplantation, if there are genetic differences between donor and recipient (Urbaniak, 2002). Alloantibodies against RBC antigens can also preexist naturally in an individual without any previous exposure to foreign RBCs and are typically of IgM type (Urbaniak, 2002). After exposure to RBCs of a genetically different individual, the recipient will produce alloantibodies (Tizard, 2012c). RBCs of the donor will be

eliminated through intravascular haemolysis and phagocytosis within the recipient. Subsequent exposure to the same antigen will result in immediate destruction of the RBCs (Tizard, 2012c).

Several authors pointed out that there are parallels between BNP and a condition in humans, namely the foetal/neonatal alloimmune thrombocytopenia (FAIT/NAIT) (Bridger *et al.*, 2011; Deutskens *et al.*, 2011; Foucras *et al.*, 2011; Witt *et al.*, 2011), which is considered to be the platelet counterpart of Rhesus Haemolytic Disease of the Newborn (RHD) (Kaplan, 2006). NAIT is caused by maternal alloantibodies, which are directed mainly against platelet-specific alloantigens of the foetus. The fetal platelet antigens, which differ from those of the mother, are inherited from the father and elicit alloantibody production in the mother. Maternal alloantibodies can cross the placenta during pregnancy due to the haemochorial placenta in humans. NAIT can already occur during a first pregnancy in healthy mothers. Newborns display petechiae, purpura or haematoma, infrequently also visceral haemorrhages and in 20% of cases intracranial haemorrhages. A thrombocytopenia and sometimes anaemia due to haemorrhages can be found (Kaplan, 2006).

Naturally occurring cases of neonatal isoerythrolysis (NI) or neonatal haemolytic disease have been observed in horses and cats. This feto-maternal incompatibility manifests itself *post natum* after colostral intake of maternal alloantibodies and is characterised by immune-mediated destruction of RBCs. In cats, this phenomenon is associated with the fading kitten syndrome (Bücheler, 1999). NI in horses is caused by alloimmunisation of the mare lacking specific erythrocyte antigens carried by the foal and inherited from its sire (Kähn *et al.*, 1991). Alloimmunisation can occur during pregnancy, parturition or blood transfusions (Kähn *et al.*, 1991).

A feto-maternal incompatibility phenomenon caused by vaccination had been observed in cattle in the past. Vaccines against anaplasmosis contained erythrocytic membranes, which stimulated alloantibody production in the vaccinated cow, if the cow had different erythrocyte antigens as contained in the vaccine. Alloantibodies were transferred to calves via colostrum and caused a haemolytic anaemia in calves that had inherited the respective erythrocyte antigens from the sire against which alloantibodies from the dam were directed (Luther *et al.*, 1985).

3.3 Previous Studies on the Aetiopathogenesis of BNP

Differential diagnoses of haemorrhagic diathesis in cattle include inherited or acquired disorders. The latter include for example infections, drug and chemical exposures or immunological causes. After the emergence of BNP, all of these differential diagnoses were investigated, and most of the

disorders could be excluded as aetiological factors for BNP. A strong evidence for an immune-mediated pathogenesis was obtained in several studies. An overview of all studies considering different factors causative for a haemorrhagic diathesis related to BNP is given hereafter.

3.3.1 Infectious Agents

In cattle, different pathogens may induce clinical symptoms similar to those of BNP. Therefore, first studies on BNP considered potential infectious agents to be involved in the pathogenesis of BNP, since many BNP-calves also revealed therapy-resistant fever, which is frequently associated with viral infections (Friedrich *et al.*, 2009c).

Infections with the BVD virus cause a variable outcome in cattle, dependent on age and immune status of the animal, as well as on the strain and biotype of the virus (Lanyon *et al.*, 2014). One of these disease syndromes is the haemorrhagic syndrome in calves, which is an acute infection caused by noncytopathic (ncp) BVD virus (Liebler *et al.*, 1995). In an experimental study, young calves were infected with a ncp field isolate of the BVD virus and clinical signs similar to those observed in BNP, e.g. internal and external haemorrhages and a thrombocytopenia, were detected 2 weeks *post infectionem* (Corapi *et al.*, 1989). Bone marrow biopsies, however, showed a marked hyperplasia of the megakaryocytes (Corapi *et al.*, 1989). This is in contrast to findings in BNP-calves, which revealed a damage of the bone marrow related to all cell lineages including the megakaryocytes (Bell *et al.*, 2010b; Friedrich *et al.*, 2009c; Kappe *et al.*, 2010; Pardon *et al.*, 2010; Schröter *et al.*, 2011). Without exception, RT-PCRs for BVD antigen were negative in all tested BNP-calves (Bell *et al.*, 2010b; Buck *et al.*, 2011; Friedrich *et al.*, 2009c; Friedrich *et al.*, 2011; Kappe *et al.*, 2010; Krappmann *et al.*, 2011; Pardon *et al.*, 2010). Moreover, all dams of affected calves had been vaccinated against BVDV (Friedrich *et al.*, 2009c; Pardon *et al.*, 2010; Schröter *et al.*, 2011). In challenge studies, no BVDV-antibodies were detected before colostrum intake, but after colostrum ingestion, calves were positive for BVDV-antibodies (Friedrich *et al.*, 2011; Schröter *et al.*, 2011). Calves that did not survive BNP had significantly higher BVDV-antibody titres compared to animals that survived the disease (Schröter *et al.*, 2011).

Bluetongue virus (BTV), or the epizootic haemorrhagic disease virus (EHDV), may cause clinical signs like reddening of the lip mucosa, oral erosions, petechiae on the lingual and buccal papillae, or echymotic haemorrhages at the hoof (Brenner *et al.*, 2011; Yadin *et al.*, 2008). PCRs for both BTV (Buck *et al.*, 2011; Kappe *et al.*, 2010; Klemt, 2010; Krappmann *et al.*, 2011; Pardon *et al.*, 2010) and EHDV (Pardon *et al.*, 2010) antigens were negative in BNP-calves. In 2008, a vaccination against the bluetongue virus (BTV) type 8 had been introduced in Germany, and there were

concerns from farmers about a possible correlation between the respective vaccine and the occurrence of BNP (Kappe *et al.*, 2010). However, BNP-cases had already been observed in 2007 (Buck *et al.*, 2011; Kappe *et al.*, 2010; Krappmann *et al.*, 2011).

Kappe et al. (Kappe *et al.*, 2010) detected circoviral DNA with very high similarity to the porcine circovirus type 2b (PCV-2) in five of 25 examined BNP-calves and in one of eight non-BNP-calves. In pigs, this virus is associated with a number of different syndromes like the postweaning multisystemic wasting syndrome (PMWS) or the porcine dermatitis and nephropathy syndrome (PDNS) (Segalés *et al.*, 2005). Many years before the occurrence of BNP, this virus had already been described in Canadian cattle with respiratory distress or aborted bovine foetuses (Nayar *et al.*, 1999). In other countries, such as the USA, PCV-2 had also been detected in cattle, whilst the exact biological impact of this virus in cattle is not understood until today (Li *et al.*, 2011). Other studies did not find any evidence that PCV-2 is involved in the pathogenesis of BNP (Bastian *et al.*, 2011; Schröter *et al.*, 2011; Willoughby *et al.*, 2010).

Friedrich et al. (Friedrich *et al.*, 2009c) and Pardon et al. (Pardon *et al.*, 2010) discussed a possible involvement of bovine parvovirus in BNP. In cats and dogs, this virus is known to cause either a panleucopenia or a pancytopenia (Hosokawa *et al.*, 1987; Weiss *et al.*, 1999). In cattle, the respective virus causes mainly diarrhoea, but no damage of the bone marrow (Durham *et al.*, 1985; Friedrich *et al.*, 2009c). Moreover, no signs of a parvovirus infection were seen in histology in BNP-calves (Pardon *et al.*, 2010).

Pathogenic bacteria were also included in analyses. A wide range of different bacteria was detected in BNP-calves, but none of them were found in all BNP-calves and were therefore associated with BNP (Kappe *et al.*, 2010; Klemt, 2010; Krappmann *et al.*, 2011). *Pasteurella multocida*, which is associated with a haemorrhagic septicaemia (Rhoades *et al.*, 1967), was only found sporadically (Kappe *et al.*, 2010) and no *Salmonella spp.* was detected (Schröter *et al.*, 2011).

3.3.2 Toxicological Agents

In literature, several toxicological agents are described, which may cause bone marrow damages and clinical signs as observed in BNP.

Furazolidone is a nitrofuran antibiotic and can cause a depletion of the bone marrow and similar clinical symptoms as BNP, but only after administration over a long period of time (Hofmann, 1972). Nitrofuranes are forbidden by law for the use in food producing animals in the European

Union (Commission Regulation (EU) No 37 / 2010). Additionally, BNP-calves tested for furazolidone were all negative (Friedrich *et al.*, 2009c; Kappe *et al.*, 2010). Similarly, chloramphenicol can cause an aplastic anaemia in cattle (Jorna and Postema, 1986), but is also forbidden by law for food producing animals in the European Union (Commission Regulation (EU) No 37 / 2010). Overdosage of the coccidiostat halofuginone can cause bloody diarrhoea (Scholtysik and Steuber, 2007). This pharmaceutical was not applied in all monitored BNP-affected herds (Pardon *et al.*, 2010), and no indication was obtained for overdosage of halofuginone in BNP-calves (Krappmann *et al.*, 2011). Other pharmaceuticals that may cause BNP-associated symptoms are sulfonamides or non-steroidal anti-inflammatory drugs. However, these drugs were applied only occasionally in connection with the treatment of BNP, and an association of these drugs with BNP was excluded by Friedrich et al. (Friedrich *et al.*, 2009c).

S-(1,2-Dichlorovinyl)-L-cysteine (DCVC) is a metabolite of trichloroethylene, which has been used in the past for the extraction of soybean oil (Kappe *et al.*, 2010). Intoxications with this agent can cause aplastic anaemia and renal injury (Lock *et al.*, 1996). However, no DCVC could be detected in BNP-calves (Friedrich *et al.*, 2009c; Kappe *et al.*, 2010).

Bracken fern or field melilot were excluded as potential toxic agents involved in BNP, too (Bell *et al.*, 2010b; Friedrich *et al.*, 2009c; Kappe *et al.*, 2010; Pardon *et al.*, 2010; Sauter-Louis *et al.*, 2012).

Analyses for mycotoxins, such as trichothecenes or mycotoxins of *Stachybotrys chartarum* and Aflatoxin B1 in forage samples, were also negative in BNP-calves (Buck *et al.*, 2011; Friedrich *et al.*, 2009c; Kappe *et al.*, 2010; Pardon *et al.*, 2010).

In an epidemiological questionnaire, a couple of farms affected by BNP as well as a few control farms could not exclude the use of rodenticides (Sauter-Louis *et al.*, 2012). The study indicated that dicumarol was used in most instances (Sauter-Louis *et al.*, 2012), which does not cause bone marrow depletion (Runciman *et al.*, 2002; Sauter-Louis *et al.*, 2012; Wang *et al.*, 2007).

3.3.3 Genetic Factors

Studies on the aetiology of BNP also involved investigations on possible genetic factors associated with BNP. Several cattle breeds were affected by BNP (Bell *et al.*, 2009; Corbière *et al.*, 2009; Friedrich *et al.*, 2009c; Pardon *et al.*, 2010), but only a small proportion of calves showed clinical signs of BNP compared to the respective whole cattle population. This was also true on individual farm level, where animals were kept under the same conditions (Foucras *et al.*, 2011; Kasonta *et*

al., 2012; Pardon *et al.*, 2010; Reichmann, 2012; Witt *et al.*, 2011). Some dams gave birth to more than one BNP-calf (Pardon *et al.*, 2010), and in challenge studies, the disease could be reproduced with colostrum from specific dams in several unrelated challenge calves (Friedrich *et al.*, 2011; Schröter *et al.*, 2012), indicating that the production of the aetiological agent may be restricted to a certain subset of dams.

For hereditary coagulopathies in cattle, inherited disorders involving coagulation factor VIII (Khalaj *et al.*, 2009) or a factor XI deficiency in Holstein cattle (Marron *et al.*, 2004) have been described. Krappmann et al. (Krappmann *et al.*, 2011) screened a well characterised crossbreed Holstein x Charolais cattle resource population with incidence of clinical BNP for mutations in factor XI. No specific mutations in the coagulation factor XI gene associated with BNP could be detected. However, the study revealed that all clinical BNP-cases were confined to one specific sire line of the F_2 resource population. All affected BNP-calves were progeny of a single F_1 Charolais x Holstein crossbred male. This accumulation of clinical BNP-cases in one specific family was statistically significant. Therefore, a genetic predisposition involved in BNP-pathogenesis is likely (Krappmann *et al.*, 2011).

A further study genotyped BNP-calves and control animals of the Holstein breed at the major histocompatibility complex (MHC) class II *DRB3* locus (Ballingall *et al.*, 2011), which is associated with autoimmune diseases and variations in immune response (Handunnetthi *et al.*, 2010). No significant differences in allele frequencies were found in BNP-calves compared to control animals (Ballingall *et al.*, 2011).

3.3.4 Idiopathic Cases of Haemorrhagic Diathesis

Sporadic cases similar to BNP and unknown aetiology were observed before the accumulation of BNP-cases in 2007 (Friedrich *et al.*, 2009c). A recently published epidemiological study revealed that 4.5% of suspected BNP-cases were born to non-PregSure® BVD-vaccinated dams, received colostrum exclusively from the own dam, and originated from farms with no history of BNP-cases. However, these calves were not further investigated for other factors that might contribute to a pancytopenia (Jones *et al.*, 2013). In literature, there are only rare reports on idiopathic haemorrhagic diathesis in calves.

Ammann et al. (Ammann *et al.*, 1996) described the case of a 14-day-old Holstein calf that displayed clinical signs of a haemorrhagic disorder like a bloody diarrhoea. This calf had been treated with trimethroprim and sulfadoxine. Haematology revealed a severe anaemia, leucopenia and thrombocytopenia, while gross pathology revealed multiple haemorrhages. These findings

were all associated with a hypocellular bone marrow, which was demonstrated in a histological examination. As possible aetiology for the condition in this calf, a drug-induced or inherited bone marrow aplasia was suspected.

Another case report was documented in 2007 in Japan (Shimada *et al.*, 2007). This case report describes an 11-day old Holstein calf, which was presented with melaena, external haemorrhages and pancytopenia, including a non-regenerative anaemia. In histology, a bone marrow hypoplasia was diagnosed, which comprised all cell lineages. Possible causes for the bone marrow aplasia, e.g. genetic factors, viral infections such as BVDV, toxic agents, chemicals or radiation were excluded. The calf had received no drugs associated with a bone marrow aplasia (Shimada *et al.*, 2007). Neither the vaccination history of the dam was mentioned in this case report, nor was the BVDV status of the dam known. This case report has a strong resemblance to BNP and a possible association of this case with BNP remains unclear.

A similar case report was published shortly afterwards, describing a 15 day old Japanese black calf with clinical findings similar to those of BNP (Fukunaka *et al.*, 2010). Haematological findings included a severe thrombocytopenia and leucopenia as well as a non-regenerative anaemia. Multiple haemorrhages were detected in necropsy, and histopathology revealed a marked bone marrow aplasia. Toxins or infections *in utero* were considered as unlikely and a viral infection with BVDV was ruled out (Fukunaka *et al.*, 2010). The authors could not exclude a possible association with BNP.

3.3.5 Evidence for an Alloimmune-mediated Pathogenesis of BNP

First investigations on the aetiopathogenesis of BNP observed that some dams gave birth to more than one BNP-calf, although not always in consecutive years (Friedrich *et al.*, 2011; Pardon *et al.*, 2010). Based on observations from farmers and findings of an emerging thrombocytopenia after colostrum intake in BNP-calves, and given the time period between colostrum ingestion and the development of clinical signs of BNP, it was suspected that colostrum might be involved as a potential aetiological agent in the pathogenesis of BNP (Friedrich *et al.*, 2011). In challenge experiments, colostrum from specific dams, which all had been vaccinated with PregSure® BVD, was then confirmed to be the key aetiological agent involved in the pathogenesis of BNP. Clinical BNP or at least haematological changes could be reproduced quite reliably in the majority of challenge calves originating from different breeds by feeding colostrum from unrelated dams that had given birth to a BNP-calf before (Friedrich *et al.*, 2011; Schröter *et al.*, 2011). These results pointed to an immune-mediated mechanism, putatively elicited by antibodies in colostrum

(Friedrich *et al.*, 2011). A different approach to demonstrate an alloimmune-mediated mechanism was described by Foucras et al. (Foucras *et al.*, 2011). In this experiment, a pool of IgGs from dams, which had given birth to a BNP-calf before, was injected i.v. into unrelated neonatal healthy calves, thereby excluding a potential effect of antibody resorption in the digestive tract after colostrum intake. Accordingly, characteristic findings associated with BNP were observed in these calves, including a cellular depletion of the bone marrow.

Further evidence of an alloimmune-mediated mechanism was then revealed in other studies. Pardon et al. (Pardon *et al.*, 2011) and Bridger et al. (Bridger *et al.*, 2011) demonstrated via flow cytometry that sera from BNP-dams contained alloantibodies that bind to calf leucocytes. The binding of alloantibodies was not age-dependent, since also older calves showed reactivity, and therefore the expression of the target antigen(s) does not seem to be age-related (Pardon *et al.*, 2011). The disease is, however, restricted to neonates after colostrum ingestion, because colostral alloantibodies are only able to pass the intestinal barrier up to 36 hours p.c.i. (Kruse, 1983). Additionally, in contrast to other species like humans, antibodies in cattle are not able to pass from the mother to the fetus during gravidity due to the epitheliochorial nature of the placenta (Bridger *et al.*, 2011; Kruse, 1983; Pardon *et al.*, 2011). In a further experiment, the amount of alloantibody-positive lymphocytes/monocytes (IgG^+-cells) in neonatal calves was monitored after intake of colostrum from BNP-dams. The percentage of IgG^+-cells mainly increased between 6 - 12 hours post colostrum intake and thereafter the severity of clinical signs of BNP correlated with the percentage of IgG^+-cells and the duration and persistence of these levels (Bridger *et al.*, 2011). Cells that were opsonised with alloantibodies induced cytophagocytosis by macrophages (Bastian *et al.*, 2011).

No alloantibodies were bound when incubating leucocytes with either serum of non-BVD-vaccinated dams or with serum from dams which had been vaccinated with an alternative BVD-vaccine to PregSure® BVD (Bastian *et al.*, 2011). Accordingly, alloantibody titres in BNP-dams were found to be significantly higher than in BVDV-unvaccinated control dams and also correlated with the severity of BNP in calves (Bridger *et al.*, 2011). The severity of BNP, however, did not correlate with the BVDV-antibody titres in these dams (Bridger *et al.*, 2011), although it had been shown that BVDV-neutralising antibody titres of BNP-dams exceeded those of PregSure® BVD vaccinated non-BNP-dams (Bastian *et al.*, 2011). Three repetitive immunisations with PregSure® BVD had been shown to induce high alloantibody titres, which differed significantly from alloantibody titres found in animals immunised with other booster vaccination schemes like three consecutive vaccinations with an inactivated BVD vaccine other than PregSure® BVD. Applying a “modified two-step vaccination” that consisted of a primary vaccination with PregSure® BVD and two

subsequent booster immunisations with a live attenuated BVD vaccine also resulted in significantly lower alloantibody titres than a vaccination scheme using only PregSure® BVD (Kasonta *et al.*, 2012).

When testing a panel of BNP-sera for alloantibody-binding to leucocytes of different individuals, leucocytes of one individual did not necessarily show a reaction with each of the BNP-dam sera (Bastian *et al.*, 2011; Bridger *et al.*, 2011). This may indicate that individual BNP-dams responded to different antigens (or different alleles of a polymorphic antigen), but also that expression of alloantigens was variable among individuals (Bastian *et al.*, 2011).

Furthermore, immunofluorescence microscopy of peripheral blood smears and bone marrow smears revealed that in comparison to non-BNP-dams, the sera of BNP-dams contained alloantibodies that bound to these cells. However, the megakaryocytes did not show a distinct staining when incubated with BNP-dam sera in this study (Pardon *et al.*, 2011). In flow cytometry studies, it was observed that BNP-associated alloantibodies bound more effectively to lymphocytes and monocytes compared to granulocytes (Bastian *et al.*, 2011; Bridger *et al.*, 2011; Pardon *et al.*, 2011). These binding patterns of BNP-associated alloantibodies to peripheral blood cell subsets were evaluated in a further study via immunofluorescence microscopy of different cell types with either BNP-dam or non-BNP-dam colostrum (Assad *et al.*, 2012). Firstly, it was shown that BNP-alloantibodies bound to 100% of all platelets and to 70% of all leucocytes. Cells of the myeloid lineage, like granulocytes and monocytes, displayed 100% reactivity with alloantibodies, whilst not all cells of the lymphoid lineage, especially the CD4+ T cells (T-helper cells), bound BNP-associated alloantibodies. Secondly, regardless of breed, the reactivity of colostral alloantibodies was identical for cells either of juvenile or of adult origin, pointing to a constant expression of the target antigen (Assad *et al.*, 2012).

The binding pattern of BNP-associated alloantibodies was also evaluated with the BVD virus and different cell lines used for vaccine production. No reaction of alloantibodies could be detected with the BVD-virus or surface antigens of all cell lines, except for the bovine kidney cell line used for the production of PregSure® BVD (Bastian *et al.*, 2011). In order to evaluate if the induction of alloantibodies was restricted only to PregSure® BVD, guinea pigs were immunised with different BVD-vaccines and only PregSure® BVD induced alloantibodies that bound to cell surface antigens of bovine leucocytes (Bastian *et al.*, 2011).

Two independent studies proposed that BNP-associated alloantibodies target the MHC class I antigen present on bovine leucocytes, but also on the Madin-Darby bovine kidney cell line (MDBK) used for the production of the inactivated vaccine PregSure® BVD (Deutskens *et al.*, 2011; Foucras

et al., 2011). MHC class I, an approximately 42 kDa protein, from the MDBK cell line was shown to be a constituent and therefore a contamination of PregSure® BVD, which might have elicited alloantibody production in BNP-dams (Deutskens *et al.*, 2011; Foucras *et al.*, 2011). After down-regulation of ß$_2$-microglobulin-coding mRNA in MDBK cells via RNA interference and consecutively reduced MHC class I expression, cell staining was reduced after incubation with BNP-dam sera (Foucras *et al.*, 2011). Immunoprecipitation with BNP-dam sera showed a reaction with MHC class I on the MDBK cell line (Deutskens *et al.*, 2011; Foucras *et al.*, 2011; Kasonta *et al.*, 2012). Sera of non-vaccinated BVDV-free dams or dams which had been vaccinated with an alternative inactivated BVD vaccine revealed no or only a very weak reactivity with MHC class I, respectively. However, some of the PregSure® BVD-vaccinated non-BNP-dams also showed reactivity with MHC class I (Deutskens *et al.*, 2011). This was confirmed by Kasonta et al. (Kasonta *et al.*, 2012) for the serum of a large proportion of animals after experimental vaccination with PregSure® BVD. Additionally, after incubation with a standardised, pooled BNP-serum, a reactivity with MHC class I on PBMCs of some BNP-dams was revealed (Deutskens, 2012).

Evidence of a massive cellular contamination of PregSure® BVD was obtained in a further study (Euler *et al.*, 2013) that compared the cell surface proteome of the MDBK cell line with the protein composition of PregSure® BVD. PregSure® BVD contained a 3.5 fold higher amount of proteins than a different, live-attenuated BVD-vaccine. Several proteins were shared between PregSure® BVD and the MDBK cell surface. MHC I was, however, one of the least abundant shared proteins in PregSure® BVD and MDBK cells and the authors instead presented some other alloantigens as candidates, that still need further validation (Euler *et al.*, 2013).

3.4 RNA-Sequencing (RNA-Seq)

In recent years, next-generation sequencing (NGS) or deep-sequencing technologies have been introduced, which offer the opportunity for high throughput sequencing at single-base resolution. In case of transcriptome sequencing, the method is termed RNA-Seq (RNA sequencing). This novel method was applied in our second study and therefore, a short overview on advantages of this method will be given.

The transcriptome is defined as the sum of all RNA molecules or transcribed loci, including mRNA, tRNA, rRNA and non-coding RNA, in a cell or particular tissues at a specific time point or condition. RNA-Seq provides the option of mapping and quantifying transcriptomes. It is not restricted to an existing genome annotation of a species. Hence, also novel transcripts or genes can be detected. This method can also reveal exon/intron boundaries or alternative splicing. Additionally, sequence

variations, for example single nucleotide polymorphisms (SNPs), can be evaluated with RNA-Seq (Lister *et al.*, 2009; Wang *et al.*, 2009). Another advantage of RNA-Seq in comparison to microarrays is its large dynamic range. Therefore, transcripts with very high or low expression levels or marked fold-changes can be detected. Finally, this method is characterised by a low technical variance. Results for both technical as well as biological replicates can be reproduced reliably (Wang *et al.*, 2009).

4 Objectives

Several questions regarding the aetiopathogenesis of BNP still remain open, and some published results demand further research. For this thesis, two main aspects concerning aetiopathogenesis and manifestations of BNP were selected for further evaluation.

In literature, several field observations or challenge studies reported on the incidence of subclinical BNP-cases. Subclinical BNP-calves display no clinical signs of BNP, but a shift in cell counts below the applied reference ranges in at least two haematopoietic cell lineages (Friedrich *et al.*, 2011; Pardon *et al.*, 2010; Schröter *et al.*, 2011; Witt *et al.*, 2011). The true incidence of subclinical BNP-cases has, however, not been thoroughly investigated. An accumulation of clinical BNP-cases in exclusively one specific sire line of an F_2 experimental resource population was revealed in a previously performed study at our institute (Krappmann *et al.*, 2011). All BNP-calves were descendants of one F_1 male. Hence, the hypothesis of a genetic predisposition being involved in clinical BNP was proposed (Krappmann *et al.*, 2011). Therefore, the present study investigated the incidence, a possible genetic predisposition, and further epidemiological factors involved in subclinical BNP (**Publication I**).

The occurrence of BNP is strongly associated with the vaccination of dams with a specific inactivated vaccine (PregSure® BVD) against the Bovine Viral Diarrhoea Virus (BVDV) (Lambton *et al.*, 2012; Sauter-Louis *et al.*, 2012). This vaccine has been shown to induce very high antibody titres against BVDV (Bastian *et al.*, 2011; Raue *et al.*, 2011), but also to elicit the production of alloantibodies in some dams (Bridger *et al.*, 2011; Pardon *et al.*, 2011). The vaccine comprises a unique adjuvant. Furthermore, it has been revealed that the vaccine is highly contaminated with proteins derived from the Madin-Darby bovine kidney (MDBK) cell line used for the production of the specific vaccine (Euler *et al.*, 2013). Due to these particular characteristics of the vaccine, the second aim of the present study was to characterise the immune response to vaccination with PregSure® BVD to gain insight into the structural and quantitative regulation of the blood transcriptome by means of an RNA-Seq approach (**Publication II**).

5 Results

5.1 Publication I

W. Demasius, R. Weikard, A. Kromik, C. Wolf, K. Müller, C. Kühn

Bovine neonatal pancytopenia (BNP): novel insights into the incidence, vaccination-associated epidemiological factors and a potential genetic predisposition for clinical and subclinical cases

Research in Veterinary Science (2014), **96**: 537-542

Research in Veterinary Science 96 (2014) 537–542

Contents lists available at ScienceDirect

Research in Veterinary Science

journal homepage: www.elsevier.com/locate/rvsc

Bovine neonatal pancytopenia (BNP): novel insights into the incidence, vaccination-associated epidemiological factors and a potential genetic predisposition for clinical and subclinical cases

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ARTICLE INFO

Article history:
Received 26 July 2013
Accepted 28 March 2014

Keywords:
Bovine neonatal pancytopenia
Genetic predisposition
Haematology
Subclinical cases
Vaccination

ABSTRACT

Bovine neonatal pancytopenia (BNP) is a haemorrhagic disease of newborn calves elicited by colostrum from specific cows. Two studies have indicated that BNP-inducing colostrum might be associated with alloantibodies directed against MHC class I in response to vaccination with a distinct inactivated viral vaccine. However, the proportion of alloantibody-producing individuals by far exceeds the proportion of clinical BNP cases in the vaccinated population. This raises the question about the incidence of subclinical, unrecognised cases and also suggests further factors involved in BNP pathogenesis, e.g., genetic predisposition. Our results on neonatal calves from a closely monitored resource population confirmed the hypothesis of a genetic predisposition for clinical BNP and suggest that the predisposition is also involved in subclinical BNP-cases. No indication was obtained for a higher frequency of subclinical BNP-cases compared with clinical cases. Neither time point nor frequency of vaccination was a relevant factor for BNP in our resource population.

1. Introduction

An increase in the number of neonatal calves showing clinical signs of haemorrhagic diathesis has been recorded in many European countries since 2007 (Gentile et al., 2009; Smolenaars and Mars, 2009; Corbiere et al., 2009; Ellis-Iversen and Colloff, 2009; Friedrich et al., 2009; Pardon et al., 2010; Sanchez-Miguel et al., 2010; Bell et al., 2010). This syndrome, clinically and in *post-mortem* autopsy characterised by severe internal and external haemorrhages, was termed "bovine neonatal pancytopenia" (BNP) at the Satellite Symposium of the European Buiatric Congress in Marseille, France in 2009. Affected calves suffer from a massive thrombocytopenia, often combined with leukopenia. Histopathological investigations of the bone marrow in clinically affected BNP-calves revealed a trilineage hypoplasia (Friedrich et al., 2009; Pardon et al., 2010).

The hypothesis of an alloimmune-mediated disease has been postulated, because BNP could be reproduced in neonatal calves by the ingestion of colostrum from unrelated dams, which had given birth to a BNP-calf before (Friedrich et al., 2011; Schroter et al., 2011; Bell et al., 2013). Furthermore, binding of alloantibodies from the sera of BNP-dams to leukocytes has been demonstrated (Pardon et al., 2011; Bridger et al., 2011), and recently, the ability of colostral alloantibodies of IgG1 subclass from BNP-dams to bind to peripheral blood cells, especially platelets, has been revealed (Assad et al., 2012). Two independent studies detected MHC class I as one possible antigen structure against which alloantibodies are directed. This was demonstrated by the binding of alloantibodies originating from BNP-dam sera to a cell surface protein (MHC class I) of leukocytes and the Madin–Darby bovine kidney cell line (MDBK), a cell line used for the production of a specific BVDV (Bovine Viral Diarrhoea Virus) vaccine (Deutskens et al., 2011; Foucras et al., 2011). Epidemiological studies have revealed a strong association between the use of this specific vaccine (PregSure® BVD, Pfizer Animal Health) and the occurrence of BNP (Sauter-Louis et al., 2012; Lambton et al., 2012).

However, there is a discrepancy between the widespread use of the distinct BVD vaccine, the high percentage of individuals producing alloantibodies in response to the vaccine and the reported low incidence of BNP-calves (Bastian et al., 2011; Kasonta et al., 2012). This might be explained by differences in the vaccination regimen between farms. An alternative explanation is a genetic predisposition required for BNP-cases that has been postulated in the past. Previous investigations at our institute (Krappmann et al., 2011) revealed an accumulation of clinical cases of BNP occurring exclusively in a specific sire line of an F_2 resource population: all BNP cases were progeny of a single F_1 male. Except for two cows, all other dams, which had given birth to a BNP-calf, had been full-siblings in the

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http://dx.doi.org/10.1016/j.rvsc.2014.03.019
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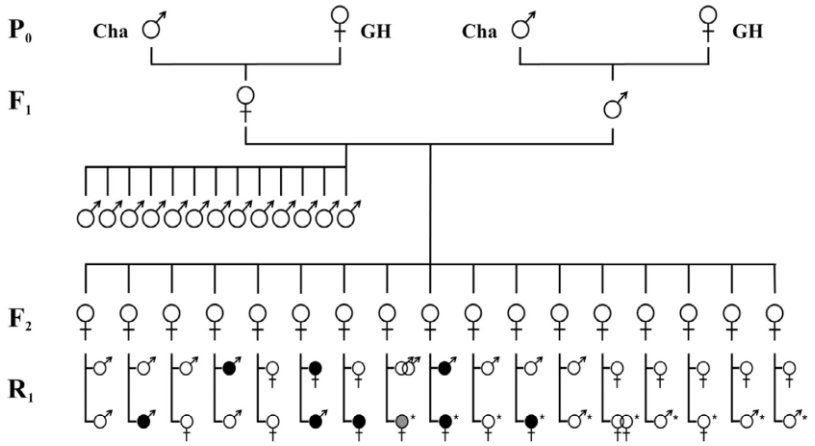

Fig. 1. Segregation of dams in a F_2 full-sib family from the BNP-affected sire line of the cattle resource population: Cha, Charolais; GH, German Holstein. The different generations of the resource population contributing to the BNP-affected F_2-familiy are designated with P_0, F_1, F_2 and R_1. Backcross calves (R_1) within the subset for parallel haematological and clinical monitoring are indicated with an asterisk. Black circles represent calves showing clinical signs of BNP; circles with a grey area represent subclinical cases. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

F_2 generation of this specific sire line (Fig. 1). Other field observations (Pardon et al., 2010) revealed the incidence of subclinical BNP-cases and a large proportion of herds with clinical BNP incidence that also had neonatal calves showing alterations in haematological parameters, but no clinical signs of BNP. This suggests that subclinical cases of BNP might be more frequent than clinical cases of BNP (Witt et al., 2011). Therefore, the objective of this study was to investigate if subclinical cases of BNP were occurring in the resource population. In the case of subclinical BNP occurring, the further objective was to investigate if these subclinical cases were also concentrated on the offspring of the specific sire line analogous to the clinical BNP-cases or if also other sire lines were affected by subclinical BNP-cases. In addition, further epidemiological factors that might be associated with BNP incidence were monitored in the F_2 resource population.

2. Methods

2.1. Animals

All calves included in this study were offspring of the experimental German Holstein × Charolais crossbred resource population herd at the FBN Dummerstorf (Kühn et al., 2002). Briefly, this population had been established by mating five unrelated Charolais sires with purebred German Holstein dams (P_0), thereby generating five independent F_1 families. For designing the F_2 generation, individuals of the families were cross-bred among each other by means of embryo transfer, resulting in full-sib and half-sib F_2 families. Our study included a total of 303 neonatal calves of all sire lines obtained from backcrossing F_2 dams of the cross-bred resource population with a single purebred German Holstein sire. All dams of experimental calves included in this study were kept under the same housing and feeding conditions on the same experimental farm and were vaccinated against the Bovine Viral Diarrhoea Virus. At the age of 12 and 13 months all dams had received at least a basic BVDV-immunisation consisting of two doses. Booster vaccinations were performed thereafter annually for all individuals at the same point in time, including pregnant and non-pregnant cows.

For the investigation of potential non-genetic factors that might interact with the PregSure® BVD vaccination and thus affect BNP incidence, 295 calves were included. The dams of those calves had received two doses of the inactivated BVDV vaccine PregSure® BVD (Pfizer) according to the manufacturer's instructions. PregSure® BVD had been introduced in March 2006 in the experimental cattle herd and had been applied until the vaccine was retracted from the European market in 2010. A subset of those 295 calves (n = 44) including all calves born between March 2011 and April 2012 was subjected to detailed parallel clinical and haematological monitoring. In addition, eight calves born in the same time interval, but originating from mothers vaccinated with a non-PregSure® BVDV vaccine, served as internal control for confirmation of blood cell count references from the literature.

All 303 calves were separated immediately after birth from their mothers and received 2–3 l of first colostrum exclusively from their own mothers no later than two hours after birth. During the whole period of postnatal investigations calves were housed in a barn with straw bedding and were fed a maternal colostrum – maternal whole milk diet three times daily until day 5 after birth. After day 5, this diet was replaced by a commercial milk replacer diet.

2.2. Clinical examinations, blood sampling, pathology

For close clinical examinations and blood sampling, a subset of 44 calves from those 295 calves born to dams with PregSure® BVD vaccination was included and also eight calves from dams with non-PregSure® BVDV vaccination.

All experimental procedures were carried out according to the German animal care guidelines and were approved and supervised by the relevant authorities of the State Mecklenburg-Vorpommern, Germany (State Office for Agriculture, Food Safety and Fishery Mecklenburg-Western Pommerania (LALLF M-V), 7221.3-2.1–005/11). All calves were monitored directly after birth until 28 days of age. Specific meticulous clinical observations of all neonatal calves were performed on days 1 to 5, day 7 and day 14 after colostrum intake, thereby paying special attention to typical clinical signs ascribed to a bleeding disorder. In particular, all mucous membranes, faeces and a possible prolonged bleeding time after venipuncture, injections or ear tagging were examined. Furthermore, blood samples were drawn at the same time. A maximum of 4 ml of blood was drawn at each point in time via venipuncture with an 18-gauge needle into an EDTA-sample tube without the use of vaccutainers. In case of clinical and haematological abnormalities,

clinical and haematological examinations were extended beyond day 14 after colostrum intake. Haematological analysis of the blood samples (specifically thrombocyte, leukocyte and erythrocyte cell count) was performed within two hours after obtaining the samples with an automatic haematology analyser (ABX Pentra 60, HORIBA). Two repetitive measurements for each sample were taken and mean values were calculated for each blood parameter. Whenever values below the lower border of reference values were obtained for blood cell counts, these were confirmed by an external certified laboratory (Metabovet/Labormedicus, Rostock, Germany). Reference ranges for thrombocytes and leukocytes were set as follows according to data for neonatal calves two weeks after birth (Tennant et al., 1974; Egli and Blum, 1998; Knowles et al., 2000; Mohri et al., 2007): Platelets 200–1200 G/l, leukocytes 4.0–12.0 G/l, erythrocytes 5.0–10.0 × 10^{12}/L. The time point for reference ranges was selected, because decline in thrombocyte and leukocyte count was observed in BNP-calves at this time (Friedrich et al., 2011; Bell et al., 2013). To avoid false positive classification of BNP-cases due to the frequently observed spurious variation of blood cell counts in neonatal calves, only calves of coincident thrombocyte and leukocyte counts below the indicated reference range at day 7, day 14 or later without clinical signs of BNP were classified as subclinical BNP-cases. The thresholds of 200 G/l for thrombocyte counts and 4 G/l for leukocyte counts are in agreement with the limits classified as indicative of BNP (Doll, 2012) and had been applied in several reports in the literature (Friedrich et al., 2011; Schroter et al., 2011).

One of the 44 calves developed a fatal bleeding disorder and had to be euthanised. The carcass was sent to the State Office for Agriculture, Food Safety and Fishery Mecklenburg-Western Pommerania (LALLF M-V) Rostock, Germany, for a pathological examination and an ELISA assay for BVDV antigen detection (IDEXX BVDV AG/Serum Plus, Ludwigsburg, Germany).

2.3. Statistical analysis

Statistical analysis was performed using the nonparametric Mann–Whitney U test in SAS, version 9.3 (SAS Institute Inc., Cary, NC, USA) after testing for normal distribution of data. Thrombocyte and leukocyte counts for each point in time of sampling were compared between calves without clinical signs of BNP from families not being affected by BNP (n = 33) and calves originating from the sire line, that had been affected by BNP in the past (n = 9). This comparison was performed to evaluate a potential genetic effect on the respective blood parameters. Calves, which developed clinical signs of BNP (n = 2) were not included in the statistical analysis. $P < 0.05$ was defined as statistically significant, $p < 0.1$ as tentatively significant.

2.4. Monitoring of further potential non-genetic factors for BNP

For each backcross calf of our resource population born to a dam vaccinated with PregSure® BVD (n = 295), the number of PregSure® BVD-vaccinations of the dam, the date of vaccination and the respective time point of vaccination relative to delivery of the calf were analysed. In addition, other veterinary treatments of the dams were monitored. Data from BNP-affected calves (n = 10) were then compared to calves without BNP (n = 285) by χ^2 test.

3. Results

3.1. Clinical findings, haematology, pathology

As expected, none of the eight calves from dams vaccinated with the non-PregSure® BVDV vaccine developed clinical BNP. In addition, none of those calves showed thrombocyte or leukocyte counts below the applied reference range at any time point.

During the time period of this study, one calf of the subset of 44 calves from PregSure® BVD-vaccinated dams developed subclinical BNP. Two weeks after birth, the calf showed thrombocyte counts and leukocyte counts below the reference range, but developed no clinical signs characteristic of BNP. This calf was a descendant of the BNP-affected sire line of the F_2 resource population and specifically of the F_2 full-sib family (Fig. 1), which had been highly affected by clinical BNP-cases in the past. Furthermore, two other calves from this specific F_2 full-sib family developed clinical and haematological changes characteristic of BNP. One of these cases was observed in February 2012 confirming that the disease was still occurring nearly two years after the last booster vaccination with PregSure® BVD. The first of these two calves survived the disease. Interestingly, a full-sib to this calf born in a previous time interval had been affected by clinical BNP and also had survived the disease. The second calf in the subset of the 44 calves developed typical signs of BNP combined with secondary infections and was euthanised on day 23 after birth *(see Materials and Methods)*. Gross pathology revealed typical findings indicative for BNP, including subcutaneous, serosal and pericardial haemorrhages, while a depleted bone marrow could be demonstrated in histopathological examinations. An ELISA test for detection of BVDV-antigen in a tissue sample was negative.

All other calves from the PregSure® BVD-vaccinated dams (n = 41) did not exhibit any clinical signs indicative for BNP and/or coincident thrombocyte and leukocyte counts below the indicated reference range up to day 14 after colostrum intake.

All calves without clinical BNP (n = 42) were included when comparing haematological parameters between those individuals from the sire line in which BNP-cases had been observed BNP (n = 9) and those individuals from other sire lines (n = 33) of the resource population. The thrombocyte counts for both groups of calves decreased after birth. Some calves, also from the non-affected sire lines, showed thrombocyte counts below the applied reference range at days 2–3 (Fig. 2). However, thrombocyte cell count started to recover at day 3 and further increased until day 7 (Fig. 3). Whereas thrombocyte numbers stabilised between day 7 and day 14 for the calves from the BNP-unaffected sire lines, calves of the affected sire line showed a second decrease in thrombocyte numbers between day 7 and day 14 after colostrum intake. Thus, thrombocyte counts on day 14 differed significantly ($P < 0.01$) when comparing the group

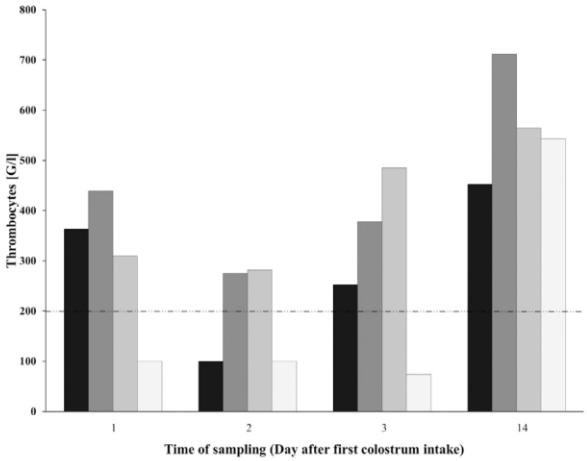

Fig. 2. Thrombocyte counts for four calves from sire lines not affected by BNP at days 1, 2, 3 and 14 after colostrum intake: The broken-dotted line indicates the lower reference value for thrombocyte counts in neonatal calves at two weeks of age. Different coloured columns represent different individuals.

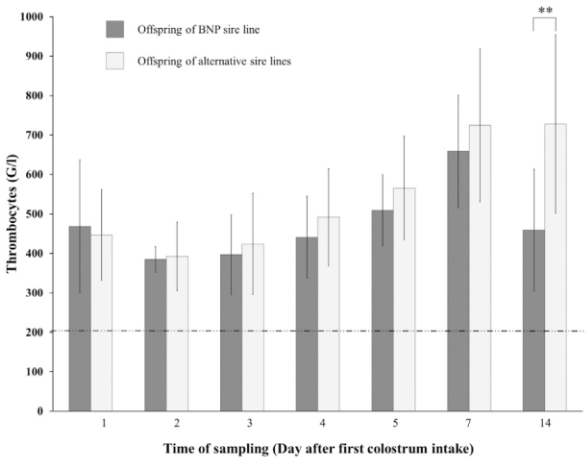

Fig. 3. Mean thrombocyte counts for each day of sampling (day after first colostrum intake): Comparison of mean thrombocyte counts (standard deviation indicated by vertical bar) between calves without clinical BNP of sire lines not affected by BNP (n = 33) and of the BNP-sire line (n = 9). **Significant difference (P < 0.01) in thrombocyte counts between the two groups. The broken-dotted line indicates the lower reference value for thrombocyte counts in neonatal calves at two weeks of age.

of clinically BNP-unaffected calves of the BNP-sire line with BNP-unaffected calves of the other sire lines. It has to be recognised that clinical BNP-calves showing the same phenomenon were not included in this analysis. Leukocyte counts in both groups showed a pattern over time that was very similar to the thrombocyte counts albeit with a time delay: The number of blood leukocytes declined after birth until day 5 and started to increase until the end of the observation period at day 14 after colostrum intake (Fig. 4). Leukocyte counts tentatively differed between the two groups of calves on day 7 (P = 0.06) with a lower leukocyte count in the calves from the affected sire line.

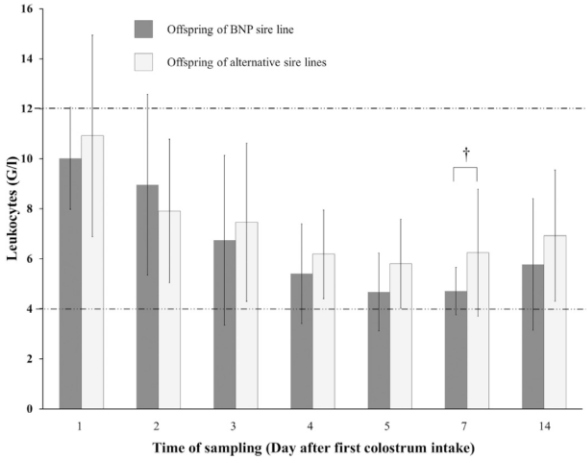

Fig. 4. Mean leukocyte counts for each day of sampling (day after first colostrum intake): Comparison of mean leukocyte counts (standard deviation indicated by vertical bar) between calves without clinical BNP of sire lines not affected by BNP (n = 33) and of the BNP-sire line (n = 9). †Tentatively significant difference (P = 0.06) in leukocyte counts between the two groups. Broken-dotted lines indicate the reference range for leukocyte counts in neonatal calves at two weeks of age.

3.2. Potential factors associated with BNP incidence

Data of the F_2 resource population from a previous study (Krappmann et al., 2011) and the present study were retrospectively evaluated since the onset of BNP-cases at the FBN Dummerstorf: Our new data set now comprised a total of 295 backcross calves born between 2007 and April 2012. This adds further independent 154 backcross calves to the initial data set from Krappmann et al. (2011). The first clinical case of BNP occurred in May 2007, 14 months after the implementation of PregSure® BVD in the experimental cattle herd. Considering that a (booster) vaccination with PregSure® BVD at a particular time during pregnancy might have an effect related to BNP, dates of (booster) vaccinations of all dams, which had given birth to a BNP-calf, were compared. Dams had been (booster) vaccinated with PregSure® BVD between 41 days up to 679 days before giving birth to a BNP-calf. Furthermore, there was no coincident date for a vaccination with PregSure® BVD of the dams, which gave birth to a BNP-calf. For instance, the first case of BNP occurred in May 2007 when some of the dams, which gave birth to a BNP-calf later on, were not yet born. Thus, the time point of booster vaccination itself or relative to parturition did not represent risk factors for the clinical and haematological observations associated with BNP. Due to the set-up of the resource population project design, cows within the F_2 resource population were kept for only two parturitions. From the total of 295 backcross calves born since 2007, 147 calves were born to heifers and 148 calves to cows in second lactation. Our data suggest that there is no difference in incidence of BNP relative to the number of cow parturitions: four BNP-calves originated from first parturitions, six BNP-calves from second parturitions (P = 0.53). Specifically within the highly affected F_2 full-sib family within the affected sire line, F_2 full-sib cows had three clinical BNP-cases from the first parturition and five BNP-cases from the second parturition (P = 0.48).

No medical treatments were differentially applied between BNP-cows and non-BNP-cows.

4. Discussion

In contrast to some previous observations, e.g. (Witt et al., 2011), subclinical cases of BNP did not occur with a high incidence in this study. There was no indication for the hypothesis that clinical BNP would be only the infrequent "tip of the iceberg" and subclinical BNP was frequent in our closely monitored research population. Yet, a genetic predisposition for the development of a subclinical BNP might be assumed, because the single subclinical BNP case that was observed in our data set occurred in one of the 11 offspring from the sire line affected by BNP. No subclinical case of BNP was observed in the 33 offspring of alternative sire lines.

Except for two individuals, the other 9 calves of the BNP affected sire line did not display any clinical signs of BNP, but revealed a decrease in thrombocyte counts between day 7 and day 14. In addition, compared to the 33 calves from the other sire lines there was a significantly decreased thrombocyte count at day 14 and a decreased leukocyte count at day 7, although the respective thrombocyte and leukocyte counts were still in ranges reported for young calves (Mohri et al., 2007). At this time, two weeks after birth, clinical BNP-calves generally start to show signs of the bleeding disorder (Friedrich et al., 2009; Pardon et al., 2010; Buck et al., 2011). Hence, our observations suggest that also those calves of the BNP-affected sire line, which did not develop clinical or subclinical BNP, but showed decreased thrombocyte counts between days 7 and 14, may have undergone an adverse effect on blood cells after colostrum intake. These data indicate that the effect of a PregSure® BVD × genotype interaction is not restricted to a clinical BNP outcome, but extends to non-clinical alterations of blood cell counts. Moreover, additional cases of BNP occurring in the sire line affected by

BNP confirm previous findings of a genetic predisposition for BNP as proposed in a previous study (Krappmann et al., 2011). These findings raise the question as to which factors are responsible for developing either a subclinical or a clinical manifestation of BNP. Possible factors could either be a predisposition on the part of the calves or other factors such as alloantibody titres in the dams or different types of alloantibodies in the colostrum.

Although the incidence of clinical BNP-cases is low considering the amount of PregSure® BVD vaccination doses sold (Kasonta et al., 2012), the high lethality (Friedrich et al., 2009; Pardon et al., 2010) and occasional high incidences on individual farms (Witt et al., 2011) can result in substantial economic losses. In addition, if subclinical BNP-cases frequently emerged within herds, this would further result in economic losses due to a decrease in health status at herd level as discussed by Sauter-Louis et al. (2012). However, our study did not provide evidence that at least in a management system, which avoided feeding of mixed colostrum, a substantial number of subclinical cases should be expected. Employing a management system of feeding mixed colostrum on BNP-affected farms might result in an increased risk of calves for haematological alterations (Witt et al., 2011). Studies on reference ranges for blood parameters in neonatal calves, and especially those applying a very close monitoring, are rare. Since reference ranges in adults differ from those in young calves (Knowles et al., 2000), reports on haematological profiles in young calves served as guideline for the reference ranges applied in this study (Tennant et al., 1974; Egli and Blum, 1998; Knowles et al., 2000; Mohri et al., 2007). A decrease in thrombocytes at days 2–3 after birth appears to be a physiological phenomenon in neonatal calves, since both groups of calves in our experiment revealed this temporal pattern, and similar observations have been published in BNP challenge experiments (Schroter et al., 2011; Bell et al., 2013). Even some individual calves from non-BNP-affected sire lines, which were clinically inconspicuous and showed high thrombocyte counts on day 14, revealed thrombocyte counts at the lower margin or below the reference range on days 2–3 (Fig. 2). Therefore, in order to detect subclinical BNP-cases, a frequent and constant monitoring of haematological parameters in the neonatal period is essential, because otherwise subclinical cases might be missed, or unaffected calves might be falsely classified as subclinical BNP-calves.

Our data indicate that the frequency of vaccinations with PregSure® BVD did not have an effect on the incidence of BNP in our resource population. This result is in line with previous data from Lambton et al. (2012). In contrast, a recent study has revealed that the vaccination regimen and frequency is likely to have an influence on the incidence of BNP (Kasonta et al., 2012). But in our data set, neither across the entire resource population nor specifically within a highly BNP-affected F_2 full-sib family, a significant effect of the number of parturitions on the incidence of clinical BNP-calves was observed, although cows at second parturition received more booster vaccinations in addition to the basic immunisation than dams at first parturition. Similar results on the distribution of BNP cases across parity or age of dam were also described in other studies (Pardon et al., 2010; Den Uijl and Smolenaars, 2012).

All dams included in our study had received at least a basic immunisation consisting of two doses of PregSure® BVD. This vaccination protocol is reported to result in higher alloantibody titters compared to a two-step vaccination programme (Kasonta et al., 2012), which consists of a primary immunisation with an inactivated BVD-vaccine like PregSure® BVD and a booster vaccination with a live-attenuated BVD-vaccine. Thus, regarding BVDV vaccination, all cows in our experiment, except for the dams of the control calves, encountered an identical vaccination protocol with the BVDV vaccine (PregSure® BVD), which has been demonstrated to be the most relevant epidemiological factor for producing BNP-inducing colostrum (Sauter-Louis et al., 2012). In spite of the identical BVDV vaccination protocol across all cows, only dams from a single specific sire line showed calves with clinical alterations associated with BNP and/or haematological effects after ingesting maternal colostrum. This further confirms the previous hypothesis from Krappmann et al. (2011) regarding a genetic predisposition for BNP, because 154 additional calves had been added to the previous data set without any BNP incidence outside the initially affected sire line.

One clinical case occurred towards the end of the study, confirming that BNP was still occurring after a large interval of 679 days since the last booster vaccination with PregSure® BVD.

Regarding BNP pathogenesis, results indicating MHC class I alleles as causal agents for inducing BNP-colostrum (Deutskens et al., 2011; Foucras et al., 2011) suggest that BNP-dams probably lack a respective epitope found on the MDBK cell line used for PregSure® BVD production. Two recent studies, which analysed the transcriptome of dams after booster vaccination with PregSure® BVD and protein composition of the specific vaccine and the cell surface proteome of the MDBK cell line, revealed an enormous (cellular) contamination of the vaccine (Euler et al., 2013; Demasius et al., 2013). The cellular proteins are likely to evoke the production of alloantibodies in dams vaccinated with the specific vaccine. However, the question arises, if MHC class I is the exclusive target antigen. The study conducted by Deutskens et al. (2011) revealed that not only serum-derived alloantibodies from PregSure® BVD vaccinated BNP-dams, but also serum-derived alloantibodies of some of the PregSure® BVD vaccinated non-BNP-dams showed a reactivity with the 44 kDa protein (MHC class I) of the MDBK cell line. Moreover, pooled serum from BNP-positive dams showed reactivity with peripheral blood mononuclear cells (PBMC) of BNP-positive dams from the resource population of the FBN Dummerstorf investigated in this study (Deutskens, 2012). A recent study by Euler et al. (2013) revealed that MHC class I was one of the least abundant proteins in the vaccine and the MDBK cell line, and the study instead presented some other possible protein candidates. If MHC class I were the only candidate against which alloantibodies are directed, this would imply that all calves developing BNP carry a MHC class I allele in common with the MDBK cell line. In several studies, however, a large proportion of calves revealed haematological changes after being fed with colostrum from unrelated BNP-dams (Friedrich et al., 2011; Schroter et al., 2011). Furthermore, feeding same quantities of pooled BNP-colostrum resulted in relatively consistent haematological findings in all challenged calves (Bell et al., 2013). It is unlikely that this high proportion of BNP-susceptible calves all shared an MHC class I allele(s) with the MDBK cell line, especially because the calves originated from different breeds. The MHC class I region is one of the most polymorphic gene regions of the bovine genome, and cattle seem to apply two principal strategies to maintain MHC class I diversity by keeping a high degree of polymorphism within MHC class I genes and by expressing a variable number and combination of those MHC class I genes (Ellis et al., 1999; Birch et al., 2006; Babiuk et al., 2007). Even in a controlled breeding programme with a small effective population size (Hayes et al., 2003) the diversity in the MHC class I region has not been diminished over 20 years as a study revealed lately (Codner et al., 2012). Hence, given this high degree of MHC variability within cattle population, it is highly unlikely that a very substantial proportion of individuals shares the MDBK MHC class I alleles. This suggests that MHC class I should not be considered as an exclusive candidate antigen. A genetic predisposition associated with BNP on the part of the dams has been further reinforced in this study, and comparisons of the genetic makeup of the MDBK cell line, BNP-positive and BNP-negative dams and also potential differential immune responses towards vaccination are likely to give a more detailed insight into the etiopathogenesis of BNP.

5. Conclusions

In conclusion, our data confirm previous findings of a genetic predisposition involved in the aetiology of clinical and potentially subclinical BNP. There is indication that a genetic predisposition of cows vaccinated with the BNP-associated vaccine results in a significant depression of thrombocyte and leukocyte count of the calves after ingesting maternal colostrum, even though this might not manifest itself as clinical or subclinical BNP and might still be within reference ranges considered normal in neonatal calves.

Our study did not reveal further factors contributing to BNP incidence. Prospective studies will have to consider genetic differences between BNP-dams and non-BNP-dams, taking structural MHC class I and other variations in vaccine contaminants as possible candidates into account.

Acknowledgements

We thank our colleagues at the FBN Dummerstorf involved in the generation and care of the F_2 resource population for their continuous support of our work. This study was financially supported by the German Ministry of Food, Agriculture, and Consumer Protection (BMELV) through the Federal Office for Agriculture and Food (BLE), grant number 2810HS27, which had no other involvement in the study.

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5.2 Publication II

Wiebke Demasius, Rosemarie Weikard, Frieder Hadlich, Kerstin Elisabeth Müller, Christa Kühn

Monitoring the immune response to vaccination with an inactivated vaccine associated to bovine neonatal pancytopenia by deep sequencing transcriptome analysis in cattle

Veterinary Research (2013), **44**:93

Demasius *et al. Veterinary Research* 2013, **44**:93
http://www.veterinaryresearch.org/content/44/1/93

VETERINARY RESEARCH

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Monitoring the immune response to vaccination with an inactivated vaccine associated to bovine neonatal pancytopenia by deep sequencing transcriptome analysis in cattle

Wiebke Demasius[1], Rosemarie Weikard[1], Frieder Hadlich[1], Kerstin Elisabeth Müller[2] and Christa Kühn[1*]

Abstract

Bovine neonatal pancytopenia (BNP) is a new fatal, alloimmune/alloantibody mediated disease of new-born calves induced by ingestion of colostrum from cows, which had been vaccinated with a specific vaccine against the Bovine Virus Diarrhoea Virus (BVDV). The hypothesis of pathogenic MHC class I molecules in the vaccine had been put up, but no formal proof of specific causal MHC class I alleles has been provided yet. However, the unique features of the vaccine obviously result in extremely high specific antibody titres in the vaccinated animals, but apparently also in further molecules inducing BNP. Thus, a comprehensive picture of the immune response to the vaccine is essential. Applying the novel approach of next generation RNA sequencing (RNAseq), our study provides a new holistic, comprehensive analysis of the blood transcriptome regulation after vaccination with the specific BVDV vaccine. Our RNAseq approach identified a novel cytokine-like gene in the bovine genome that is highly upregulated after vaccination. This gene has never been described before in any other species and might be specific to ruminant immune response. Furthermore, our data revealed a very coordinated immune response to double-stranded (ds) RNA or a dsRNA analogue after vaccination with the inactivated single-stranded (ss) RNA vaccine. This would suggest either a substantial contamination of the vaccine with dsRNA from host cells after virus culture or a dsRNA analogue applied to the vaccine. The first option would highlight the potential risks associated with virus culture on homologous cells during vaccine production; the latter option would emphasise the potential risks associated with immune stimulating adjuvants used in vaccine production.

Introduction

Vaccination regimes are a powerful strategy to protect our animal populations against microbial diseases (e.g., [1]). However, application of vaccination regimes requires a comprehensive knowledge about all potential vaccination effects. Bovine neonatal pancytopenia (BNP) is a new disease of new-born calves characterised by extreme haemorrhages, thrombocytopenia, leukocytopenia, and cellular depletion of the bone marrow [2]. BNP ends lethally in the vast majority of cases, and no specific treatment is available. Recent studies convincingly revealed that BNP is an alloimmune/alloantibody mediated disease induced by ingestion of colostrum from cows vaccinated with a specific vaccine (PregSure®) against the Bovine Virus Diarrhoea Virus (BVDV) [3-5]. This inactivated vaccine directed against a single stranded (ss)RNA virus is distinguished by an extremely high production of specific BVDV antibodies, which is potentially due to the unique adjuvant included in the vaccination dose. Alloantibodies induced after vaccination with the PregSure® BVDV vaccine bind to MHC class I cell surface proteins of leukocytes and also of the Madin-Darby bovine kidney (MDBK) cell line, which had been used for production of the specific BVDV vaccine [6,7]. Thus, the hypothesis is that contaminating MHC class I antigens from the bovine MDBK cell line in the vaccine act as alloantigens and elicit the production of the pathogenic alloantibodies. However, the exact pathogenesis of BNP is still not fully elucidated. For example,

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experimental vaccinations in several studies showed a much higher proportion of individuals with alloantibodies than reported BNP cases in the population relative to the large number of vaccinated individuals [8]. Furthermore, no formal proof of a specific causal MHC class I allele has been provided yet. The specific nature of the vaccine composition can be elucidated by comprehensive knowledge about quantitative and structural regulation of the blood transcriptome after vaccination with the specific BNP-associated vaccine, which will provide novel insights into the immune response to the vaccine.

Deep sequencing of a transcriptome by next generation RNAseq offers the tool for a precise and truly holistic analysis of the expressed loci within cells and tissues [9]. Recent studies showed that it outperforms previous methods for transcriptome analysis due to its large dynamic range and low technical variance [10]. In addition, RNAseq is not restricted to the known genome annotation but enables identification of novel, previously unknown functionally relevant loci in the genome as recently exemplified by the discovery of a novel human interferon gene [11]. This property to add information to an existing genome annotation is valuable especially in genomes with no high-quality annotation like in many livestock species including cattle.

Recently, a transcriptome analysis of peripheral blood mononuclear cells in calves using deep sequencing reported a major IFNγ/IL22 response to vaccination directed against Mycobacterium bovis [12]. Furthermore, KEGG pathways *Cytokine – cytokine receptor interaction*, *Cell cycle*, *Prion diseases* and *p53 signalling pathway* were significantly modulated. This demonstrates that RNAseq experiments are a useful tool for monitoring the immune response to vaccination.

To our knowledge, we present the first whole blood transcriptome analysis of a livestock species in response to a virus-based vaccine applying deep RNA sequencing. The results provide a comprehensive catalogue of the immune response to the specific vaccine indicating a major reaction to RNA virus infection and an activation of T cell response. In addition, a new cytokine-like gene with strong protein-coding potential was identified for the first time, which was highly upregulated after vaccination, and which has not yet been described in cattle or any other species before.

Materials and methods

Animals

All experimental procedures were carried out according to the German animal care guidelines and were approved and supervised by the relevant authorities of the State Mecklenburg-Vorpommern, Germany (State Office for Agriculture, Food Safety and Fishery Mecklenburg-Western Pommerania (LALLF M-V), 7221.3-2.1-005/11). The study included 12 lactating and non-lactating cows aged three to five years. Except one Holstein cow, all individuals were F_2 cows from a German Holstein × Charolais crossbred population [13]. All cows were kept under the same dairy cow conditions on the experimental farm of the FBN Dummerstorf. All cows had received a basic double vaccination with an inactivated BVDV vaccine (PregSure®, Pfizer, Berlin, Germany) according to the manufacturer's recommendations and at least one booster vaccination 15 months prior to our experiment. Thus, the immune response monitored in our experiment is a recall response to a previously encountered vaccine. Four of the cows had calves that had developed a clinical BNP. For this study, jugular blood was taken immediately prior a further booster vaccination with PregSure® and 14 days later. The time point day 14 was selected due to the documented relevance of alloantibodies for BNP and because antibody production after a PregSure® booster vaccination could be assumed to have reached a plateau at this date [14]. After sampling, 2.5 mL blood was immediately transferred to PAXgene blood RNA tubes (PreAnalytiX, Hombrechtikon, Switzerland). Samples were frozen and stored at −80 °C according to the manufacturer's instructions until further processing.

For RT-PCR confirmation of RNAseq data regarding XLOC_032517, tissue samples and blood samples from four additional F_2-cows from the German Holstein × Charolais crossbred population were analysed. All individuals had not received a vaccination with PregSure®, but had been vaccinated with an alternative inactivated BVDV vaccine. Tissue samples were taken immediately after slaughter and snap frozen in liquid nitrogen and stored at −80 °C until further processing.

Sample preparation

Whole blood RNA was isolated by the PAXgene Blood RNA Kit (PreAnalytiX, Hombrechtikon, Switzerland) according to the manufacturer's instructions and stored at −80 °C. Residual genomic DNA was carefully eliminated by on-column digestion using twice the amount of RNAse-free DNase I solution the manufacturers recommended in the protocols. The samples were monitored for RNA concentration with the NanoDrop 1000 system (Peqlab, Erlangen, Germany). RNA integrity was determined on the Bioanalyzer 2100 (Agilent, Böblingen, Germany). Potential sample contamination with genomic DNA was meticulously checked by PCR with genomic primers according to [15]. Those samples showing traces of contamination were again treated with DNAse I and purified according to the RNAeasy Min Elute Cleanup protocol (Qiagen, Hilden, Germany) to carefully remove all residual DNA. Only RNA samples without detectable DNA contamination were used for further processing in RNAseq and locus-specific RT-PCR experiments.

Demasius et al. Veterinary Research 2013, 44:93
http://www.veterinaryresearch.org/content/44/1/93

Total RNA from tissue samples was extracted as has been described previously [16]. Madin-Darby bovine kidney cells (MDBK) were grown in Eagle's Minimal Essential Medium (EMEM) (Sigma-Aldrich Chemie, Steinheim, Germany) supplemented with 2 mM L-glutamine (Biochrom AG, Berlin, Germany), 1% non-essential amino acids (NEAA) (Biochrom AG, Berlin, Germany) and 10% heat-inactivated fetal calf serum (FCS) (PAN-Biotech GmbH, Aidenbach, Germany). Cells were maintained at 37 °C and 5% CO2. Total RNA was prepared from the MDBK-cells using Trizol (Invitrogen, Darmstadt, Germany). The RNA pellet was resuspended in 50 μL RNase-free water and stored at −80 °C until further processing. Check for DNA contamination and DNase treatment of total RNA from tissues and MDBK cells were performed as described for blood samples.

Library preparation and sequencing

RNAseq libraries were prepared from 1 μg total RNA using the Illumina TruSeq RNA library preparation kit (Illumina, San Diego, USA) with indexed adapter sequences to enable sample multiplexing during cluster generation and sequencing by synthesis. For each individual, two libraries were prepared: from sampling before and 14 days after vaccination resulting in a total of 24 different TruSeq RNA libraries for sequencing. The RNAseq libraries were monitored for insert size with the Bioanalyzer 2100 (Agilent, Böblingen, Germany) and for highly repetitive sequences by cloning a subset of the libraries into a plasmid vector and sequencing of 40 randomly selected clones from each library.

Taking advantage of the index adaptors, individual mixes for each lane of the flow cells were prepared for sequencing by pooling libraries from 12 samples for each mix. Mixes were equally distributed on three flow cells to avoid technical bias of results. Paired-end sequencing with 2 × 61 cycles was performed on an Genome Analyzer GAIIx (Illumina, San Diego, USA) using a PhiX control. The resulting reads were demultiplexed using CASAVA v1.8. All demultiplexed reads of one sample from the different mixes and flow cells were merged into a single fastq file and checked for quality (base quality scores, adaptor contamination, repetitive sequences) using FastQC [17]. The fastq files passing quality threshold served as input for further analyses.

Sequence assembly and locus annotation

Reads were aligned to the bovine reference genome UMD3.1 [18] using Bowtie/TopHat 2.03 [19] options. Tophat enables spliced read alignments. For guided alignment options, we supplied TopHat with the bovine gene model annotation from Igenome ([20], NCBI version, accession date 12/06/2012). The guided alignment option employs the reference annotation in a first alignment step using Bowtie to map reads against a virtual transcriptome generated from the annotation data and subsequently converts the mapped reads to genome mapping. The remaining reads failing to map to the virtual genome will then be further processed for spliced alignment against the genome. This strategy takes advantage of the existing annotation, but keeps also aligned reads mapping to previously unannotated transcription units of the genome.

The resulting BAM file from read alignment was filtered using SAMtools [21] for reads that showed more than two mismatches to the reference genome. Furthermore, for those reads with more than one reported alignment, only the alignment with the lowest query hit index was kept to avoid multiple counting of reads during expression analysis.

The filtered BAM file was submitted to transcript assembly using Cufflinks 2.02 options [19]. Each sample was first analysed individually for transcript assembly. The Igenome gene annotation (see above) was provided to guide the assembly. This enables the output of novel genes and isoforms in addition to the provided reference transcripts. The resulting .gtf-files containing the information on transcript assembly for each sample were merged using Cuffmerge with the Igenome reference annotation and the bovine Ensembl gene annotation release 66 [22]. This final .gtf-file was used for locus and transcript quantification using Cuffdiff 2.02 with the bovine UMD3.1 as reference genome assembly. Transcript and locus assemblies were visualised for inspection of the BAM files and the final annotation files with the Integrative Genomics Viewer [23].

Differential expression analysis

The estimated number of fragments originating from each locus in the final annotation file was obtained using the Cuffdiff option of Cufflinks. The resulting read-group-tracking file was filtered for the estimated fragment count per locus for each individual and each time point. This served as input for tests of differential expression using edgeR [24]. In contrast to Cuffdiff, edgeR provides the option to include systematic effects, e.g., vaccination or animal effect in the model for differential expression by fitting linear models. To test for differential locus expression, a negative binomial distribution of read counts was assumed. Only loci with an expression level exceeding 0.1 counts per million reads (cpm) in each of the 24 samples were included. After calculation of the normalisation factor, the effect of the vaccination on differential gene expression was calculated with the model: counts = individual + treatment. Tests for statistical significance included accounting for multiple testing by calculating the false discovery rate (FDR) according to [25]. Only differences in expression with a significance threshold of $q < 0.05$ were considered statistically significant.

Demasius *et al. Veterinary Research* 2013, **44**:93
http://www.veterinaryresearch.org/content/44/1/93

Pathway analysis

For pathway analysis, only loci were included with a gene annotation and a statistically significant differential expression ($q < 0.05$) prior vs. after vaccination. The initial gene annotation from the transcript assembly process in Cufflinks was supplemented by BLAST search of sequences from unknown loci with the bovine NCBI Refseq sequences (accession date 21/11/2012). The final list of differentially expressed genes was analysed for affected pathways using GOseq [26] and Ingenuity pathway analysis [27]. For GOseq analysis, gene acronyms were translated into Ensembl IDs using the Biomart tools [28]. Due to the poor functional annotation of the bovine genome, we used the human Ensembl annotation for pathway analysis in GOseq. In the GOseq analysis, the length bias characteristic for RNAseq data is accounted for by using the Wallenius distribution to approximate the true null distribution. GOseq was subsequently applied to test KEGG pathways [29] for over- or underrepresentation in the set of differentially expressed genes. The respective *p*-value for over- or underrepresentation is calculated from the null distribution. The significant KEGG pathway maps were inspected for significantly differentially expressed genes.

Ingenuity pathway analysis was applied to identify biological functions, canonical pathways, networks and upstream regulators involved in the response to vaccination with PregSure®. For this purpose, the list of differentially expressed genes was investigated including the respective $\log_{\text{fold change}}$ to indicate the direction and quantity of differential expression. For identification of activation or inhibition of upstream regulators, a threshold for the activation z-score calculated in the IPA analysis of $|\text{z-score}| > 2$ was applied.

RT-PCR confirmation of RNAseq results

The structure and expression of the novel locus XLOC_032517 were confirmed by locus-specific RT-PCR analysis. RNA from blood samples were investigated from all animals prior and after vaccination. In addition to individuals vaccinated with PregSure®, expression of XLOC_032517 was monitored in blood samples from three animals of the Charolais × German Holstein resource population, which had not been vaccinated with the specific BVDV vaccine. Furthermore, also a collection of tissues from a cow of the F_2 resource population not vaccinated with the specific BVDV vaccine and also the MKBK cell line were investigated for XLOC_032517 expression.

One primer pair (TC_85490_F1/ TC_85490_R2, Additional file 1) was designed to amplify all exons of the XLOC_032517 transcript under the following PCR conditions: initial denaturation at 94 °C, 35 cycles of amplification at 62 °C annealing and 45 s for extension. The obtained PCR fragments were excised from the agarose gel, purified using the Nucleospin Extract II kit (Macherey and Nagel, Düren, Germany) and sequenced on a capillary sequencer (ABI310, Life Technologies, Darmstadt, Germany; MEGABACE 1000, GE Healthcare Europe, Freiburg, Germany). The obtained sequences were aligned to the sequence of XLOC_032517 as obtained by RNAseq using Bioedit [30].

Differential quantitative expression of XLOC_032517 was confirmed by quantitative real time RT-PCR using primers TC_85490_F3/ TC_85490_R3 essentially as described by [15] except for cDNA synthesis performed with oligo dT and random hexamer primers. Two reference genes (EIF3K, MTG1; Additional file 1) for normalisation were obtained from the RNAseq data by selecting loci with low normalised variance of cpm values across all samples and with expression levels in range with XLOC_032517. Statistical analysis of quantitative expression between time points was carried out with the SAS MIXED procedure analogous to the analysis of the deep sequencing data including the time point relative to vaccination as fixed and the animal as random effect in the model. Statistical analysis of differential quantitative expression between the 12 animals vaccinated with PregSure® (sample from day 0 of our experiment) and three cows that had not encountered PregSure® vaccination was calculated using SAS GLM.

Results

Read mapping

After demultiplexing and merging of reads, 33.1 – 45.7 million paired-end fragments (two reads per fragment) were obtained per sample (Table 1) amounting to a total of 953 259 252 million fragments for the entire experiment. After alignment and filtering, 57.8 – 79.7 million reads per sample (a total of 1 665 651 857 reads for the entire experiment) were submitted to further analysis. This dataset represented 85.1-89.0% of all reads indicating a high proportion of mapped reads and a high uniformity between samples for both, the number of mapped reads and also the percentage of mapped reads relative to all reads.

Transcript annotation and quantification

Analysis of expression revealed that 28 690 loci exceeded an expression level threshold of 0.1 cpm (counts per million reads) in at least one sample, and 18 181 loci exceeded this threshold in all samples. 4596 (25.3%) of those loci with expression level > 0.1 cpm in all samples had no previous locus annotation in the Igenome NCBI annotation file.

The five loci with the highest cpm across all samples and time points were serine dehydratase (SDS), ribosomal S18 RNA (RN18S1), CD74 molecule, major histocompatibility complex (class II invariant chain, CD74), bovine major

Demasius *et al. Veterinary Research* 2013, **44**:93
http://www.veterinaryresearch.org/content/44/1/93

Table 1 Overview of the alignments of reads per sample.

Individual	Sampling	Breed	Total number of fragments	Uniquely counted, mapped reads with < 3 mismatches	% of reads uniquely mapping, < 3 mismatches	% of reads mapping to globin clusters
cow 1	before vacc	Cha × GH F_2	44 776 690	77 918 387	87.01	1.04
cow 1	after vacc	Cha × GH F_2	44 627 365	78 360 907	87.79	2.01
cow 2	before vacc	Cha × GH F_2	43 990 812	77 993 272	88.65	0.07
cow 2	after vacc	Cha × GH F_2	44 636 963	77 742 662	87.08	0.23
cow 3	before vacc	Cha × GH F_2	40 798 596	71 377 966	87.48	0.10
cow 3	after vacc	Cha × GH F_2	37 816 325	65 542 541	86.66	0.11
cow 4	before vacc	Cha × GH F_2	35 447 535	61 851 369	87.24	0.18
cow 4	after vacc	Cha × GH F_2	39 030 040	69 141 268	88.57	0.16
cow 5	before vacc	GH	34 889 160	61 093 146	87.55	0.40
cow 5	after vacc	GH	33 543 345	59 711 466	89.01	1.33
cow 6	before vacc	Cha × GH F_2	42 232 327	73 237 641	86.71	0.10
cow 6	after vacc	Cha × GH F_2	41 569 088	71 882 069	86.46	0.26
cow 7	before vacc	Cha × GH F_2	40 572 312	70 148 894	86.45	3.06
cow 7	after vacc	Cha × GH F_2	44 155 006	77 074 387	87.28	0.92
cow 8	before vacc	Cha × GH F_2	35 704 593	61 371 734	85.94	0.51
cow 8	after vacc	Cha × GH F_2	37 375 624	65 532 890	87.67	0.86
cow 9	before vacc	Cha × GH F_2	33 125 544	57 825 014	87.28	0.37
cow 9	after vacc	Cha × GH F_2	38 731 113	67 755 124	87.47	0.05
cow 10	before vacc	Cha × GH F_2	36 740 555	62 541 431	85.11	1.10
cow 10	after vacc	Cha × GH F_2	38 024 182	67 142 881	88.29	7.14
cow 11	before vacc	Cha × GH F_2	36 549 423	63 893 567	87.41	2.17
cow 11	after vacc	Cha × GH F_2	39 502 827	69 593 787	88.09	0.56
cow 12	before vacc	Cha × GH F_2	45 701 139	79 680 037	87.18	0.16
cow 12	after vacc	Cha × GH F_2	43 718 688	77 239 417	88.34	1.18
Total			953 259 252	1 665 651 857		

Cha, Charolais; *GH*, German Holstein; vacc, vaccination.

histocompatibility complex class I (BoLA) and β2 microglobulin (B2M), the latter three representing major determinants of the histocompatibility complex in cattle. However, it has to be considered that the cpm value does not take transcript length into account, which results in an up-bias of longer transcript compared to short transcripts.

Human transcriptome analysis by RNAseq is impaired by a high proportion of reads mapping to the human globin cluster [31]. Thus, a specific depletion of the RNA samples for globin sequences to enhance the complexity of the human RNAseq libraries is required. Initial Sanger sequencing of individual cloned fragments from our bovine blood RNAseq libraries identified only single clones containing sequences with homology to the bovine globin clusters (data not shown). After sequencing the RNAseq libraries on the Illumina Genome Analyzer GAIIx, we looked for reads mapping to the bovine α haemoglobin cluster on BTA25 (region 190 kb – 219 kb) or to the β haemoglobin cluster on BTA15 (region 48.990 Mb – 49.080 Mb). The percentage of reads mapping to these chromosomal areas [0.07 to 7% of all reads (Table 1)] is substantially lower compared to human studies (60%, [32]) and indicates that the complexity of the bovine RNAseq libraries is not compromised by a huge proportion of reads generated from the bovine globin clusters.

Differential expression due to vaccination

Analysis of the vaccination effect on blood RNA expression yielded 2901 differentially expressed loci at a significance threshold of $q < 0.05$ (Figure 1, Additional file 2). 2578 of the significantly differentially expressed loci had an official annotation or could be identified by BLAST search against the NCBI Refseq data set. For 323 differentially expressed loci at a $q < 0.05$ threshold (11.1% of all differentially expressed loci), there was no bovine gene annotation available. Upregulation of expression after vaccination was observed for 1879 loci, whereas 1022 loci showed a higher expression prior to vaccination.

Our RNAseq analyses highlighted a strong upregulation of the previously unknown locus XLOC_032517 after

Demasius *et al. Veterinary Research* 2013, **44**:93
http://www.veterinaryresearch.org/content/44/1/93

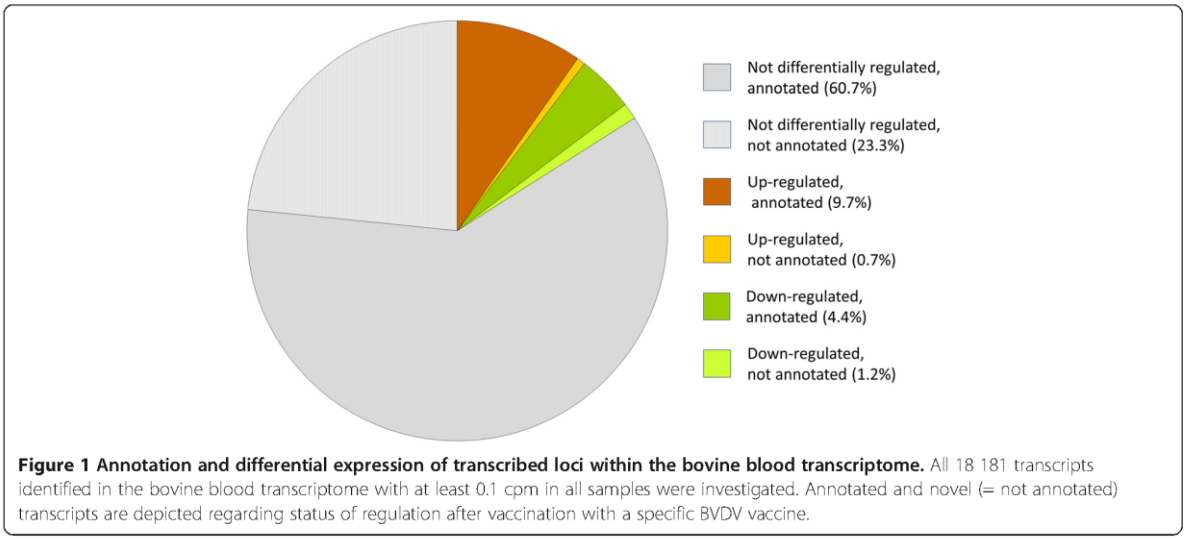

Figure 1 Annotation and differential expression of transcribed loci within the bovine blood transcriptome. All 18 181 transcripts identified in the bovine blood transcriptome with at least 0.1 cpm in all samples were investigated. Annotated and novel (= not annotated) transcripts are depicted regarding status of regulation after vaccination with a specific BVDV vaccine.

vaccination in all samples (Figure 2A). In fact, XLOC_032517 is on top of the list (Additional file 2) of differentially expressed loci regarding statistical significance and second regarding fold change. In spite of the very strong regulation of XLOC_032517 expression after vaccination, there is no functional annotation of this locus neither in bovine nor in any other eukaryote genome. Locus specific RT-PCR analysis and subsequent sequencing of the obtained product approved the sequence of the transcript. Quantitative real-time PCR analysis confirmed a highly significant ($p < 0.0001$) 3.51 fold upregulation of XLOC_032517 expression after vaccination compared to the time point prior vaccination (Figure 2B). XLOC_032517 transcripts were also detected in blood samples of cows not vaccinated with the PregSure® vaccine (Figure 3). Three cows, which had never encountered PregSure® vaccination showed a significantly lower XLOC_032517 expression ($p = 0.008$) compared to the 12 cows, which had received at least three PregSure® vaccinations. Expression analyses of bovine tissues showed a strong XLOC_032517 expression in kidney, liver, lung, spleen and lymph node, a weak to moderate expression in thyroid gland, skeletal muscle, adrenal gland, brain, subcutaneous fat and mammary gland. No expression could be detected in the pituitary gland and in the MDBK cell line used for vaccine production.

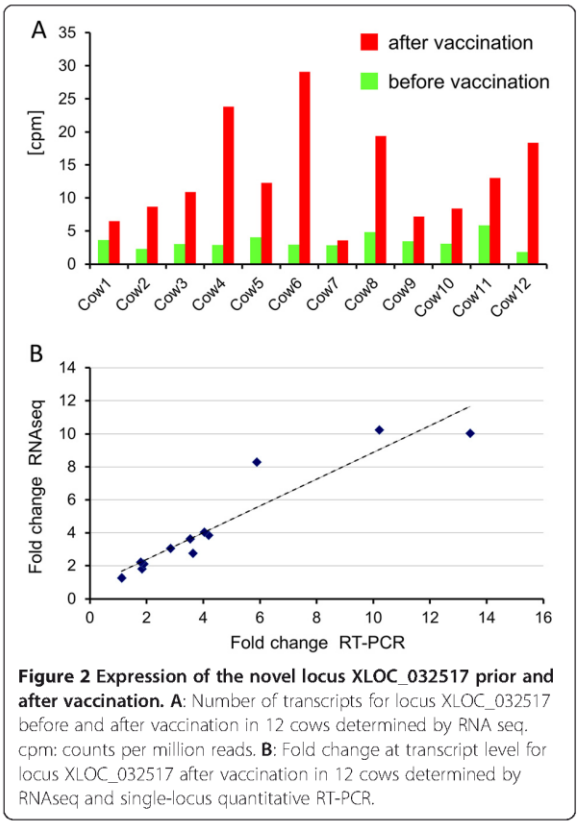

Figure 2 Expression of the novel locus XLOC_032517 prior and after vaccination. A: Number of transcripts for locus XLOC_032517 before and after vaccination in 12 cows determined by RNA seq. cpm: counts per million reads. **B**: Fold change at transcript level for locus XLOC_032517 after vaccination in 12 cows determined by RNAseq and single-locus quantitative RT-PCR.

The bovine locus XLOC_032517 showed a transcript length of 765 bp (Genbank Accession Nr. KF051797) and comprises 4 exons on chromosome 7 (Figure 4) in the region of 23.69 Mb between CSF2 and LOC789264. Analysis of a potential translation of the transcript sequence into amino acid sequence yielded an open reading frame from nt 48 to 542. Thus, the predicted bovine protein encoded by XLOC_032517 has 165 amino acids and a molecular weight of 18558.56 Daltons.

Due to lack of functional annotation for XLOC_032517 in public databases, bioinformatic analyses searched for similarity to sequences in other species to obtain information on the potential functional relevance

Demasius *et al. Veterinary Research* 2013, **44**:93
http://www.veterinaryresearch.org/content/44/1/93

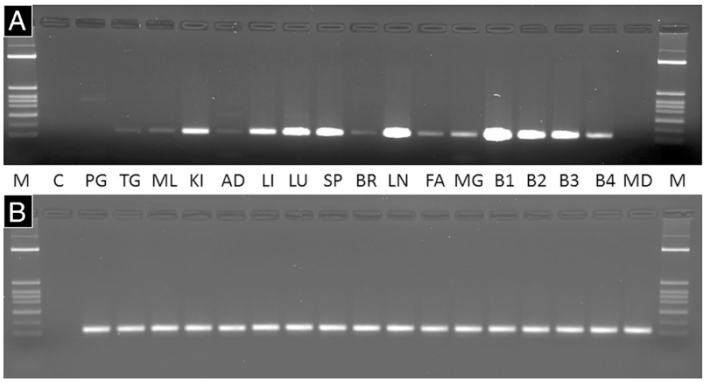

Figure 3 Expression of XLOC_032517 in blood and other bovine tissues. Detection of RT-PCR products from the XLOC_032517 locus **(A)** and the reference gene EIF3K **(B)** in cDNA from whole blood of cows, in bovine tissue samples and the MDBK cell line. M: Molecular weight marker, C: negative control without template, PG: pituitary gland, TG: thyroid gland, ML: skeletal muscle, KI: kidney, AD: adrenal gland, LI: liver, LU: lung, SP: spleen, BR: brain, LN: lymph node, FA: fat, MG: mammary gland, B1: blood sample from cow vaccinated with BVDV vaccine PregSure®, B2-B4: blood sample from cows vaccinated with an alternative BVDV vaccine, MD: MDBK cell line.

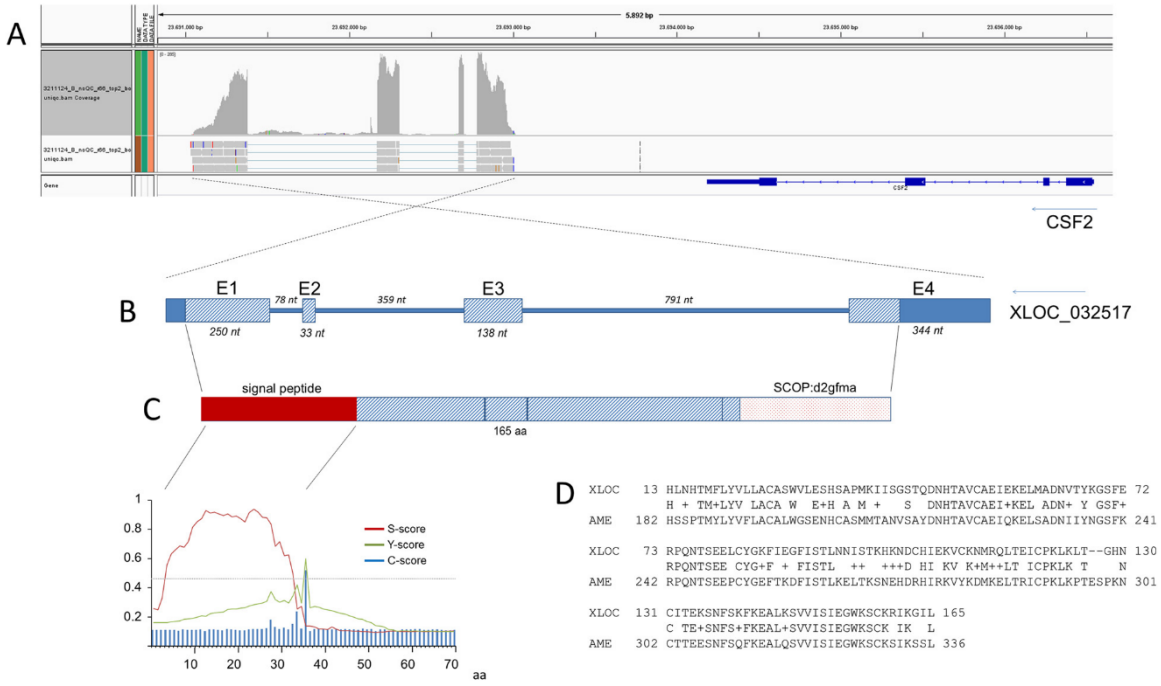

Figure 4 Structural features of the novel bovine locus XLOC_032517. A: Assignment of reads in the genomic region BTA7:23 691 000 Mb – 23 697 000 Mb visualised by Integrative Genome viewer. Annotation and orientation of the bovine CSF2 gene is indicated according to the NCBI Btau 6.1 assembly [33]. **B**: Genomic structure of XLOC_032517. Boxes indicate exons (E1-E4). Solid blue colour indicates the predicted 5'and 3'UTR, shaded pattern indicates an open reading frame with the predicted coding sequence. **C**: Structure of the predicted XLOC_032517 protein with signal peptide (red colour) and d2gfma motif (red dotted pattern). Significant evidence of the signal peptide motif is obtained by calculation of S-, Y-, and C-scores with SignalP [34]. **D**: Homology of the predicted XLOC_032517 amino acid sequence (XLOC) with the predicted great panda LOC100473502 amino acid sequence (AME) as determined by BLASTP [33].

Demasius *et al. Veterinary Research* 2013, **44**:93
http://www.veterinaryresearch.org/content/44/1/93

of the locus. BLAST search at NCBI [33] with the XLOC_032517 sequence detected similar genomic sequences in a conserved exon-intron structure (essentially exons 1, 2, and 4) in the pig, dog, human and great panda genomes (Additional file 3). The sequence mapped *in silico* in an analogous chromosome interval flanked by the same neighbouring genes CSF2 and P4HA2. However, in those species there was also no functional annotation indicated for the respective chromosomal regions. Further genomic sequences with partial similarity to XLOC_032517 were found for sheep, lama, cat and horse (either from NCBI Refseq genomes or Whole Genome shotgun contigs deposited at NCBI). Searching the Ensembl data base [35] for cDNAs similar to XLOC_032517 identified no strong similarity to any known cDNAs in mammals. The CLTA-004 gene from human or great panda displayed only a weak similarity (< 70% across >130 bp) in this database. Further similarity search in the NCBI sequence repository brought forward five functionally non-annotated caprine sequences established by transcriptome shotgun assembly from blood (e.g., Accession Nr. JO333249.2). The identified caprine sequences showed a high similarity to XLOC_032517 (96% identity across 713 bp). BLASTP search for similar protein sequences revealed similarity only between the predicted amino acid sequence of the bovine XLOC_032517 and a hypothetical protein XP_002912997.1 of the LOC100473502 locus in the great panda, which had been predicted by bioinformatic analyses.

Missing functional annotation for sequences homologous to XLOC_032517 required further screening of the XLOC_032517 transcript for potential functionally relevant features. SignalP analysis [36] of the amino acid sequence of the predicted protein revealed a significant indication of a signal peptide cleavage site after amino acid (aa) 35 and a signal peptide region comprising aa 1 to 35 (see Figure 4). Furthermore, SMART analysis [37] indicated the additional features IL14/IL13 (aa 14 – 123) and CSF2 (aa 36 – 158). Although the CSF2 feature was below a significant threshold, the features IL14/IL13 and CSF2 would suggest a cytokine function of XLOC_032517, which fitted the prediction of a signal peptide for XLOC_032517 and also the feature SCOP: d2gmfa indicating a 4-helical cytokine [38,39]. Similarity to CSF2 is also supported by the genomic organisation of XLOC_032517, which is located adjacent to the CSF2 – IL3 cluster of colony stimulating factors on bovine chromosome 7 (BTA7). XLOC_032517 shares a similar four-exon structure with CSF2 and, like CSF2 and IL3, is placed in telomere – centromere orientation on the chromosome.

Identification of differentially expressed annotated genes

Among the list of annotated differentially expressed loci in our experiment, many groups of genes with relevance in the immune response were highlighted. Specifically, interleukin signalling was affected indicated by differential expression of several interleukin receptor genes (IL18R1, IL21R, IL2RA, IL7R, and IL9R). Interleukin 1 (IL1) response is promoted after vaccination due to the upregulation of the IL1 receptor ligand IL1RL1 and the downregulation of IL1RN (receptor antagonist) and of IL1R2 (non-signalling receptor, $q = 0.058$). Furthermore, five members of the group of suppressors of cytokine signalling are upregulated after vaccination (SOCS2, SOCS4, SOCS5, SOCS6, SOCS7), and five ABC transporters (ABCB8, ABCB9, ABCB10, ABCD3, ABCE1) involved in the transport of molecules across extra- and intra-cellular membranes and particularly important in antigen presentation (ABCB8, ABCB9, [40]) were all upregulated. Upregulation was also observed for four genes encoding caspases (CASP3, CASP7, CASP8, CASP8AP2). Several chemokine receptors (CCR2, CCR4, CCR5, and CCR7) showed significantly differential expression as did inhibitors of nuclear factor of kappa light polypeptide gene enhancer in B cells (NFKBID, NFKBIL1, NFKBIZ). The three STAG genes (STAG1, STAG2, and STAG3), members of the cohesion complex [41], are all co-ordinately upregulated. In addition, structural maintenance of chromosome (SMC) condensins SMC2, SMC3, SMC4, SMC5, SMC6 and NCAPG, a non-structural maintenance of chromosomes condensin, are all higher expressed after vaccination compared to prior vaccination.

Pathway analysis

Bioinformatic pathway analyses of the list of significantly differentially expressed genes (Additional file 2) after vaccination revealed a large number of significantly enriched pathways, biological functions and upstream regulators. Most of these pathways had a close association to immune response. Our data indicated a coordinated immune response to viral dsRNA sequences as well as an upregulation of T cell response and a response to an alloantigen.

KEGG pathways affected by vaccination

GOseq pathway analysis of differential expression revealed 29 significantly over-represented KEGG pathways ($p < 0.05$, Additional file 4). The *Ribosome* pathway stood out as the most significantly affected pathway. Furthermore, the list of the 10 most significantly enriched KEGG pathways also comprised *Allograft rejection*, *Graft-versus-host disease*, *Cytokine-cytokine receptor interaction*, *Natural killer cell mediated cytotoxicity*, and *RIG-I-like receptor signalling* indicating a major immune response to an alloantigen and a response to an RNA virus. Other significantly enriched KEGG pathways with an impact on immune response were the *MAPK signalling* pathway, the *T cell receptor signalling, the Toll-like receptor signalling, the Fc epsilon*

Demasius et al. Veterinary Research 2013, 44:93
http://www.veterinaryresearch.org/content/44/1/93

RI signalling and *the antigen processing and presentation* pathway.

The KEGG pathway analysis confirmed that after vaccination the individuals responded to a viral RNA antigen due to overrepresentation of differentially expressed genes from the RIG-I-like receptor signalling pathway (Additional file 5) and upregulation of DDX58 encoding RIG I, a sensor of dsRNA [42]. Furthermore, the KEGG pathway downstream to the *RIG-I-like receptor signalling, MAPK signalling*, was confirmed as significantly affected by the vaccination in our transcriptome analysis. For the *MAPK signalling* pathway, differentially expressed genes of almost all parts of the pathway were observed.

Biological functions affected by vaccination

IPA analysis indicated that main biological functions affected by vaccination (Additional file 6) can be separated into four main groups: biological functions indicating an activation of gene expression, biological functions related to blood cell development and differentiation, biological functions involved in cell death, cell cycle and survival, and the biological function of ubiquination. Specifically, the functions *differentiation of blood cells*, *development of blood cells* and *development of leukocytes* or *quantity of T lymphocytes* (Additional file 6) showed a highly significant overrepresentation of genes. Other categories of biological functions affected by vaccination comprise *infectious disease, cell mediated immune response, humoral immune response, immunological disease, cellular assembly and organisation, cellular development, cellular function and maintenance, haematological system development and function, lymphoid tissue structure and development, protein synthesis* and *posttranslational modification*.

Canonical pathways affected by vaccination

A total of 178 different canonical pathways were found significantly overrepresented as a response to vaccination (Additional file 7) in the IPA analysis. The list included many pathways known to be crucial during immune response. The canonical pathways highlight two key mechanisms in response to vaccination: response to dsRNA or a dsRNA analogue and upregulation of T cell receptor signalling.

In addition to the RIG I signalling pathway identified in KEGG, IPA showed a significant enrichment of differentially expressed loci for main mechanisms of protection against RNA viral infection: *EIF2 signalling* and TLR3 signalling within the *TLR signalling pathway*. Further pathways related to virus infection that are significantly enriched with differentially expressed loci are *NFkB activation by viruses* and *virus entry via endocytic pathways*.

The IPA analysis highlighted the *EIF2 signalling* (Additional file 8) as the most significantly enriched pathway seen as an effect of vaccination. In our data set, the EIF2AK2 gene encoding PKR and EIF2A encoding phosphorylate translation initiation factor EIF2 α are significantly upregulated after vaccination. Also several other kinases and translation initiation factors in the *EIF2 signalling* pathway showed a higher expression level after vaccination compared to the status prior vaccination.

Within the canonical pathway *Toll-like receptor signalling*, specifically the string starting with TLR3, is significantly overrepresented in the list of differentially expressed genes after vaccination (Additional file 9). The TLR3 receptor is a specific receptor for dsRNA [43]. The respective gene is upregulated after vaccination. Furthermore, several genes downstream in the *TLR signalling* pathway that lead to transcription of proinflammatory cytokines are also co-ordinately upregulated (IRAK4, TAB2, MAP3K7 encoding TAK1, MAPK8 encoding JNK1 and FOS encoding c-fos).

Besides response to dsRNA or a dsRNA analogue, the upregulation of *T cell receptor signaling* is a second major regulatory response to vaccination identified in our data set by Goseq analysis of the KEGG database (see above) as well as by IPA analysis of canonical pathways (Additional file 10). The CD3G gene encoding a member of the T cell receptor cluster as well as ZAP70 encoding a protein kinase activated by TCR clustering are both upregulated. In addition, all three major downstream signaling pathways of ZAP70 (NF-AT, Ras and also PKC) contain several significantly upregulated molecules. Examples are PPP3CA and PPP3CB encoding peptides belonging to the PP2B complex and the NFAT5 gene. Support for the hypothesis of a ZAP70 downstream regulation is also supported by the canonical pathways *role of NFAT in regulation of immune response* and *PKCʘ signaling in T lymphoctes* being significantly enriched with differentially expressed genes. Upregulation of T cell receptor signaling is also confirmed by the pathway *CD28 signaling in T helper cells* being significantly enriched with differentially expressed genes. Most of those genes are upregulated, only MHC class II DOB is downregulated.

In contrast to T cell activation, the IPA analysis indicated a downregulation of B cell activation 14 days after vaccination in our data: Most of the genes in the significantly affected canonical pathway *B cell development* are downregulated (Additional file 11), and key molecules at the start points of B cell receptor signal transduction are also downregulated. CD79A and CD79B act as signal transducers of the B cell receptor in B cells, and both showed a higher expression prior to vaccination compared to 14 days after vaccination. Not only the initial signal transducers CD79A/B, but also a co-stimulator for B cell receptor signalling, CD19, is downregulated.

A further signalling receptor required for B cell activation, CD40 showed a significantly lower expression after

Demasius *et al. Veterinary Research* 2013, **44**:93
http://www.veterinaryresearch.org/content/44/1/93

vaccination. Cell surface molecules of the entire process of B cell development from stem cell to plasma cell are all downregulated with the exception of IL7-R and CD80/CD86. However, the latter molecules also appear on the surface of non-B cell antigen presenting cells and are not specific to the B cell lineage.

The canonical pathway *NFκB signalling* is significantly enriched for differentially expressed genes after vaccination. Specifically genes in the signalling cascade of NFκB for B cell maturation are co-ordinately downregulated (Additional file 12) adding further evidence towards suppression of B cell maturation.

Major upstream regulators

Pathway analysis identified major upstream regulators based on expected causal effects between upstream regulators and target genes and predicts activation or inhibition of those regulators. Highly significantly affected upstream regulators in our data set were v-myc myelocytomatosis viral related oncogene (neuroblastoma derived, MYCN), the T cell receptor complex TCR, the CD40 ligand (CD40LG), CD28, E2F transcription factor 1 (E2F1), interleukin 2 (IL2), transforming growth factor beta 1 (TGFB1), CD3, and microRNAs miR-30c-5p, miR-155-5p and miR-124-3p (Table 2, Additional file 13).

Discussion

Our study investigated the recall response to vaccination with the vaccine associated with BNP. The potential relevance of a recall response to this vaccine for BNP is underlined by data indicating that an increased frequency of vaccination in an individual is associated with increased alloantibody titres [8].

Identification of a novel locus significantly related to immune response

Applying the holistic deep sequencing approach to the entire bovine blood transcriptome, we identified 4596 previously unknown transcribed loci in the bovine genome. This confirms the power of the RNAseq approach to reveal novel functional elements in immune response. A prime example for the potential relevance of these new functional elements is the gene XLOC_032517, a previously unknown locus with the most significant difference of all investigated loci regarding expression related to vaccination with a specific BVDV vaccine. The new gene could be detected in bovine blood samples of divergent origin and also in a variety of tissues except pituitary gland. The level of expression varied between tissues with those tissues showing the strongest expression, which have a particular relevance during immune response to invading pathogens (e.g. lymph node, spleen, and lung).

There is no annotation of the locus in any other species, and also no transcripts with homology to XLOC_032517 were identified in any database except for goat. This might indicate that the XLOC_032517 is specific to ruminant immune response, although a predicted protein from *Ailuropoda melanoleuca* (XP_002912951.1) has a partial similarity to the predicted XLOC_032517 protein. The partially conserved exon-intron-structure of homologous sequences in the human and other genomes might point forward to an evolutionary process for XLOC_032517 to become transcriptionally active or silenced, respectively.

Due to lack of any previous data on XLOC_32517, its functional role was completely unknown. Bioinformatic search for functionally relevant structural features of locus XLOC_032517 added further pieces of evidence that the new gene is involved in immune response due to its similarity with structural features identified in CSF2 and IL14/IL13 and its predicted affiliation to the 4-helical cytokine superfamily. Studies from human indicate that CSF2 is expressed in human leukocytes especially upon activation [44]. Interestingly, only for CSF1, expression could be detected in our data set, whereas no expression of the other genes encoding colony stimulating factors (CSF2, CSF3 or IL3) was observed. This pattern is in agreement with the data set from O'Loughlin et al. [45], who performed an RNAseq experiment on calf leukocytes before and after weaning. It has to be evaluated, if XLOC_032517 is a bovine functional surrogate to CSF2. CSF2 is not expressed in bovine blood cells in our data set, although there is a significant and coordinated upregulation after vaccination of several genes encoding proteins of a specific transcription factor complex (calcineurinA-calcineurinB-Runx1: PPP3CA, PPP3CB, RUNX1) relevant for up-regulation of CSF2 [46]. In summary, the *in silico* analyses advocate that the predicted protein encoded by XLOC_032517 might be a new member of the colony stimulating factor family in cattle. Further studies will have to confirm the predicted protein coding potential of XLOC_032517 and the expression pattern in different specific leukocyte populations.

Immune response to dsRNA or a dsRNA analogue after vaccination

The pathway analysis of the expression data from whole blood transcriptomes highlighted a very specific immune response to viral dsRNA or a dsRNA analogue after vaccination (Figure 5). KEGG as well as IPA pathway analyses confirmed that the individuals responded to virus RNA demonstrated by overrepresentation of differentially expressed genes from key pathways of innate recognition of viruses: the *RIG-I-like receptor signalling* pathway, the *TLR/TLR3 signalling* pathway, and the *EIF2 signalling via PKR* [47].

The *EIF2 signalling* pathway is the most significantly vaccination-affected canonical pathway according

Demasius *et al. Veterinary Research* 2013, **44**:93
http://www.veterinaryresearch.org/content/44/1/93

Table 2 List of the 20 most significant upstream regulators identified as involved in gene regulation after vaccination as determined by IPA analysis.

Upstream regulator	Molecule type	Predicted activation state[1]	*p*-value of overlap	Number of target molecules in dataset
miR-30c-5p (and other miRNAs w/seed GUAAACA)	mature microRNA	Inhibited	1.75E-07	29
MYCN	transcription regulator	Inhibited	4.08E-07	52
TCR	complex		1.02E-06	57
miR-155-5p (miRNAs w/seed UAAUGCU)	mature microRNA	Inhibited	1.97E-06	34
CD40LG	cytokine	Activated	2.06E-06	67
CD28	transmembrane receptor	Inhibited	6.13E-06	62
mir-210	microRNA		1.58E-05	16
E2F1	transcription regulator	Activated	8.88E-05	52
miR-124-3p (and other miRNAs w/seed AAGGCAC)	mature microRNA	Inhibited	1.23E-04	38
HOXA9	transcription regulator		1.33E-04	34
IL2	cytokine	Activated	1.77E-04	58
CD3	complex	Inhibited	4.26E-04	98
miR-291a-3p (and other miRNAs w/seed AAGUGCU)	mature microRNA	Inhibited	4.55E-04	18
SMARCC1	transcription regulator		7.17E-04	4
YWHAQ	other		7.37E-04	5
SHOX	transcription regulator		7.37E-04	5
TGFB1	growth factor	Activated	7.99E-04	187
FSH	complex		1.64E-03	60
TMBIM6	other		1.68E-03	6
miR-17-5p (and other miRNAs w/seed AAAGUGC)	mature microRNA	Inhibited	1.94E-03	10

[1] For identification of activation or inhibition of upstream regulators, a threshold for the activation z-score calculated in the IPA analysis of |z-score| > 2 was applied.

to IPA analysis. *EIF2 signalling* is affected by dsRNA-viral infection via PKR. The endpoint of the *EIF2 signalling* pathway is the regulation of translation. The EIF2AK2 gene encoding PKR and EIF2A encoding EIF2 α are both upregulated after vaccination. After viral contact the PKR is activated by autophosphorylation after binding to dsRNA [47]. The activated form of the PKR can phosphorylate translation initiation factor EIF2 α, which in turn inhibits protein synthesis via suppression of protein synthesis complex formation. Downregulation of protein translation is a method of protecting the individual against virus replication by preventing viral structural protein synthesis [48]. A downregulation of translation is also confirmed by analysis of biological functions affected by vaccination (Additional file 6). Phosphorylation of EIF2A via PRK has also been shown to mediate apoptosis by increasing caspase 3 [49], which is in agreement with the CASP3 upregulation after vaccination in our data set. Further support for the hypothesis of a downregulated protein synthesis after vaccination is obtained from *Ribosome* being by far the most significantly affected KEGG pathway after vaccination (Additional file 4). Specific analysis of the respective genes in that pathway indicated that the vast majority of ribosomal protein encoding genes, which showed a significantly differential expression after vaccination, was all concordantly downregulated (e.g., 45 of the total of 46 differentially expressed RPL and RPS genes, Additional file 2).

The second mechanism significantly affected by vaccination, which protects the individual specifically against RNA viral infection, is the RIG I signalling pathway (Figure 5) [42,50]. The gene DDX58 encoding RIG I is upregulated ($p = 0.058$). RIG I acts as a cytoplasmic sensor of viral nucleic acids [42] and induces pro-inflammatory cytokine and interferon I expression. Posttranslational polyubiquination of RIG I is pivotal for activation of RIG I. We found increased ubiquitination of proteins in the list of significantly affected biological functions (see Additional file 6) after vaccination. Activated ubiquitination might thus be relevant in our experiment for RNA sensor signalling in addition to its known role during proteasomal degradation, e.g. of viral peptides for MHC class I presentation [51].

The third mechanism of protection against viral dsRNA (Figure 5) identified in response to vaccination in our dataset is the activation of the TLR3 signalling pathway known to upregulate the synthesis of proinflammatory cytokines after TLR3 activation by dsRNA [43,47].

Demasius *et al. Veterinary Research* 2013, **44**:93
http://www.veterinaryresearch.org/content/44/1/93

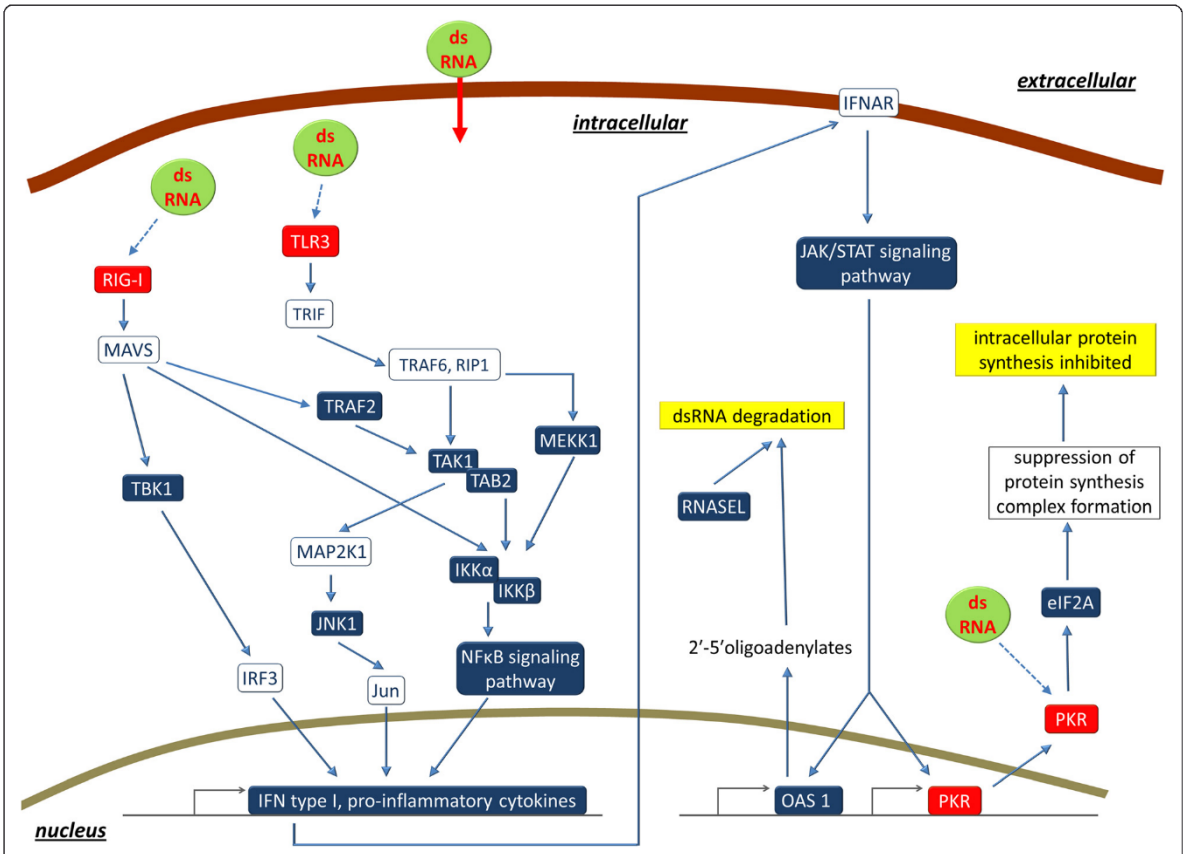

Figure 5 Model for the coordinated response to dsRNA after vaccination with the inactivated vaccine PregSure® against ssRNA BVD virus. Coloured boxes indicate differentially expressed genes or significantly affected pathways when comparing gene expression before and after vaccination. Red boxes indicate intracellular sensors of dsRNA; blue boxes indicate other genes or pathways. Red arrow indicates transport of dsRNA into the cell, the blue dotted lines indicate activation of dsRNA sensors by dsRNA, and the blue solid lines indicate other regulatory or activating effects.

The cytokine activation might relate to a further machinery of protection against flaviviral invasion that is upregulated after vaccination: RNA degradation (Figure 5). The OAS1 gene is upregulated in our data set. OAS1 is induced by α interferons and activates RNASE L by synthesising 2',5'-oligoadenylates (2-5As) [48]. RNASE L degrades viral RNA and inhibits viral replication. This mechanism can be induced by interferon type I signalling: a respective upregulation of IFNA16, member of the antiviral IFNα family, was detected in our data set. Alternatively, OAS1 is able to act as sensor for dsRNA directly. Interestingly, those two Interferon-type I-induced antiviral pathways that also act by direct recognition of dsRNA (OAS1, PKR, [48]) are significantly affected by BVDV vaccination in our data set. In contrast, those other interferon-type I-induced antiviral pathways (MX1, ADAR) not affected by vaccination, lack dsRNA sensor activity and are exclusively dependent on IFN alpha signalling [48].

Obviously the upregulation of the protection against dsRNA is no short term effect, because significantly differential expression could be detected 14 days after application of the vaccine suggesting a long lasting release of dsRNA or dsRNA analogue molecules from the injection site. The differential expression of the dsRNA sensors PKR, TLR3, and DDX58 (Figure 5) and the affected downstream pathways in response to vaccination implicate that the cells monitored in the blood must have had contact with dsRNA. One explanation might be the roaming of cells that incorporated viral dsRNA at the injection site. However, PregSure® is an inactivated vaccine against the BVD virus, an ssRNA pestivirus belonging to the Flaviviridae [52]. Thus, the vaccine should only contain ssRNA. This raises the question as to the origin of the coordinated viral-dsRNA-sensor upregulation in blood cells after vaccination. Due to the inactivated virus in the vaccine, intracellular virus replication after infection known to

generate dsRNA by-products [47] should be excluded. Thus, the vaccine itself must have contained dsRNA or a dsRNA analogue. The dsRNA or the analogue could either originate from cellular remnants of the host cells after virus cultivation for vaccine production or were intentionally added to the vaccine as adjuvant. Vaccine contamination fits the hypothesis of a massive contamination of the PregSure® vaccine with components of the MDBK host cells, a bovine kidney epithelial cell line, as indicated by [53]. This massive vaccine contamination with bovine cellular contaminants could also be responsible for the massive production of alloantibodies in cows producing BNP inducing colostrum. Alternatively, the vaccine might have been modulated by addition of a dsRNA analogue, e.g. polyinosinic:polycytidylic acid (poly I:C), a toll-like receptor (TLR) agonist that mimics the immunostimulatory properties of dsRNA, which has been suggested to improve vaccination effects (e.g., [54]).

Coordinated regulation of groups of genes relevant for immune response

In addition to the coordinated activation of dsRNA response mechanisms, the concordant differential expression of several members from groups of immune - relevant genes further indicated a coordinated regulation of a variety of immune response components to vaccination. The promoted interleukin 1 (IL1) response is one example, It is supported by the differentially expressed nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitors (NFKBID, NFKBIL1, NFKBIZ) that are known to be involved in the induction of inflammatory genes activated through TLR/IL1 signalling [55].

Another example for the concordant regulation of several genes from one immune-relevant family is the upregulation of SOCS genes, which might represent a negative feedback to interleukin receptor upregulation according to SOCS' known inhibitory effects on cytokine signalling [56].

Furthermore, upregulation of members of the cohesion complex and also SMC and non-SMC condensins that are essential during mitosis suggest a promotion of cell division.

T cell and B cell response

In our data set, we observed a coordinated upregulation of T cell receptor signalling upon vaccination. Signal transduction goes via CD3, and subsequently ZAP70, which are both upregulated. ZAP70 is known to be involved in protein kinase C θ and calcineurin-induced transcriptional activity [57]. Three major downstream signalling pathways of ZAP70 (NF-AT, NFκB and also JUN/FOS, [58-60]) contain several significantly upregulated molecules. For example, in the *NF-AT signalling* pathway, PPP3CA and PPP3CB belonging to the PP2B complex are upregulated as well as NFAT5, encoding a factor of activated T cells important for induction of gene expression: the NFAT5 protein belongs to the group of nuclear factors that are crucial for inducible gene transcription during immune response [61].

There is a coordinated regulation between genes presumably acting in antigen presenting cells and genes presumably acting in the T cell populations: the gene CD3G encoding a protein within the T cell receptor is upregulated after vaccination. The CD80 and CD86 genes encoding proteins on the surface of antigen presenting cells also show a higher expression after vaccination. The interaction of CD80 with ligands, e.g. CD28 or CD152 is important for T cell communication with the antigen-presenting cells [62]. CD28 is highlighted as a major upstream regulator in IPA analyses. In addition, the MHC class II gene DO also expressed in antigen presenting cells is downregulated after vaccination. In humans, the DO heterodimer expressed in antigen presenting cells (macrophages, dendritic cells) suppresses peptide loading of MHC class II molecules by inhibiting HLA-DM [63]. Upregulation of CD80 and downregulation of Bola DOB in our data set indicates an activation of antigen presentation in the antigen presenting cells after vaccination.

A potential effector mechanism after T cell activation is the induction of apoptosis in the antigen presenting cells via caspase: initiator caspase CASP8 and its interacting protein CASPA8AP2 as well as the effector caspases CASP3 and CASP7 are all upregulated. The hypothesis of induced apoptosis is also supported by the significantly affected canonical pathway *mitochondrial dysfunction* suggesting activation of the intrinsic or mitochondrial pathway of apoptosis and by the increased expression of a member of the tumor necrosis factor (ligand) superfamily, TNFSF10 (TRAIL). TRAIL has been shown to trigger the activation of MAPK8/JNK, CASP8, and CASP3 [64].

The hypothesis of T cell induced apoptosis fits also the upregulation of GZMA expression encoding granzyme A after vaccination. Granzyme A synthesis and transmission to target cells is a mechanism of cytotoxic T cells killing antigen presenting target cells via the intrinsic apoptosis pathway [65]. In addition to cytotoxic T cells, granzyme is also produced by Natural killer (NK) cells. These cells might also contribute to the elevated transcript level of the GZMA gene, because there is a significant upregulation of genes encoding NK cell lectin-like receptors [KLRK1 and LOC100294723 (Killer cell lectin-like receptor subfamily B member 1B allele A-like)]. Interestingly, NKG2 encoded by KLRK1 recognizes non-classical MHC class I proteins like polymorphic MICA and MICB produced by stressed cells [66]. MICA is a stress-induced self-antigen frequently expressed in virus-infected cells. This result adds further indicators for a

Demasius *et al. Veterinary Research* 2013, **44**:93
http://www.veterinaryresearch.org/content/44/1/93

specific immune response to virus after vaccination with the BVDV vaccine.

In contrast to T cell activation, surprisingly B cell activation 14 days after vaccination with the BVDV vaccine seems to be suppressed. In addition to signal transducing peptides CD79A, CD79B and CD19, also cell surface molecules along the entire process of B cell development from stem cell to plasma cell are downregulated (IgM, IgD, Mu). Deactivation of B cells is also supported by the downregulation detected for TNFRSF13C. TNFRSF13C is a receptor for B cell-activating factor (BAFF), which is known to enhance B-cell survival in vitro and is a regulator of the peripheral B cell population [67]. The indication on suppression of B cell response at transcriptome level 14 days after vaccination is particularly unexpected, because the vaccine is known for high production of neutralising BVD antibodies [14]. The reason for the lack of B cell activation at this time point is unknown. It might be speculated that there is a negative feedback at the level of transcriptional regulation to counteract a high B cell response prior to day 14 post vaccination.

Alloimmune response

KEGG pathway analysis indicated the *allograft rejection* pathway as significantly enriched with differentially expressed genes. This would fit the hypothesis of a substantial immune response to an alloantigen from the BVDV vaccine [3-5]. However, close examination of the expression of key mediators of alloantigen response indicated that there was no coordinated pattern of expression indicating an activation of this pathway. On the one hand, CD80 and CD86 expressed on professional antigen presenting cells (e.g., macrophages) are both upregulated and MHC class II DO (inhibitor of antigen binding to MHC, see above) is downregulated. This would suggest an activation of alloantigen response. On the other hand, CD40 was downregulated, but for CD40LG, one of the major upstream regulators identified in our data set (see Table 2), activation was predicted. CD40 is relevant for B cell activation [68], which is important for antibody mediated alloantigen response and also for macrophage mediated cytotoxic alloantigen response.

Comparison to previous studies

Comparing our data with a previous study applying deep transcriptome sequencing in cattle [12] on the immune response to vaccination against Mycobacterium bovis showed that there was only a limited overlap of affected pathways between that study and our data. Only the *cytokine – cytokine receptor interaction* pathway was significantly overrepresented with differentially expressed genes in both studies. However, it has to be considered that Bhuju and colleagues [12] used a very different kind of vaccine directed against an intracellular bacterial pathogen, investigated specifically PBMC leukocyte expression and used a bovine genome annotation restricted to genes in the previous Btau4 assembly. In contrast, our study monitored the effect of an inactivated ssRNA virus vaccine in whole blood samples immediately stored after collection without any preparatory manipulation. Vartanian and colleagues [31] summarised the advantages of analysing whole blood compared to specific cell fractions. In addition, we specifically generated a comprehensive annotation of the bovine genome for our analysis. We did not find any equivalent further studies of vaccination effects of inactivated viral vaccines with deep sequencing techniques of blood transcriptomes in mammals. Whereas there are multiple studies on immune response to anti-viral vaccination at transcriptome level using microarrays in human, chicken, pigs or aquaculture (e.g. [69]), to the author's knowledge respective data are missing in cattle for vaccinations similar to our regime.

Conclusions

RNAseq of blood samples is restricted to the analysis of those transcript-carrying cells roaming in the blood stream. Any cellular regulation involved in immune response restricted to the periphery is not captured by this technique. However, our analyses demonstrated that a comprehensive regulation of the immune response to vaccination can be detected by our approach.

Our data showed a very coordinated immune response to dsRNA or a dsRNA analogue after vaccination with the specific, inactivated BVDV vaccine associated to BNP. This indicates a massive contamination of the vaccine, because the vaccine is inactivated and directed against BVDV, an ssRNA virus. Alternatively, a dsRNA mimicking agent, e.g., polyinosinic:polycytidylic acid (poly I:C), a toll-like receptor (TLR) agonist might be constituent of the vaccine adjuvant. The vaccine used in our experiment had been demonstrated to generate BNP-associated alloantibodies, which are probably directed against cellular contaminations of the vaccine. A contamination of the vaccine by MDBK host cell proteins has been demonstrated [53]. However, our data did not indicate a major, coordinated response to an alloantigen after vaccination. Thus, in contrast to the immune response to dsRNA or an analogue, there is no clear mechanism identified for activated alloantigen response after vaccination with the BVDV vaccine from our data set. However, we did observe a previously unknown locus highly upregulated after vaccination with potential cytokine features. It will have to be investigated in future studies, if this novel gene is specifically related to the vaccine used in this experiment and also to BNP or if it is a gene with a general function in immune response.

Demasius *et al. Veterinary Research* 2013, **44**:93
http://www.veterinaryresearch.org/content/44/1/93

Additional files

Additional file 1: List of primers used for genomic confirmation and differential expression of the novel locus XLOC_032517. The table lists the sequences of all primers used for confirmation of structure and differential expression of the new locus XLOC_032517.

Additional file 2: List of differentially expressed loci prior vs. after vaccination at a threshold of q < 0.1. The table provides a comprehensive list of all loci showing differential expression in response to vaccination at a significance threshold of q < 0.10. LogFC: log fold change between expression levels prior and after vaccination, logCPM: log cpm; gene: official locus name or previously unknown locus (= no annotat.), locus: chromosomal position of the expressed locus.

Additional file 3: Conservation of exon-intron structure in the human genome for a sequence homologous to XLOC_032517. The figure provides the result from a BLAST search with the XLOC_032517 transcript (Genbank Accession No. KF051797) against all human genome assemblies deposited at NCBI.

Additional file 4: List of significantly overrepresented KEGG pathways established by GOseq analysis from the list of significantly differentially expressed genes after vaccination. The table lists all significantly affected KEGG pathways with the respective p-values for overrepresentation of genes showing a significantly different expression in response to vaccination.

Additional file 5: KEGG pathway RIG I like receptor signalling. The overview of the KEGG RIG I like receptor signalling pathway indicates significantly affected downstream KEGG pathways and differentially expressed genes after vaccination with the PregSure® vaccine obtained from GOseq analyses. All non-differentially expressed genes have a white background, all upregulated genes have an orange-red background and all downregulated genes have a green background. All differentially expressed genes or significantly affected pathways share the red frame. Solid red frames indicate results obtained from the KEGG data base, dashed red frames highlight significantly affected biological functions as indicated by IPA analysis.

Additional file 6: List of biological functions significantly affected by vaccination as determined by IPA analysis. The folder "categories of affected function" summarises the affected biological functions into categories according to IPA analysis. Furthermore, the range of p values for overrepresentation and the number of differentially expressed loci in each category is indicated. The folder "affected_biological_functions" lists the individual biological categories with a significant overrepresentation of differentially expressed genes. Furthermore, the predicted activation status and the number of differentially expressed genes assigned to the respective function are indicated.

Additional file 7: List of canonical pathways significantly affected by vaccination as determined by IPA analysis. The table lists all significantly affected canonical pathways obtained by IPA analysis with the respective p-values for overrepresentation of genes showing a significantly different expression in response to vaccination. The ratio provides the proportion of genes in the respective canonical pathway, which are significantly differentially expressed after vaccination, relative to all genes in the pathway. In addition, the proportion of upregulated and downregulated genes in the respective pathway and a full list of all differentially expressed genes assigned to the respective pathway are indicated.

Additional file 8: EIF2 signalling canonical pathway significantly affected by vaccination. The figure highlights all elements within the IPA canonical pathway EIF2 signalling differentially expressed after vaccination. Orange/red elements: upregulated after vaccination, green elements: downregulated after vaccination. Colour intensity reflects the different fold change of expression.

Additional file 9: TLR signalling canonical pathway significantly affected by vaccination. Elements within the IPA canonical pathway TLR signalling differentially expressed after vaccination are indicated. Orange/red elements: upregulated after vaccination, green elements: downregulated after vaccination. Colour intensity reflects the different fold change of expression.

Additional file 10: T cell receptor signalling canonical pathway significantly affected by vaccination. Elements within the IPA canonical pathway T cell receptor signalling differentially expressed after vaccination are indicated. Orange/red elements: upregulated after vaccination, green elements: downregulated after vaccination. Colour intensity reflects the different fold change of expression.

Additional file 11: B cell development canonical pathway significantly affected by vaccination. Elements within the IPA canonical pathway B cell development differentially expressed after vaccination are indicated. Orange/red elements: upregulated after vaccination, green elements: downregulated after vaccination. Colour intensity reflects the different fold change of expression.

Additional file 12: NFκB signalling canonical pathway significantly affected by vaccination. Elements within the IPA canonical pathway NFκB signalling differentially expressed after vaccination are indicated. Orange/red elements: upregulated after vaccination, green elements: downregulated after vaccination. Colour intensity reflects the different fold change of expression.

Additional file 13: List of target molecules for predicted upstream regulators of differential expression after vaccination as determined by IPA analysis. The file provides a list of predicted upstream regulators obtained by IPA analysis from the data set of differentially expressed genes. In addition, for all upstream regulators, all respective differentially expressed target genes are indicated.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
WD carried out the experiment, contributed to data collection and analysis, and participated in drafting the manuscript; RW participated in the design of the study, conceived the qRT-PCR experiments and participated in drafting the manuscript; FH participated in data analysis and drafting the manuscript; KM participated in conceiving the study and drafting the manuscript, and CK participated in conducting the experiment, carried out the data analysis, conceived the experiment and prepared the manuscript. All authors read and approved the final manuscript.

Acknowledgements
We thank our colleagues at the FBN Dummerstorf involved in the generation and care of the SEGFAM F2 resource population for continuous support of our work. The excellent technical assistance of Simone Wöhl, Antje Lehmann and Marlies Fuchs and the valuable discussions with Prof. Dr T. Rümenapf (Vienna), Dr Juliane Günther and Dr Ronald Brunner (FBN Dummerstorf) are thankfully acknowledged. The MDBK cell line was kindly provided by Prof. Dr T. Rümenapf (Vienna). This study was funded by the German Ministry of Food, Agriculture, and Consumer Protection (BMELV) through the Federal Office for Agriculture and Food (BLE), grant number 2810HS27.

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Received: 17 May 2013 Accepted: 24 September 2013
Published: 7 October 2013

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doi:10.1186/1297-9716-44-93
Cite this article as: Demasius *et al.*: Monitoring the immune response to vaccination with an inactivated vaccine associated to bovine neonatal pancytopenia by deep sequencing transcriptome analysis in cattle. *Veterinary Research* 2013 **44**:93.

6 Discussion

Since the emergence of BNP in 2007, several studies focussed on different factors potentially involved in the aetiopathogenesis of the disease. Although these studies remarkably shed light onto the complex aetiopathogenesis of this alloimmune disease, a number of questions regarding the pathogenesis require further investigation or have emerged from published findings. The fundamentals for the objectives deduced for this study were limited data concerning subclinical BNP cases (Pardon *et al.*, 2010; Witt *et al.*, 2011) and the hypothesis of a genetic predisposition involved in clinical BNP (Krappmann *et al.*, 2011). Furthermore, the immune response to vaccination with the specific inactivated BVD-vaccine associated to BNP (PregSure® BVD) had not been characterised in detail before. It was only known that this vaccine elicited significantly higher neutralising antibody titres compared to other commercial BVD-vaccines (Bastian *et al.*, 2011; Raue *et al.*, 2011), and additionally evoked BNP-associated pathogenic alloantibodies (Bridger *et al.*, 2011; Pardon *et al.*, 2011). Results from our studies confirm that a genetic predisposition is required for clinical and potentially subclinical BNP, and provide evidence that the incidence of subclinical BNP cases is not higher than for clinical BNP cases. Analysis of the blood transcriptome regulation after booster vaccination with PregSure® BVD applying an RNA-Seq approach revealed a very coordinated immune response to double-stranded (ds) RNA, although the inactivated vaccine is directed against a single-stranded (ss) RNA virus. This indicates a contamination of the vaccine with dsRNA, or the inclusion of a dsRNA analogue into the vaccine. Additionally, the study identified a novel gene with cytokine-like features, which is highly upregulated after vaccination in all animals. These results are comprehensively discussed hereafter in the context of the aetiopathogenesis of BNP.

All our studies were performed on animals of the experimental German Holstein x Charolais crossbred resource population at the FBN Dummerstorf, except for one German Holstein cow also kept within the resource population. This resource population provided a good basis for the investigations, because this population had been kept throughout under constant housing, feeding and management conditions including identical vaccination protocols for all cows. All dams had received at least a basic vaccination with PregSure® BVD, consisting of two doses given according to the manufacturer's instructions. Booster vaccinations were performed annually. PregSure® BVD presents the main risk factor for producing BNP colostrum (Lambton *et al.*, 2012; Sauter-Louis *et al.*, 2012), and the respective vaccination regime is known to induce high alloantibody titres (Kasonta *et al.*, 2012). All dams included in the second study (Demasius *et al.*, 2013) had been classified into three groups according to results previously reported by

Krappmann et al. (Krappmann *et al.*, 2011) and according to findings of our first study (Demasius *et al.*, 2014). Firstly, dams, which gave birth to clinical BNP-calves, were included. They all originated, except for the German Holstein cow, from one specific sire line and were fullsibs. The second group comprised fullsib-dams from the same sire line which in contrast had given birth to clinically healthy calves, but which nonetheless showed haematological deviations from the average of the peer group. Thirdly, a group of control cows from alternative sire lines and with healthy calves was included.

Our first study (Demasius *et al.*, 2014) closely monitored all newborn calves of the resource population for the incidence of subclinical BNP and other epidemiological factors involved in clinical and subclinical BNP. Other studies which had reported on the incidence of subclinical BNP (Pardon *et al.*, 2010; Witt *et al.*, 2011) considered calves with haematological alterations and without clinical signs as suspected subclinical cases after only a single blood sampling. Although literature on haematological profiles of neonatal calves is sparse, reports on healthy calves show that they may display transient blood cell counts below reference ranges (Egli and Blum, 1998). Similar findings were also reported from BNP challenge studies (Friedrich *et al.*, 2011; Schröter *et al.*, 2011). The very close and frequent clinical and haematological monitoring of a subset of calves included in our study enabled exact classification of subclinical cases and discrimination of subclinical BNP-calves from healthy calves that showed only transient thrombocytopenia or leucopenia. Moreover, all calves included in our study had received colostrum exclusively from their own dams, since feeding of mixed colostrum on BNP-affected farms may result in an increased risk for calves to develop haematological alterations (Witt *et al.*, 2011). Our study could not confirm the hypothesis that subclinical BNP cases occur more frequently than clinical BNP cases. However, the single observed subclinical BNP case in our study occurred in the one specific sire line of the resource population in which an accumulation of clinical BNP cases had been observed in a previous study (Krappmann *et al.*, 2011). This previous study had proposed the hypothesis of a possible genetic predisposition involved in clinical BNP (Krappmann *et al.*, 2011). The present study could confirm this hypothesis of a genetic predisposition involved in clinical BNP, as two further clinical BNP cases exclusively occurred in this one specific sire line. For the single subclinical BNP case, a genetic predisposition can also be assumed, as this case was also confined to the BNP-affected sire line. Moreover, healthy calves of this BNP-affected sire line revealed a decrease in thrombocytes between day 7 and day 14 after colostral intake and significantly decreased thrombocyte counts on day 14 as well as decreased leucocyte counts on day 7, compared to healthy calves of other sire lines. Although blood cell counts were still above ranges reported for young calves in other studies (Mohri *et al.*, 2007), these calves must have ingested an agent via colostrum which had a decreasing effect on cell numbers of certain blood

cell lineages. Considering published literature on BNP, it can only be speculated about the factor(s) responsible for different manifestations of the disease at this stage. These factors could be variable alloantibody titres in dams, different types of alloantibodies produced by dams and different alloantibody combinations ingested by calves, or also a predisposition on the part of the calves. Yet, our results suggest a genetically determined reaction of the dam towards PregSure® BVD vaccination. Our study indicates that subclinical BNP occurs with a low incidence. For confirmation of this hypothesis, large scale studies with close clinical and haematological monitoring should be performed in different cattle herds vaccinated with PregSure® BVD. But since a genetic predisposition also seems to be involved in subclinical BNP, the true incidence for subclinical BNP might be similarly low as for clinical BNP. This would be in agreement with the reported low incidence for clinical BNP (Kasonta *et al.*, 2012; Pardon *et al.*, 2010) relative to the number of PregSure® BVD-vaccinated animals or the number of vaccination doses sold (Kasonta *et al.*, 2012). Knowledge about the true incidence of subclinical BNP is useful, because subclinical BNP might result in a decrease of health statuses of calves (Sauter-Louis *et al.*, 2012). On farms with high incidence rates of BNP, subclinical cases were assumed to be the reason for an increase in medical treatments (Reichmann, 2012), which can result in substantial economic losses.

Our study did not reveal further epidemiological factors contributing to BNP. With regard to PregSure® BVD vaccinations, neither the time point itself nor the proximity to parturition were risk factors for clinical or haematological alterations associated with BNP. These results are in line with findings of other epidemiological studies (Jones *et al.*, 2013; Lambton *et al.*, 2012). Also, the frequency of PregSure® BVD vaccination did not present a risk factor, which was also shown by Lambton et al. (Lambton *et al.*, 2012). However, the findings of Lambton et al. (Lambton *et al.*, 2012) are in contrast to those of Jones et al. (Jones *et al.*, 2013), the latter revealing that dams of second or more lactation were at higher risk of having a BNP-calf, because these dams had received more doses of PregSure® BVD compared to first lactation dams. Repetitive vaccinations with PregSure® BVD result in higher alloantibody titres (Kasonta *et al.*, 2012). However, experiments also showed that some animals already developed alloantibodies after receiving only a single dose of PregSure® BVD (Kasonta *et al.*, 2012). Finally, different vaccination regimes may also account for differing alloantibody titres in dams (Kasonta *et al.*, 2012). Our study applied for all cows an identical PregSure® BVD vaccination scheme according to the manufacturer's instructions, which had been reported to result in higher alloantibody titres compared to a two-step vaccination regime (Kasonta *et al.*, 2012). Excluding further potential epidemiological factors for BNP in the experimental resource population of the FBN Dummerstorf focuses factors involved in the aetiopathogenesis of BNP to a genetic predisposition of the dam required for

producing BNP-colostrum (Demasius *et al.*, 2014; Krappmann *et al.*, 2011) in combination with the main risk factor, PregSure® BVD vaccination (Lambton *et al.*, 2012; Sauter-Louis *et al.*, 2012).

Reports that contaminating MHC class I antigen, originating from the MDBK cell line used for vaccine production of PregSure® BVD, is responsible for production of pathogenic alloantibodies in some dams (Deutskens *et al.*, 2011; Foucras *et al.*, 2011) would match with the hypothesis of a possible genetic predisposition required for producing BNP-colostrum (Demasius *et al.*, 2014; Krappmann *et al.*, 2011). Related dams, as found in our experimental setup, are more likely to display the same MHC class I haplotypes. Dams that differ in MHC class I genotype from the MHC class I genotype of the MDBK cell line will respond to the MHC class I antigen with alloantibody production. The low incidence rate observed for clinical BNP-calves (Kasonta *et al.*, 2012; Pardon *et al.*, 2010) might therefore be explained by diversity in the MHC class I region (Kelley *et al.*, 2005), and the genetically determined MHC class I makeup carried by dam and calf. However, considering different strategies that exist to achieve and maintain diversity in the MHC region, and especially those applied in cattle, the MHC class I hypothesis has to be questioned. The strategies for maintaining diversity in the MHC class I region include polymorphic genes and/or varying gene numbers and combinations in different haplotypes (Kelley *et al.*, 2005; Parham *et al.*, 1995; Trowsdale and Parham, 2004). Cattle apply a combination of both strategies. So far, the existence of six classical MHC class I genes (gene 1 – 6) has been proposed (Hammond *et al.*, 2012). Cattle show region configuration polymorphism, expressing between one and three of the six genes per haplotype (Birch *et al.*, 2006; Codner *et al.*, 2012; Ellis *et al.*, 1999). Additionally, high degrees of polymorphisms can be found between individuals (Babiuk *et al.*, 2007). Considering these strategies applied by cattle for maintaining diversity in the MHC class I region, it is unlikely that the large proportion of observed non-BNP-dams shared (an) MHC class I allele(s) with the MDBK cell line. This assumption is underlined by findings that alloantibodies against MHC class I can be found in sera of some non-BNP-dams (Deutskens *et al.*, 2011) as well as in a large proportion of animals after experimental vaccination (Kasonta *et al.*, 2012). The high degree of diversity in the MHC class I also contradicts the hypothesis of causal MHC class I alleles, because clinical BNP or at least haematological alterations could be reproduced frequently with BNP-colostrum in almost all challenge calves (Bell *et al.*, 2013; Friedrich *et al.*, 2011; Schröter *et al.*, 2011). It is unlikely that all of these unrelated challenge calves shared MHC class I alleles with the MDBK cell line. In our resource population, sera of all dams included in the second study, regardless of BNP status, contained alloantibodies directed against MHC class I on MDBK cells (Deutskens, 2012). This also included the control cows from non-BNP affected sire lines. Calves of these dams which were monitored in our first study (Demasius *et al.*, 2014) did not show any clinical or haematological alterations consistent with BNP. Our unpublished results have revealed

that these control cows, which were included in our second study, do not share classical MHC class I alleles with the MDBK cell line, except for one control cow, which shares a single MHC class I allele with the MDBK cell line. Consequently, also control dams differing from the MDBK cell MHC class I genotype might respond to allogeneic MHC class I of the MDBK cell line with alloantibody production. Exclusion of MHC class I as single causal agents for BNP would eradicate further puzzling observations regarding the MHC class I hypothesis that had previously arisen. These findings reported that BNP-associated alloantibodies bind to a target antigen expressed on platelets and all cells of the myeloid lineage, but not to all cells of the lymphoid lineage (Assad *et al.*, 2012). However, MHC class I expression in humans is found on nearly all nucleated cells, and especially lymphoid tissues reveal high expression levels of MHC class I (Agrawal and Kishore, 2000). If the ubiquitously expressed MHC class I would be the target antigen in calves, then alloantibody-related damages should not only be detected in haematological tissue in histopathology (Friedrich *et al.*, 2009b; Pardon *et al.*, 2010). As discussed in Euler et al. (Euler *et al.*, 2013), a transfusion-related syndrome in humans, known as transfusion-related acute lung injury (TRALI), is characterised by a high frequency of MHC class I alloantibodies. However, these are only weak triggers with low fatality. Not all antigens crucial for transfusion have been identified yet (Euler *et al.*, 2013). Prominent MHC class I alloantibody production in TRALI patients might overshadow other possible candidates, which might be the case in BNP as well (Euler *et al.*, 2013).

Observations regarding challenge studies in calves suggest that potentially more than one antigen might induce BNP-associated alloantibody production. While feeding similar volumes of colostrum from a single BNP-dam to challenge calves resulted in different degrees of severity for clinical findings in these calves (Friedrich *et al.*, 2011; Schröter *et al.*, 2011), ingestion of mixed colostrum increased the incidence of clinical BNP as well as the mortality rate (Schröter *et al.*, 2011). Feeding a standardised quantity from the same pooled colostrum to each challenge calf resulted in consistent alterations found in all challenge calves (Bell *et al.*, 2013). If there are multiple causal antigens involved in BNP-associated alloantibody production, pooling colostrum increases the chance that a wider spectrum of different alloantibodies may be found in the colostrum as compared to colostrum of a single dam. It has to be considered, however, that if multiple alloantibodies would be directed against multiple different antigens, this will result in a dilution effect of each specific alloantibody in mixed colostrum and might not exert BNP in calves anymore. Indications for a massive contamination of PregSure® BVD with proteins derived from the MDBK cell line was revealed in the study by Euler et al. (Euler *et al.*, 2013). Therefore, there is more than one potential protein candidate proposed which might elicit pathogenic alloantibody production. Results of our second study (Demasius *et al.*, 2013) provided further evidence of a

massive contamination of the vaccine. We could observe a very coordinated immune response to dsRNA or a dsRNA analogue 14 days after booster vaccination with PregSure® BVD across all dams included in the study. This is revealed by upregulated, immune response-related genes and overrepresented pathways in KEGG (Kyoto Encyclopedia of Genes and Genomes, http://www.genome.jp/kegg/pathway.html) and Ingenuity pathway analysis (IPA, Ingenuity® Systems, http://www.ingenuity.com), especially by the *RIG-I-like receptor signaling pathway* (KEGG) and the string starting with the Toll-like receptor (TLR) 3 in the overrepresented canonical pathway *TLR signaling pathway* (IPA). TLR3 is a receptor for dsRNA (Zhang *et al.*, 2013). Based on these results, we proposed a model for a very coordinated response to dsRNA after PregSure® BVD vaccination (Figure 5, (Demasius *et al.*, 2013)). These results are striking, because PregSure® BVD is an inactivated vaccine against the BVDV, an ssRNA pestivirus belonging to the *Flaviviridae* (Peterhans *et al.*, 2010). We could not identify differentially expressed genes or enriched pathways related to a response to ssRNA, for example upregulation of receptors for ssRNA like TLR7 or TLR8 (Heil *et al.*, 2004). Since the vaccine is inactivated, there should not be viral replication occurring anymore. During replication, dsRNA may occur temporarily as a replication intermediate (Kato *et al.*, 2006). Therefore, the source for dsRNA may have originated from the MDBK host cell line used for virus cultivation during vaccine production. This would be another proof of the massive contamination of the vaccine. Alternatively, a dsRNA analogue was added to PregSure® BVD, for example polyinosine-polycytidylic acid (poly(I:C)). Poly(I:C) can be detected by both RIG-1 as well as TLR3 (Kato *et al.*, 2006). This synthetic dsRNA analogue has been shown to increase immunostimulatory effects (Thim *et al.*, 2012) and could be an example for a potentially added adjuvant to PregSure® BVD.

These findings also draw attention to the unique nanoparticle-based adjuvant contained in PregSure® BVD and to potentially further adjuvants added. According to the instruction leaflet, PregSure® BVD contains the adjuvants Quil A, cholesterol, Amphigen Base and drakeol 5 (liquid paraffin). Quil A is a saponin which originates from the bark of *Quillaja saponaria*, a Chilean tree (Singh and O'Hagan, 2003). This saponin has been shown to intercalate into cell membranes and to have haemolytic properties. To reduce these cytotoxic effects, Quil A is combined with phospholipids and cholesterol (Singh and O'Hagan, 2003). This results in nano-complex formation of about 40 nm, termed immune stimulatory complexes (ISCOMs), which carry the respective antigens (Sjölander *et al.*, 1997). ISCOMs are recognised by APCs (Singh and O'Hagan, 2003). Adjuvants can have a major influence on the type of immune response elicited after vaccination (Sjölander *et al.*, 1997). Quil A showed the ability to efficiently induce humoral and cell-mediated immune responses. In mice, antigen-specific antibodies of all IgG subclasses are induced. Additionally, Quil A induces both Th1-like as well as Th2-like immune responses, characterised by

IFN-γ and IL-4 production, respectively (Sjölander *et al.*, 1997). The dose of the BVDV-antigen (structural glycoproteins E1 and E2, (Pfizer Animal Health Technical Bulletin, 2004)) bound to the ISCOM is not known. PregSure® BVD is known to elicit significantly higher neutralising antibody titres compared to other commercial BVD-vaccines (Bastian *et al.*, 2011; Raue *et al.*, 2011). These antibody titres even exceed titres observed in naturally infected animals (Bastian *et al.*, 2011). It can be assumed that these findings are due to the very potent adjuvant contained in PregSure® BVD, which elicits multiple types of immune responses. Considering the aetiopathogenesis of BNP and the massive cellular contamination of the vaccine (Euler *et al.*, 2013), it can be assumed that this potent adjuvant represents a further driving factor in alloantibody production towards an antigen, that has yet to be characterised.

In our second study (Demasius *et al.*, 2013), we found a strong upregulation of a previously unknown locus, XLOC_032517, with cytokine-like features, which was highly upregulated across all samples 14 days after booster vaccination with PregSure® BVD. No functional annotation of this locus could be found in cattle or any other species. Detection of this novel transcript highlights the capacity of RNA-Seq in comparison to other methods applied for transcriptome analysis (Wang *et al.*, 2009). Whole blood samples of non-PregSure® BVD vaccinated animals also showed expression of XLOC_032517, however, at significantly lower levels compared to PregSure® BVD-vaccinated animals. In future studies, it has to be evaluated if upregulation of XLOC_032517 is specifically linked to PregSure® BVD vaccination, or can also be found after application of other immunostimulatory agents, for example after vaccination with other commercial BVD vaccines. Moreover, it should be determined which specific blood cells express XLOC_032517, and it should be determined, if the transcript is translated into a protein. Finally, it has to be evaluated, if expression of XLOC_032517 is specific to immune response in ruminants.

Different immune response pathways and expression of specific cytokines are regulated by T-helper cells (Th cells) (Kidd, 2003). Th1 cells elicit a cell mediated (CMIR) type 1 response to intracellular pathogens, while the type 2 response is an antibody mediated immune response (AMIR) to extracellular pathogens. In cattle, a type 2 response is associated with production of IgG1 (Estes and Brown, 2002). Colostrum-derived BNP alloantibodies were shown to be exclusively of IgG1 subclass (Assad *et al.*, 2012). Several studies deal with AMIR or CMIR and could classify animals into high, average or low immune responders, accordingly (Begley *et al.*, 2009; Heriazon *et al.*, 2013; Hine *et al.*, 2012). Besides these phenotypic classifications, heritabilities for both AMIR and CMIR have been established as moderate and high, respectively (Heriazon *et al.*, 2013). In our first study (Demasius *et al.*, 2014), we could demonstrate an accumulation of BNP-dams in one specific sire line of our resource population and a genetic predisposition required for

the production of pathogenic BNP-colostrum. It has been shown that some dams sporadically produce alloantibodies against MHC class I after only one dose of PregSure® BVD (Kasonta *et al.*, 2012), and that BNP-dams show higher alloantibody titres than non-BNP-dams (Bastian *et al.*, 2011). These findings suggest that these dams were high AMIR. A recent study that evaluated humoral immune response to human chorionic gonadotropin (hCG) in lactating dairy cows revealed that most cows showed a humoral immune response to hCG after two to three injections of hCG (Giordano *et al.*, 2012). However, one cow already showed an antibody response against hCG after a single exposure, while some animals did not develop any detectable antibody responses after three injections of hCG (Giordano *et al.*, 2012). Similar immune responses towards a contaminating antigen in PregSure® BVD can be assumed. Therefore, regardless of (a) further yet unidentified specific antigen(s) responsible for alloantibody production in BNP, genetically determined differences in humoral immune responses in dams vaccinated with PregSure® BVD have to be additionally considered regarding the aetiopathogenesis of BNP.

7 Conclusions and Outlook

The emergence of the feto-maternal incompatibility phenomenon BNP in recent years and published results relating to the aetiopathogenesis of this disease have highlighted the risks involved in producing vaccines on homologous cell lines. In case of inadequate purification during vaccine production, contaminating antigens of the host cell line in combination with very potent adjuvants in the vaccine may result in fatal diseases as observed for BNP. Although PregSure® BVD has been retracted from the market and BNP will thus no longer occur, elucidation of the exact aetiopathogenesis of BNP is essential, because this may have implications for the general safety of vaccines, including vaccines for use in humans. Immunisation with specific vaccines and subsequent transcriptome analysis can give insights into the structural and quantitative regulation of the transcriptome in specific tissues or cells after vaccination. In our study the novel method of RNA-Seq on RNA derived from whole blood after booster vaccination with PregSure® BVD was applied. This represents an approach independent of any proposed hypotheses at a time where the exact aetiopathogenesis of BNP is not fully uncovered. Contradictory observations regarding the MHC hypothesis raise the question, if MHC class I should be regarded as a single causal antigen responsible for BNP-associated alloantibody production. It has been shown that PregSure® BVD is highly contaminated with proteins derived from the producer cell line MDBK, which implies that a whole panel of further potential antigens has to be validated in the future. We obtained indication on a previously postulated potential contamination of the vaccine with residues of the MDBK cell line, because we found a very coordinated immune response towards dsRNA after booster vaccination, although PregSure® BVD is directed against an ssRNA virus. Alternatively, a dsRNA analogue might have been added to the vaccine in order to improve the immunostimulatory properties of the vaccine. In addition, we could reveal a strong upregulation of a cytokine-like gene in all animals after booster vaccination with PregSure® BVD, which has never before been described in any other species. Prospective studies will have to evaluate whether this novel gene is specifically linked to an immune response after PregSure® BVD vaccination. Finally, we could confirm a genetic predisposition as a requirement for the production of either BNP- or non-BNP colostrum in dams vaccinated with PregSure® BVD.

Based on these results and other findings regarding the aetiopathogenesis of BNP, future studies will have to consider genetically determined differential immune responses in cows after vaccination. Analyses for differential gene expression between BNP and non-BNP-dams after PregSure® BVD vaccination will give further insights into these mechanisms. Thereby, the very potent features of the unique adjuvant contained in PregSure® BVD and its potential effects have

to be kept in mind. Knowledge about expression patterns of antigens on haematopoietic stem cells and peripheral blood cells targeted by BNP-associated alloantibodies can also aid to the understanding of the aetiology of BNP. Finally, an exact description of MHC class I alleles expressed by BNP-dams, non-BNP-dams and the MDBK cell line will provide evidence, if MHC class I can be regarded as single causal agent for BNP-associated alloantibodies. Puzzling observations and published findings regarding the MHC hypothesis have highlighted that the exact aetiopathogenesis of BNP has not been fully elucidated yet.

8 Summary

Investigations on selected aspects involved in the aetiology of bovine neonatal pancytopenia (BNP)

Bovine neonatal pancytopenia (BNP) is a recently emerged alloimmune disease affecting neonatal calves and is strongly associated with the vaccination of dams with a specific inactivated BVDV (Bovine Viral Diarrhoea Virus) vaccine (PregSure® BVD, Pfizer Animal Health). The vaccine comprises a unique adjuvant based on nanoparticles and has been shown to be highly contaminated with cellular proteins of the vaccine producer cell line MDBK (Madin-Darby bovine kidney). The disease is induced by ingestion of colostral alloantibodies of certain PregSure® BVD-vaccinated dams. The hypothesis that BNP-associated alloantibodies might be directed against MHC class I has been proposed. Currently, there are published observations, which contradict this hypothesis and which suggest that the aetiopathogenesis of BNP has not been fully elucidated yet. The aims of this study were to gain more insights into selected factors and underlying mechanisms involved in BNP. The hypothesis of a genetic predisposition for clinical BNP had been proposed in a previous study, but the incidence and potential genetic predisposition for subclinical cases had not been investigated thoroughly before. Therefore, a well-defined cattle resource population was monitored for the incidence, vaccination-associated epidemiological factors and a possible genetic predisposition involved in subclinical BNP. Prominent immune responses to PregSure® BVD vaccination had been reported. This study characterised the immune response to a booster immunisation with PregSure® BVD to obtain insights into the structural and quantitative regulation of the blood transcriptome after vaccination by means of deep sequencing transcriptome analysis. Furthermore, this approach should facilitate insights into the composition of the vaccine.

The previously proposed hypothesis of a genetic predisposition required for giving birth to clinical BNP-calves could be confirmed in this study. Our results suggest that a genetic predisposition is potentially also involved in subclinical BNP and additionally required for a significant decrease of thrombocytes and leucocytes in healthy calves without manifestation of clinical or subclinical BNP. In the monitored cattle resource population, the frequency of subclinical BNP did not exceed the frequency of clinical BNP-cases. Further vaccination-associated epidemiological factors, e.g. frequency of vaccination or time point of vaccination relative to parturition, could be excluded.

Transcriptome analysis 14 days after booster vaccination with PregSure® BVD highlighted a very coordinated immune response to double-stranded (ds) RNA, although the inactivated vaccine was directed against a single-stranded (ss) RNA BVD virus. The source of the dsRNA is unknown and could have originated from the MDBK cell line, which would underline the contamination of the vaccine with host cells from vaccine production. Alternatively, a dsRNA analogue was potentially added to improve the efficacy of the vaccine. Finally, this study identified a cytokine-like gene, which was highly upregulated across all animals after booster vaccination. This gene has not been described before in any other species. It has to be evaluated if upregulation of this gene is specific for ruminant immune response and linked to PregSure® BVD vaccination. In conclusion, this study confirmed that a genetic predisposition of PregSure® BVD-vaccinated dams is required for producing BNP-colostrum. Therefore, prospective studies will have to consider genetically determined differential immune responses between BNP- and non-BNP-dams towards exogenous proteins combined with very potent adjuvants. Regarding the MHC class I hypothesis, published observations raise the question, if MHC class I should be considered as single causal agent for BNP-associated alloantibodies. This requires further research. Potential allogeneic effects of a whole panel of contaminating proteins contained in the vaccine still have to be validated.

9 Zusammenfassung

Untersuchungen zu ausgewählten Aspekten, die an der Ätiologie der Bovinen Neonatalen Panzytopenie (BNP) beteiligt sind

Die Bovine Neonatale Panzytopenie (BNP) ist eine vor kurzem aufgetretene Alloimmunerkrankung neugeborener Kälber, die stark assoziiert ist mit der Impfung mit einer bestimmten, inaktivierten BVDV (Bovines Virusdiarrhoe-Virus) Vakzine (PregSure® BVD, Pfizer Animal Health) bei Muttertieren. Die Vakzine enthält ein spezifisches, auf Nanopartikeln basierendes Adjuvans. Es konnte nachgewiesen werden, dass der Impfstoff hochgradig mit zellulären Proteinen der MDBK-Zellline (Madin-Darby bovine kidney) kontaminiert ist, die für die Impfstoffherstellung verwendet wurde. Die Erkrankung wird ausgelöst durch die Aufnahme kolostraler Alloantikörper bestimmter PregSure® BVD-geimpfter Muttertiere. Es ist die Hypothese aufgestellt worden, dass BNP-assoziierte Alloantikörper gegen MHC Klasse I gerichtet sind. Gegenwärtig lassen sich publizierte Beobachtungen finden, die im Widerspruch zu der MHC Klasse I-Hypothese stehen und die zeigen, dass die Ätiopathogenese der BNP noch nicht vollständig geklärt ist. Ziele der vorliegenden Studie waren daher, weitere Einblicke in ausgewählte Faktoren und zugrunde liegende Mechanismen, die an der BNP beteiligt sind, zu gewinnen. In einer vorherigen Studie war die Hypothese einer möglichen genetischen Prädisposition für die klinische BNP aufgestellt worden. Über die Inzidenz und eine mögliche genetische Prädisposition für die subklinische BNP lagen noch keine umfassenden Studien vor. Daher wurde eine gut charakterisierte Ressourcenpopulation auf die Inzidenz, impfassoziierte epidemiologische Faktoren und eine mögliche genetische Prädisposition für subklinische BNP untersucht. Schließlich war nach PregSure® BVD-Impfungen über prominente Immunreaktionen berichtet worden. Die vorliegende Studie charakterisierte die Immunantwort nach einer PregSure® BVD-Auffrischimpfung auf Transkriptomebene mittels eines RNA-Seq-Ansatzes, um Einblicke in die strukturelle sowie quantitative Regulation des Blut-Transkriptoms sowie weitere Hinweise auf die Impfstoffzusammensetzung zu erhalten.

Die in einer früheren Studie aufgestellte Hypothese einer genetischen Prädisposition für das Hervorbringen von klinischen BNP-Kälbern konnte in dieser Studie bestätigt werden. Die hier gewonnenen Ergebnisse legen nahe, dass für eine subklinische BNP ebenfalls eine genetische Prädisposition erforderlich ist sowie auch für einen signifikanten Abfall von Thrombozyten- und Leukozytenzahlen bei klinisch gesunden Kälbern, die keine Manifestation einer klinischen oder

subklinischen BNP zeigen. In der untersuchten Ressourcenpopulation gab es keinen Hinweis auf eine höhere Frequenz subklinischer Fälle im Vergleich zu klinischen BNP-Fällen. Weitere impfassoziierte epidemiologische Faktoren wie Anzahl an Impfungen oder Zeitpunkt einer Impfung während der Trächtigkeit konnten ausgeschlossen werden. Die Transkriptomanalyse 14 Tage nach Auffrischimpfung mit PregSure® BVD konnte eine koordinierte Reaktion gegen doppelsträngige (ds) RNA aufzeigen, obwohl ein inaktivierter Impfstoff gegen das einzelsträngige (ss) BVD-RNA-Virus eingesetzt worden war. Der Ursprung der dsRNA ist unbekannt. Entweder stammte die dsRNA aus der MDBK-Zelllinie, was die Kontamination der Vakzine mit der für die Impfstoffherstellung verwendeten Zelllinie bestätigen würde oder es wurde ein dsRNA-Analogon dem Impfstoff zugefügt, um die immunstimulatorischen Eigenschaften der Vakzine zu verbessern. Schließlich konnte in der vorliegenden Studie bei allen Tieren ein signifikant hochreguliertes Gen mit zytokinartigen Eigenschaften 14 Tage nach Booster-Impfung identifiziert werden. Dieses Gen war zuvor bei keiner anderen Spezies beschrieben worden. Zukünftige Untersuchungen sollten klären, ob die Hochregulierung dieses Gens spezifisch für eine Immunantwort beim Rind ist und ob ein Zusammenhang mit einer PregSure® BVD-Impfung besteht. Abschließend konnte diese Studie bestätigen, dass eine genetische Prädisposition seitens PregSure® BVD-geimpfter Muttertiere für die Produktion von BNP-Kolostrum erforderlich ist. Daher sollten zukünftige Studien genetisch determinierte Unterschiede in der Immunantwort auf exogene Proteine in Kombination mit potenten Adjuvanzien zwischen BNP-Kühen und nicht-BNP-Kühen berücksichtigen. Hinsichtlich der MHC Klasse I – Hypothese werfen publizierte Beobachtungen die Frage auf, ob MHC Klasse I als einziger, kausaler Kandidat für BNP-assoziierte Alloantikörper angesehen werden kann. Hierzu bedarf es weiterer Untersuchungen. Die potenziellen allogenetischen Effekte einer Vielzahl kontaminierender Proteine in der Vakzine sollten validiert werden.

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11 Acknowledgements

I would like to thank my mentor Prof. Dr. Christa Kühn for offering me the opportunity to work on this fascinating topic and the excellent scientific supervision.

I thank Prof. Dr. Eckhard Wolf for the acceptance of this thesis at the Faculty of Veterinary Medicine at the Ludwig-Maximilians-Universität München and for the critical review of this manuscript.

Furthermore, I want to thank all members of the Genome Physiology Unit at the FBN: Prof. Dr. Christa Kühn, Dr. Rosemarie Weikard, Dr. Juliane Günther, Simone Wöhl, Antje Lehmann, Philipp Widmann, Andreas Kromik, Jacqueline Knaust, Oda Haufft, Kristin Krappmann, Bärbel Pletz and Emma Schröder. I am particularly thankful to Simone Wöhl and Antje Lehmann for their patient and friendly initial technical training and the excellent technical assistance. I specially thank Philipp Widmann for the helpfulness and discussions and Andreas Kromik for helping me with taking samples. The technical assistance of Marlies Fuchs is greatly acknowledged.

I specially want to thank Frieder Hadlich for the help with bioinformatic questions and for the motivating and positive working atmosphere in the office.

I would like to thank Dr. Bernd Stabenow and all members of the experimental cattle facility of the FBN Dummerstorf for their support. My special thanks go to Sylvia Krumbiegel and Astrid Dethloff for their tireless commitment and reliability.

The financial support of the German Federal Ministry for Food and Agriculture (BMEL) and the FBN Dummerstorf is greatly acknowledged.

I would like to thank all my friends and my brother for the support during the last few years. Special thanks to Marcel, Sarah, Danuta, Kai-Uwe, Martina, Michael and Tomas.

For the proof reading, critical review and help with formatting of this manuscript I would like to thank Dr. Achim Salamon and my parents.

I want to explicitly thank my partner Achim for enriching my life and the great support during the last few months, especially in situations, where only hope was left.

My deepest gratitude goes to my parents for their continuous love and support and for making my education possible. Without you, I would not be there, where I am today.