Transmission and pathogenesis of hepatitis E virus infection in European wild boar and domestic pigs, and the establishment of a small animal model for hepatitis E

Thesis

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by
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to my family
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<td>RT-PCR</td>
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<tr>
<td>SLA</td>
<td>swine leukocyte antigen</td>
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<tr>
<td>SPF</td>
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<td>virus-like particles</td>
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1. Introduction

A perorally transmitted non-A, non-B hepatitis virus was first suspected in 1980 during an outbreak of acute viral hepatitis in India. In 1990, the etiological agent of the disease was defined as hepatitis E virus (HEV), a name adopted later by other authors. Previously, it was assumed that HEV transmission occurs by the human-to-human route only, and primarily by water supplies contaminated with fecal excretions of humans. Therefore, the next major advance was the discovery of a closely related virus among pigs in the USA, initially named as swine HEV, which was genetically different from the previously recognized genetic groups of human HEV. Especially the detection of similar HEV strains in swines and humans suggested zoonotic transmission routes for HEV. Nowadays, it is widely accepted that HEV causes hepatitis E in humans especially in developing countries, but sporadic and autochthonous cases do also occur in industrialized nations.

HEV is a non-enveloped, positive-stranded RNA virus and member of the genus *Hepevirus* in the family *Hepeviridae*. To date, four major mammalian genotypes (HEVgt1 to gt4) have been identified, but HEV-related viruses have also been detected in rats, rabbits, different carnivores, bats, moose, camels, chicken and salmonid fish. While HEVgt1 and gt2 are exclusively found in humans, HEVgt3 and gt4 are zoonotic and commonly infect both humans as well as other mammalian species like pigs, wild boar and deer. HEV is unique among the known hepatitis viruses, in that it has an animal reservoir. HEVgt1 and gt2 are responsible for the majority of HEV infections in humans in endemic areas of Asia, Africa and Mexico. In contrast, HEVgt3 and gt4 have been identified with increasing frequency in sporadic and autochthonous human cases in Europe, the USA, China and Japan. Sources are mainly contaminated raw or undercooked meat derived from domestic pigs and wild boar, as well as deer. This was confirmed by the molecular characterization of HEV sequences recovered from food products and from affected patients. In Europe, main reservoirs of HEV are pigs and wild boar confirmed by serological and molecular analyses. In Germany, wild boar is discussed as one of the main sources of human autochthonous infections. Moreover, cross-species infections with HEVgt3 and gt4 have been demonstrated experimentally. However, not all sources of human infections have been identified thus far and in many cases, the origin of HEV infection in humans remains unknown. Understanding HEV interspecies
and intraspecies transmission is needed to implement efficient prevention and control measures.

HEV infections in humans normally lead to an acute, often self-healing disease, but cases of fulminant liver failure and extrahepatic manifestation have also been described. Especially pregnant women with concomitant HEV infection are at high risk. However, chronic infections are also observed in patients co-infected with human immunodeficiency virus and in immunosuppressed patients. The swine and other mammalian animal species infected by HEV generally remain asymptomatic, whereas chickens infected by avian HEV may develop a disease known as hepatitis-splenomegaly syndrome. Both viral as well as host factors determine the course of HEV infection, but mechanisms leading to different outcomes are only partially understood.

Several studies have been performed in domestic pigs by intravenous or contact transmission of domestic pig-derived HEV characterized by subclinical HEV infection and mild histopathological signs of hepatitis. Aside from effective horizontal HEV transmission among experimentally infected domestic pigs, phylogenetic studies also indicate natural HEV transmissions from domestic pig to wild boar. Conversely, little is known about the course of HEV infection in European wild boar and their role in HEV transmission to domestic pigs to date. Experimental challenge studies have not been carried out yet in wild boar. The present study therefore aimed on characterization of the pathogenesis of a wild boar-derived HEVgt3 strain in experimentally infected European wild boar, and to detect possible horizontal transmissions to domestic pigs (manuscript I). In humans, the infection with HEV can lead to microscopically visible hepatic lesions, particularly during chronic HEV manifestation. Pathological lesions in wild boar have not been investigated yet. Hence, the question arose whether histological examination of porcine liver would also show inflammatory or degenerative processes associated with wild boar-derived HEVgt3 infections. Compared to common domesticated swine breeds, the miniature pig offers several breeding and handling advantages. Miniature pigs have been used already in several fields of biomedical research as feasible animal models, but HEV infection studies have never been carried out in this pig breed. Therefore, the second goal of this first study was also to assess the manifestation of HEV infection in miniature pigs under experimental conditions, and to prove their suitability as a model for HEV infection as well.
Data on the cellular immune response following an HEV infection in humans are sparse, while data on pigs are missing completely to our knowledge. Thus, the second study aimed to characterize cellular immune responses in experimentally HEVgt3 infected wild and domestic swine and to determine the influence of dexamethasone-induced immunosuppression on porcine HEV pathogenesis (manuscript II). To answer this question, comparative analyses of cellular and humoral immune responses, viral replication and microscopically visible hepatic lesions were performed. Therefore, dexamethasone-treated and non-treated wild boar were inoculated intravenously with HEVgt3. In order to prove horizontal HEVgt3 transmission and determine differences in the susceptibility to HEVgt3 infection in terms of the immune status, dexamethasone treated and non-treated domestic pigs were kept in contact to infectious feces derived from the intravenously inoculated wild boar. Additionally, the immunosuppressive effect of dexamethasone was monitored in non-infected control pigs.

While acute HEV infections in swine have been studied in more detail, little is known about persistent HEV infections in pigs possibly leading to chronic hepatitis E. Consequently, another purpose of the third study was to prove the occurrence of persistent HEV infection also in swines (manuscript III). If possible, the course and infectivity of porcine chronic hepatitis E should be characterized, and its suitability as an animal model for human chronic hepatitis E infection assessed.

Wild boar are extremely limited in their availability for experimental studies, and proper handling is difficult. Moreover, infection studies in pigs under high containment conditions are very expensive. As a consequence, animal numbers in experiments are often quite low. Moreover, the biological diversity in common swine breeds needs to be appropriately considered for the interpretation of results. The development of a suitable small animal model for hepatitis E would therefore be a major progress for studying HEV biology, especially in respect to the investigation of immunopathogenetical mechanisms, and for the evaluations of novel therapeutics and vaccines. Therefore, the fourth study aimed to assess the susceptibility of different wild-type and knock-out mice, Wistar rats, and European rabbits to wild boar-derived HEVgt3 as determined by the analysis of HEV replication and anti-HEV antibody responses (manuscript IV). Moreover, the influence of dexamethasone treatment in rats on their susceptibility to wild boar-derived HEVgt3 was analyzed. Additionally, the protective ability of a HEV vaccine candidate in HEVgt3 inoculated rabbits was assessed in a proof of
principle approach.

The objective of this thesis was to provide insight into the HEV pathogenesis in European wild boar and to characterize the transmissibility of the wild boar-derived HEVgt3 infection to domestic pigs. Moreover, a suitable small animal model for porcine HEV infection should be established. With respect to the HEV perpetuation in its reservoirs and possible public health risk, especially regarding wild boar as a main source of human autochthonous infection, these studies contribute to the understanding of the dynamics and biology of this zoonotic disease.
2. Literature review

2.1 Historical background of hepatitis E

A perorally transmitted non-A, non-B hepatitis virus was first suspected by Khuroo in 1980 during an outbreak of acute viral hepatitis in the Kashmir Valley, India [3]. Most patients were 11 to 40 years old, and cases occurred in villages with a common water source. The outbreak was characterized by a high occurrence rate and mortality among pregnant women. The mode of spread of the epidemic, length of incubation, clinical features and biochemical test results of the patients studied were similar to that of hepatitis A. A few months later, Wong et al. reported the results of retrospective serological testing of sera stored since a large outbreak of hepatitis that had occurred in New Delhi during 1955 to 1956, and two smaller outbreaks in Ahmedabad (1975 to 1976) and Pune (1978 to 1979) in the Western part of India [4]. In this study, none of the specimens deriving from the three outbreaks showed evidence of acute hepatitis A and only a few had markers of acute hepatitis B, providing valuable support to the existence of an enteric non-A, non-B hepatitis agent [4]. This paved the way for the discovery of a new hepatitis agent. However, a recent re-review of monographs published in the 19th century identified several descriptions on hepatitis E-like outbreaks already in the last decade of the 18th century, mainly in Western Europe and several of its colonies [5]. Teo et al. described also that one of the first reported putative hepatitis E outbreaks occurred in 1794 in the Palatinate Luedenscheid, Germany [5]. However, a detailed description of the course of this type of hepatitis was not available until 1983. Typical acute hepatitis was reproduced by an experimental infection of a human volunteer after oral administration of pooled stool extracts from presumed cases of epidemic non-A, non-B hepatitis [6]. Hence, spherical virus-like particles (VLPs) were visualized by immune electron microscopy in the human volunteer’s stool samples collected during preclinical and early postclinical phases [6]. Furthermore, cynomolgus monkeys inoculated with a stool suspension from the volunteer showed excretion of similar VLPs, liver enzyme elevation and histological changes of hepatitis, fulfilling Koch’s postulates [6]. In 1990, the etiological agent of the disease was defined as hepatitis E virus (HEV), a name adopted later by other authors [7]. Reyes et al. identified also similar genomic sequences in clinical specimens obtained from several geographical regions at different time-points [7]. As a result of the successful sequencing of the entire HEV genome the first immunogenic epitopes were identified [8,9]. Hence, the
development of serological assays able to detect anti-HEV antibodies was realized [10]. In most patients, the infection with HEV was shown to be associated with the appearance of anti-HEV IgM by the time of development of disease, and anti-HEV IgG responses occurred shortly thereafter [10]. Before specific serological tests were introduced, the diagnosis of hepatitis E was only based on the exclusion of serological markers of HAV and HBV infections in combination with the monitoring of epidemiological features [1]. Up to then it was assumed that HEV transmission occurs by the human-to-human route only, and primarily by water supplies contaminated with human fecal excretions. The next major advance was the discovery of a closely-related virus among pigs in the USA, named as swine HEV, which was genetically different from the two previously recognized genetic groups of human HEV [11]. Simultaneously, a few indigenous human cases of hepatitis E were identified in the USA, and genomic sequences of these human HEV isolates most closely resembled those from the swine HEV [12-14]. This prompted studies for HEV-like viruses among several animal species around the world and among human cases. In 1999, serological evidence for HEV infection in swine was also reported in the Australian wild boar [15]. In the same year, a HEV-related agent in chickens which had appeared already in the 1980s in Australia in chickens [16] was further characterized. Several studies led to the discovery of hitherto unsuspected zoonotic transmission of the virus, leading to a major shift in the understanding of HEV. Especially the detection of similar HEV strains in swine and humans suggested additional zoonotic transmission routes for HEV. In the following years, further studies indicated that mainly sporadic hepatitis E cases in industrialized countries are caused by zoonotic transmission of HEV. Domestic pigs, wild boar, deer and other mammals were identified as possible HEV reservoirs [17,18]. Nevertheless, novel HEV-related viruses were also found in different rat species, rabbits, ferrets, minks, fox, several bat species and moose, and a distantly related agent was described in salmonid fish species [1]. In any event, their zoonotic potential has not yet been elucidated sufficiently and their impact on human hepatitis E epidemiology is mostly unknown. Based on recent findings, new taxonomic grouping schemes of the viruses within the family Hepeviridae were suggested previously [1,19].
2.2 **Taxonomic considerations within the family *Hepeviridae***

Current published descriptions of the taxonomical classification within the family *Hepeviridae* are contradictory in terms of the designation of species and genotypes. According to the actual taxonomy stated by the International Committee on Taxonomy of Viruses, the family *Hepeviridae* comprises only the single genus *Hepevirus* [20]. This genus comprises the single species HEV, which is formed by HEV genotype (gt) 1 to gt4. Rabbit HEV is considered as belonging to gt3 and rat HEV is listed as a tentative additional species of the genus *Hepevirus*. The species avian HEV is comprised within the family *Hepeviridae*, but not assigned to a genus so far. Up to now, other recently discovered viruses are not considered in the classification scheme. Therefore a consensus classification system for the family *Hepeviridae* is currently unavailable, but a new taxonomic scheme has recently been proposed [19]. It is suggested that the family is divided into the genera *Orthohepevirus* (*Orthohepevirus* A with isolates from the human, pig, wild boar, deer, mongoose, rabbit and camel; *Orthohepevirus* B with isolates from the chicken; *Orthohepevirus* C with isolates from the rat, greater bandicoot, Asian musk shrew, ferret and mink; *Orthohepevirus* D with isolates from the bat) and *Piscihepevirus*. A phylogenetic tree based on the full-length genomic sequences with genotype classification of known animal strains of HEV is depicted in Figure 2.1.

![Figure 2.1 Phylogenetic tree of the different HEV species.](image)

Full-length (or near: moose partial) sequences were aligned using Muscle and the bootstrap consensus tree was constructed with the neighbor joining method (MEGA). Light blue circle indicate strains with cross species transmission capability in experimental models. Reprinted from Pavio et al. 2015 [21].

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2.3 **Morphology and genome organization of HEV**

HEV is a non-enveloped small virus with a diameter of 27–32 nm. Although HEV particles present in feces and bile are non-enveloped, those in circulating blood and culture supernatant have been found to be covered with a cellular membrane, similar to enveloped viruses [22]. A similar membrane hijacking has been reported for HAV, a picornavirus, with broad implications for viral egress mechanisms and host immune responses [23]. The HEV genome is a positive-sense, single-stranded RNA composed of approximately 7200nt, which is capped and polyadenylated (Figure 2.2) [8,24]. The HEV genome comprises a 5′ untranslated region (UTR), three open reading frames (ORF1, ORF2 and ORF3) and a 3′ UTR followed by a poly-A tail [25]. ORF1 encodes non-structural proteins, including a methyltransferase, papain-like cysteine protease, macrodomain, helicase and RNA-dependent RNA polymerase [26,27]. Between the protease and the macrodomain, a hypervariable region containing a proline-rich hinge was found [28]. ORF2 and ORF3 overlap, and their proteins are translated from a bicistronic subgenomic RNA that is 2.2 kb in length into the structural proteins [29,30]. ORF2 encodes the viral capsid protein of 660 amino acids (aa) that is responsible for virion assembly [31], interaction with target cells [32,33], and immunogenicity [34]. The ORF2 protein consists of three linear domains: the shell domain (aa 129 to 319), the middle domain (aa 320 to 455), and the protruding domain (aa 456 to 606), harboring the neutralizing epitope(s) [35-38]. The ORF3 protein is a small protein of approximately 113 to 114 aa which is thought to act as an adaptor to link the intracellular transduction pathways, reduce the host

![Figure 2.2 Organization of the HEV genome.](image)

Non-structural proteins are translated from ORF1 while the ORF2 and ORF3 structural proteins are translated from a single subgenomic RNA. UTR, untranslated region; Y, Y-domain; PCP, papain-like cysteine protease; HVR, hypervariable region; X, macro domain; RdRp, RNA-dependent RNA polymerase. Reprinted from Debing et al. 2014 [2].

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inflammatory response and protect virus-infected cells [39]. Recently, it was found that ORF3 proteins play an important role in virion egress from infected cells [40-43].

2.4 HEV replication cycle

Although understudied, the HEV replication cycle seems typical for a single-stranded RNA virus of positive polarity (Figure 2.3) [2]. The virus particle first binds to heparan sulfate proteoglycans on the host cell membrane [33], transfers to its so far unknown cellular membrane receptor(s) and the resulting complex is internalized through clathrin-mediated endocytosis [44]. Subsequently, the capped viral genome is released from the virion during the uncoating process and directly translated by the host cell ribosomal machinery. The non-structural proteins thus generated an environment which allows the replication of the viral

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genome, probably in specific viral replication complexes, and the production of subgenomic RNA that is translated into the structural proteins from ORF2 and ORF3. Full-length RNA progeny is assembled with ORF2 capsid protein into viral particles which are subsequently released from the cell in a non-lytic fashion [2,45]. During release, HEV particles probably acquire a lipid bilayer (envelope) and associated ORF3 protein which are removed later by bile salts and enteric proteases respectively [43,46]. It was previously demonstrated that an intact PSAP motif in the ORF3 protein is required for the formation and release of membrane-associated HEV particles with ORF3 proteins on their surface [41]. Moreover, the tumor susceptibility gene 101 and the enzyme activities of vacuolar sorting proteins are involved in the release of HEV virions, indicating that HEV utilizes the multivesicular body pathway to release HEV particles, which is promoted by the cellular mechanism of endosomal sorting complexes required for transport [42]. Furthermore, membrane-associated HEV particles are released together with internal vesicles through multivesicular bodies by the cellular exosomal pathway [47]. Additionally, hepsin reported to function as an inhibitor of several tumors in humans, was confirmed to have specific interactions with the ORF3 protein [48].

The lack of an efficient cell culture system for HEV has greatly hampered detailed analyses of this virus [22]. Several cell culture systems, especially the human hepatocellular carcinoma cell line PLC/PRF/5 and the human lung carcinoma cell line A549, repeatedly have been shown to support the replication of HEVgt3 and gt4 strains, although with rather low efficiency [22]. The cell lines A549 and HepG2/C3A were shown to support the assumption of replication of HEVgt1 strains, albeit with low efficiency [49,50]. In addition, three-dimensional cell culture systems and porcine cell cultures have been used for isolation of gt3 strains [51,52]. Moreover, an in vitro model was developed to replicate human HEV in primary cynomolgus macaque hepatocytes [53]. Recently, pig-derived HEV replicated in primary human hepatocytes as well [54]. Distinct patient-derived gt3 strains carrying insertions in their hypervariable ORF1 region have recently been shown to replicate more efficient in cell culture [55,56]. Jirintai et al. reported successful propagation of rabbit HEV in human liver- and lung-derived carcinoma cell cultures [57]. When liver homogenates obtained from wild rats (Rattus rattus) in Indonesia were inoculated onto human hepatocarcinoma cells, the rat HEV replicated efficiently in PLC/PRF/5, HuH-7 and HepG2 cells [58].
2.5 Host association and virus transmission of HEV

HEV is the etiological agent of acute hepatitis E, an infection considered to be endemic in many developing countries in Africa and Asia. HEV is transmitted primarily by the fecal–oral route and has been reported to occur as large waterborne epidemics and small outbreaks in developing areas [59]. Over the last decade, an increasing number of sporadic locally acquired cases occurred in several high income countries, in which it is often not possible to establish the route of acquisition of infection [21]. There are currently four well-characterized genotypes (gt) of HEV in mammalian species, although numerous novel strains of HEV likely belonging to either new genotypes or species have recently been identified. HEVgt1 and gt2 are only observed in human infections, whereas gt3 and gt4 are known to infect humans and an expanding host range of animal species, respectively [1,18]. Hepatitis E is considered a zoonotic infection with domestic pig and wild boar serving as the main reservoir for human infections [21]. Viruses related to HEV have been identified in several other animal species including chicken, mongoose, deer, rabbit, mink, fox, camelids, ferret, bat, and fish. The known geographical distribution of mammalian HEV, avian HEV and HEV-related viruses is shown in Figure 2.4.

Fecally contaminated drinking water seems to represent the major vehicle for HEVgt1 and gt2 transmission, but direct transmission of human HEV by contact with HEV-containing feces may occur. Contrarily, the transmission routes of other hepeviruses are not so clearly understood. Zoonotic transmission of HEVgt3 and gt4 is primarily connected with the consumption of HEV-contaminated meat and meat products, whereas human infections by contact with pigs seem to occur sporadically as well. Understanding HEV interspecies transmission is needed to implement efficient prevention and control measures. Figure 2.5 depicts a summary of the results of experimental cross-species infection trials.

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Figure 2.4 Geographical distribution of hepeviruses.
The distribution of human epidemic HEV genotypes 1 and 2 (A), zoonotic HEV genotypes 3 and 4 (B), and HEV-like viruses detected in rabbits, chicken, rats, bats, ferrets, minks and fox (C) is shown. Reprinted from Johne et al. 2014 [1].
2.5.1 Mammalian HEV

2.5.1.1 Human genotypes 1 and 2
HEVgt1 and gt2 have caused epidemics and outbreaks of hepatitis E in tropical and some subtropical regions usually due to transmission by fecal contamination of water supplies [60,61]. Until now, HEVgt1 and gt2 have only been found in humans and in environmental specimens contaminated with human excrements [60,61]. In outbreaks, person-to-person spread of HEVgt1 and gt2 is thought to be uncommon. However, recent studies from Uganda showed that household factors may be more important than previously believed [62]. The major age group affected by gt1 and gt2 infections ranges between 15 and 30 years and clinical symptoms are normally characterized by an acute self-limiting hepatitis [63]. High mortality rates up to 26.9% were described in pregnant women, with most fatal cases in the third trimester [64,65]. The high mortality observed during pregnancy seems to be unique for

![Figure 2.5 Summary of the results of experimental cross-species infection trials.](chart.png)
HEVgt1 and gt2 infections, and is under controversial discussion [63,64]. Many hepatitis E outbreaks caused by HEVgt1 were described in different countries of the Asian and African continent. Large outbreaks were recorded between 1986 and 1988 in China [66] and in 2008 in India [67]. Additional outbreaks caused by HEVgt1 were reported in Algeria, Chad, Namibia and Sudan [68-70]. HEVgt2 was first detected by the analysis of samples from a hepatitis E outbreak in Mexico between 1986 and 1987, and was subsequently designated as the Mexican isolate of HEV [71,72]. Thereafter, hepatitis E cases caused by HEVgt2 were rarely reported, but recently there were cases from Namibia, Nigeria and Sudan [69,70]. Initially, HEVgt1 was experimentally successfully transmitted to cynomolgus macaques and tamarins [73]. Thereafter other non-human primate species were used repetitively as suitable animal models for human hepatitis E caused by HEVgt1 and gt2 [74-77]. Interestingly, trials to induce severe liver injury in gt1 infected pregnant rhesus monkeys failed [78]. Transmission of HEVgt1 and gt2 isolates to pigs by intravenous inoculation was not successful [79]. Although an earlier study indicated susceptibility of laboratory rats to gt1 infection [80], recent trials to infect laboratory rats with gt1 or gt2 failed [81,82]. Another study in C57BL/6 mice demonstrated that animals intravenously inoculated with gt1 were not susceptible to HEV [83]. Remarkable genetic heterogeneity within HEVgt1 and gt2 exists [84]. Based on complete as well as partial genomic sequences several HEV subtypes have been described. Lu et al. defined the HEV subtypes 1a to 1e and 2a to 2b [85], but more recent analyses could not confirm the presence of genetically distinct subtypes [84,86]. Anyhow, differences in the virulence of distinct subtypes were suggested previously [87,88].

2.5.1.2 Animal-associated zoonotic genotypes 3 and 4

In most cases of autochthonous hepatitis E in developed countries the source and route of infection cannot be identified. However, the evidence suggests that most cases may be due to consumption of uncooked or undercooked HEV contaminated pork or game meat [89-91]. The pig is considered as primary host for HEVgt3 and gt4, and both genotypes are found in pigs throughout the world [63]. HEVgt3 was discovered in 1997 when samples of domestic pigs from the USA were analyzed [11]. Later an HEV isolate closely related to this pig virus was detected in a hepatitis E patient from the USA [12,13]. Until now, several HEVgt3 strains have been detected in humans and different animal species distributed worldwide [92]. HEVgt3 is responsible for the majority of human infections in industrialized countries in
Europe, Japan and the USA [92-94]. Recently, HEVgt3 strains have also been detected in pigs from the African continent [95]. In 1998, HEVgt4 was first identified in specimens from sporadic human hepatitis cases from Taiwan [96], and thereafter also in pigs from the same geographical region [97]. Simultaneously, HEVgt4 strains were identified in hepatitis patients from China and the complete genome was sequenced [98,99]. In China, HEVgt4 represents the most commonly detected HEV strain in humans and pigs [100-102], but HEVgt4 strains are also endemic in Japan [103,104]. Recently, the HEVgt4 was detected in Europe which may indicate an actual spread of this genotype to another continent as well. HEVgt4 was found in pigs from The Netherlands and in autochthonous human hepatitis E cases from France and Italy [105-107]. Serological studies suggested that most human infections with HEVgt3 and gt4 are asymptomatic [63]. Symptomatic HEV infections have primarily been seen as sporadic acute hepatitis cases. However, small outbreaks caused by HEVgt3 respectively gt4 have been described as well [107,108]. Contrary to HEVgt1 and gt2 infections, these genotypes can cause hepatitis especially in the middle-aged and elderly. In addition, males were found approximately 4-fold more often diseased than females [63]. Previously, chronic infections in immunocompromised transplant patients or the manifestation of neurological disorders have been associated with HEVgt3 infection [109-111]. The main transmission routes of HEVgt3 and gt4 are presumed to be zoonotic and foodborne [94]. In Europe, Japan and the USA, HEV-specific antibodies were frequently detected in domestic pigs underlining their role as a source of HEV infection [92,112-114]. Case studies in Japan and France indicated foodborne routes of HEVgt3 transmission originated from the ingestion of HEV-containing meat or sausages prepared from wild boar, sika deer or pig [90,115,116]. Recently, hepatitis E cases due to the ingestion of pork meat and entrails containing HEVgt4 were described in Japan [117]. Raw pig liver sold in grocery stores in Hokkaido was also tested for the presence of HEV. Interestingly, two HEV isolates from pig livers were identical with isolates obtained from hepatitis E patients [91]. The thermal stability of HEV has been investigated. HEV remains viable even after heating to 56 °C for 60 min with 1% of infectious viral particles remaining [49]. Heating to temperatures of 71 °C for 20 min is required to fully inactivate the virus [118]. Direct contact with pigs is another possible route of HEV transmission. Seroprevalence studies in the USA showed that pig handlers and veterinarians were more likely to be anti-HEV IgG positive indicating
previous exposure, compared with the normal population [119]. Other routes such as environmental transmission or transmission by blood/blood products may also be important [120]. As recently suggested for porcine heparin products, swine-derived products used in human medicine may also pose a risk of HEV transmission [121]. Moreover, the risk of using infected pig manure on farmland also remains to be determined. There is currently no evidence to suggest that person-to-person spread occurs with HEVgt3 and gt4. The host range of HEVgt3 and gt4 is broad and natural infections have been detected in individuals belonging to the order Artiodactyla, in mongoose, monkey and rats [92,122,123]. Single reports of HEVgt4 detection in cattle and sheep exist [94]. Experimental inoculation of non-human primates using pig-derived HEVgt3 and gt4 strains confirmed the capability of zoonotic transmission [17,124]. Pigs have repeatedly been shown to be susceptible for experimental infection by intravenous inoculation with HEVgt3 and gt4 isolates [79,125]. Moreover, experiments to infect laboratory rats with gt3 were not successful [81,82,126]. Injection of transcripts of a HEVgt4 cDNA into the liver of rats led to transient seroconversion [127]. This genotype was also shown to be infectious for Balb/c nude mice [128]. Another study in C57BL/6 mice demonstrated that animals intravenously inoculated with gt3 and gt4 were not susceptible to HEV [83]. Experimental infection of rabbits with HEVgt3 and gt4 strains resulted in seroconversion; however, virus shedding was dependent on the strain used [129]. Experimental infection of Mongolian gerbils with HEVgt4 was successful as well [130]. For HEVgt3 and gt4 high sequence variability has been reported. Nucleotide sequence divergences of up to 27.1% were found for HEVgt3 and 19.9% for HEVgt4 isolates based on complete genome sequences [84]. In the subtype classification system suggested by Lu et al., the subtypes 3a to 3j and 4a to 4g were defined [85]. However, recent analyses using more strains and larger genomic regions could not consistently confirm this subgrouping [84,86]. Some studies tried to link specific nucleotide substitutions present in genetically divergent HEVgt3 and gt4 strains to the severity of the disease caused by them [131-133]. By comparative genome sequence analyses, two silent mutations in HEVgt4 isolates and one amino acid exchange within the helicase domain of HEVgt3 strains have been identified as presumed indicators of increased virulence of strains derived from mild and severe clinical cases [131,132].
2.5.1.3 **Wild boar-associated novel genotypes**

Although a consensus classification system for HEV genotypes is currently unavailable, HEV variants from Japanese wild boar (*Scrofa scrofa leucomystax*) have provisionally been classified into two novel genotypes (gt5 and gt6) [19]. Recent studies in wild boar indicate the presence of different genetic lineages of presumably boar-indigenous HEV strains. In 2011, a HEV strain divergent from gt1 to gt4 was reported from a wild boar sample collected in Japan [104]. Subsequently, in a survey among wild boar that were also captured in Japan, 4.2% of the animals were found with ongoing HEV infections, whereas one possessed a novel HEV variant [134]. Further genetic analyses suggested to place these strains into novel genotypes [84,86]. The geographical distribution, degree of variation as well as the host range and zoonotic potential of these novel HEV variants remain unclear as these isolates have only been found once in single animals. It can be assumed that the presence of several HEVgt3 and gt4 strains and the novel distinct isolates in wild boar indicates that this animal species represents a major reservoir for HEV [1].

2.5.1.4 **Rabbit HEV**

In 2009, a new HEV was isolated from farmed rabbits in China [135]. Subsequently, the virus was detected in farmed rabbits from other regions of China [136-138], Mongolia [57], France [139] and the USA [140-142]. Rabbit HEV was also found in wild rabbits from France [139]. A HEV strain closely related to rabbit HEV was detected in a human hepatitis E patient from France, indicating a possible zoonotic transmission of rabbit HEV to humans [139]. The rabbit HEV strains isolated to date show 73–77%, 70–76%, 75–82%, 71–77% identity to the genotypes 1, 2, 3, 4 respectively, at the nucleotide level and 53–65% identity to avian HEV isolates [143]. Phylogenetic analyses revealed that rabbit HEV isolates are most closely related to HEVgt3 [142,144], although some authors have suggested that they represent a novel genotype [135,145]. Anyhow, in phylogenetic trees all known rabbit HEV strains form a cluster separated from HEVgt3 [146], thus indicating a separate evolution of the viruses in the different hosts. Sequence and phylogenetic analyses revealed that a rabbit HEV strain isolated in the USA is a distant member of the zoonotic HEVgt3, thus raising a concern for potential zoonotic human infection. A unique 90-nucleotide insertion within the X domain of the ORF1 was identified in the rabbit HEV, and this insertion may play a role in the species tropism of HEV [141]. Interestingly, HEV sequences of a human strain in France and rabbit
strains are closely related sharing a 93-nucleotide insertion [139]. A recent study indicated that rabbit HEV belongs to the same serotype as human HEV [147]. Experimental inoculation of rabbits with rabbit HEV led to seroconversion, fecal virus shedding, viremia and elevated liver enzyme levels [129,148]. Evidence of chronicity was also observed in experimentally rabbit HEV infected SPF rabbits as persistent fecal shedding and elevated liver enzymes were observed for more than six months after infection [149]. Under experimental conditions, rabbit HEV has been shown to infect non-human primates as they developed typical hepatitis [150], thus indicating a zoonotic potential of the rabbit HEV. Pigs intravenously inoculated with rabbit HEV strains developed transient viremia and sporadic virus shedding [140]. Moreover, rabbits could be experimentally infected with human HEVgt4 originated from patients with acute hepatitis E [129,148]. Experimental infection of rabbits with HEVgt3 and gt4 strains resulted in seroconversion, but virus shedding was dependent on the strain used [129]. Findings indicate that rabbits may serve as a small animal model for several HEV strains, but it remains to be determined whether the rabbit model also applies for wild boar-derived HEVgt3 infections. A study in China found no evidence of natural cross-species infection between pigs and rabbits [136].

2.5.1.5 Rat HEV

By the development of a hepevirus-specific broad-spectrum RT-PCR rat HEV was first detected in Norway rats (Rattus norvegicus) caught in Germany [151]. A primer walking-based approach resulted in the determination of the entire genome sequence of two strains, which showed genome sequence similarities of only 49.5–55.9% to avian HEV strains and HEVgt1 to gt4 strains, respectively [152]. These investigations revealed the existence of three additional putative open reading frames in rat HEV [152]. Since then, rat HEV strains were detected from wild rats in the USA, Vietnam, Indonesia and China [81,153-155], suggesting that rat HEV infection is widely distributed in wild rats around the world. The multiple detection of distinct HEV strains in different rat species resulted in the assumption of a rat host specificity of this virus type [152,155-157]. However, rat HEV-related sequences was recently found in the Greater Bandicoot rat (Bandicota indica) and the Asian musk shrew (Suncus murinus), which might suggest a broader host range or spillover infections [153,158]. Previous detection of HEV-specific antibodies in other rodent species may indicate the existence of further, antigenically related HEV strains [159-162]. The full-length genomes of
rat HEV from Germany and Vietnam were determined [152,156,157], and partial sequences of rat HEV detected from USA and Indonesia have been reported [81,155]. Nucleotide sequence analyses suggest that the genome of rat HEV is genetically diverse [156,157]. Furthermore, the detection of a HEVgt3 from various species of wild rats in the USA has recently been reported [123], suggesting that rats could be a host for mammalian HEV as well. However, another report indicated that HEVgt1, gt2 and gt3 failed to infect Sprague-Dawley rats (Rattus norvegicus) [81]. At this time, the susceptibility and infectivity of human HEV in rats still remains controversial. Wistar rats (Rattus norvegicus) could be experimentally infected with a human HEV isolate (genotype not known) in earlier studies [80]. Contrarily, in a more recent study [82] Wistar rats were resistant to intravenously inoculated HEVgt1 originated from a cynomolgus monkey, HEVgt3 collected from a domestic pig, or from wild boar-derived HEVgt4 isolates. The zoonotic potential of rat HEV is controversially discussed. Experimental infections of rhesus monkeys [81] and domestic pigs [140] with rat HEV were not successful. Contrarily, the inoculation of the virus into laboratory rats led to seroconversion and virus shedding [81,82]. A hepatotropism of the virus was found in experimentally and naturally infected rats, although obvious clinical symptoms were not recorded [82,152]. Interestingly, the inoculation of nude rats with rat HEV led to persistent infections [82]. To assess the zoonotic potential of rat HEV serological tools capable of differentiating rat HEV-specific antibodies from those specific for HEVgt3 have been developed as well [156]. A sero-epidemiological study in German blood donors and forestry workers revealed that a few sera of forestry workers showed reactivity with rat HEV-derived antigen [163]. Additionally, a higher reactivity with recombinant rat HEV antigen than with the corresponding HEVgt3 antigen has also been detected in pig sera collected in Germany [113]. Johne et al. 2014 suggested that these results might be explained by rare human and pig infections by rat HEV or by infections with an antigenically related hepevirus [1].

2.5.1.6 Bat HEV

In 2012, Drexler et al. initially described the existence of novel hepeviruses in bats based on a screening in feces, blood and liver collected from several bat species using a hepevirus broad-spectrum RT-PCR [164]. In this study, HEV was detected in African, Central American, and European bats, forming a novel phylogenetic clade in the family Hepeviridae. Bat hepeviruses
were highly diversified and comparable to human HEV in sequence variation, and no
evidence for the transmission of bat hepeviruses to humans was found [164]. Although high
viral loads were found especially in the liver, it is not known whether bat HEV infection
causes any disease in bats [164]. No data are currently available on the ability of experimental
transmission of bat HEV strains to bats or other mammals. Full-genome analysis of one
representative virus, as well as sequence- and distance-based taxonomic evaluations
suggested that bat hepeviruses constitute a distinct genus within the family Hepeviridae [164].
Drexler et al. concluded that this may imply that hepeviruses invaded mammalian hosts not in
recent times, and underwent speciation according to their host restrictions [164]. Moreover
the author assumed that human HEV-related viruses in animals might represent secondary
acquisitions of human viruses. This is in contrast to the assumption that animal precursors
were causally involved in the evolution of human HEV [164].

2.5.1.7 Novel HEV genotype in camelids
In a molecular epidemiology study of HEV in dromedaries in Dubai, United Arab Emirates, a
virus was detected in fecal samples from camels. Hence, comparative genomic and
phylogenetic analyses revealed a previously unrecognized HEV genotype. Complete genome
sequencing of two strains showed >20% overall nucleotide difference to known HEV strains
[165]. Further genetic analyses suggested to place this novel strain into the genus Orthohepevirus A and genotype 7 [86].

2.5.1.8 Novel HEV-related virus in moose
Lately, a novel virus was detected in a sample collected from a Swedish moose (Alces alces). The virus was suggested to be a member of the Hepeviridae family, although it was found to be highly divergent from common HEV genotypes (HEVgt1 to gt4) [166]. Lin et al. suggested that this moose-related HEV may be important as a potential unexplored HEV transmission pathway for human infections, because moose are regularly hunted for consumption in Scandinavia [166]. However, it remains to be investigated whether this novel strain has a zoonotic potential. In comparison with existing HEV genotypes, the moose HEV genome showed a general nucleotide sequence similarity of 37-63% and an extensively divergent putative ORF3 sequence [166]. Moreover, phylogenetic analysis revealed that the moose HEV formed its own branch between the HEVgt1 to gt4, and other divergent animal HEV strains [166]. The sequence divergence between the moose-derived sequences and those
from deer-derived HEVgt3 may indicate a specific association of the detected strain with the moose, or may be alternatively explained by a spillover infection from a so far not identified reservoir [166]. It is not known whether the moose-related HEV causes any disease in moose. Interestingly, the animal was found to be emaciated, had a myocardial injury and infections by Anaplasma phagocytophilum and other agents [166].

2.5.1.9 Putative carnivore HEV strains

By a next generation sequencing approach first molecular evidence of carnivore-borne hepeviruses was obtained for household pet ferrets (Mustela putorius) in 2012 [167]. Thereafter, a distinct hepevirus strain designated fox hepevirus was identified in fecal samples from red foxes [168]. Lately, in farmed mink from Denmark a further hepevirus was identified, but was not detected in wild-living animals [169]. Phylogenetic analysis showed that the mink-associated virus was clearly distinct from, but closely related to recently reported ferret and rat HEV variants [167,169]. Until now, only the complete genome of ferret HEV has been analyzed, whereas from mink hepevirus only a short-sized segment of ORF1 (261 nt), and for fox hepevirus segments of ORF 1 (362 nt) and ORF2 (295 nt) have been explored [1]. Johne et al. suggested the ferret as the most likely reservoir of ferret HEV, and the mink being the reservoir for another carnivore hepevirus [1]. Accordingly, the detection of fox HEV in fecal samples of foxes from a region in The Netherlands may also suggest the fox as a virus reservoir [1]. The authors pointed out as well, that this novel hepevirus may also originate from a prey species, or a so far unknown rodent-borne hepevirus, which only run through the gastrointestinal tract of the foxes [1]. Currently, the clinical impact of infections with the carnivore-associated hepeviruses in their putative reservoirs is not known. Less is reported about the current situation in pets. A recent study reported a sporadic acute hepatitis E case of a 47-year-old man whose pet cat was tested positive for anti-HEV antibodies [170]. Lately, the seroprevalence of HEV infection among pet dogs and cats in China has been investigated. The overall HEV prevalence in 658 dog and 191 cat serum samples was 21.12% and 6.28%, respectively [171].

2.5.2 Avian HEV

Up to now, three genotypes (gt1 to gt3) and a putative new gt4 have been proposed for avian HEV [172-176]. Phylogenetic analysis of the full or nearly complete genome of avian HEV
strains indicated the presence of gt1 in Australia and Korea, gt2 in the USA, gt3 in Europe and China and, more recently, a novel gt4 has been described in Hungary and Taiwan [172-176]. Additional putative genotypes comprising isolates from North America and Europe have been identified suggesting high diversity within avian HEV [177]. First, the avian HEV was associated with big liver and spleen disease in Australia [16] and hepatitis-splenomegaly syndrome in North America [178]. Later, avian HEV infection was associated with disease outbreaks in chicken flocks worldwide [172,177,179-182]. Chickens affected by hepatitis-splenomegaly syndrome typically have enlarged liver and spleen accompanied by a drop in egg production and high mortality rates [182]. Characteristic histopathological changes may include massive coagulative necrosis and non-specific hepatitis with a wide distribution of the avian HEV through the liver [180,181]. Based on serological data, avian HEV is widespread in chicken flocks with seropositive rates of approximately 71% in the USA, 90% in Spain and 57% in Korea [176,179,183]. However, the role of avian HEV in hepatitis-splenomegaly syndrome is unclear, as the virus has also been detected in flocks with no history of this syndrome [176,183,184]. Differences in virus strain, virus dose, diet and age were suggested as potential co-factors for the manifestation of the full spectrum of clinical hepatitis-splenomegaly syndrome [181]. Nevertheless, avian HEV strains recovered from healthy chickens in normal flocks and previously considered to be avirulent were only slightly attenuated in an experimental infection model [185]. Recently, a study determined if recombinant capsid antigen derived from an avian HEV strain can induce cross-protection against mammalian HEVgt3 challenge in a pig model. Although the protection was not complete in all pigs, the results from this study revealed a certain cross-protection of the avian HEV capsid antigen against mammalian HEVgt3 [186].

2.5.3 Fish hepevirus

In 1988 a virus was initially isolated from a cutthroat trout named cutthroat trout virus (CTV) [187], and was recently found to be similar to mammalian and avian hepeviruses regarding morphology and genome organization [188]. Using a salmon embryo-derived cell culture system, a slow and focal cytopathic effect could be demonstrated upon CTV replication [187]. Batts et al. detected a broad geographical distribution of this virus in the western part of the USA, including additional trout species [188]. The genome sequence similarity of the prototype CTV isolate to mammalian HEVgt1 to gt4, rat HEV and avian HEV was found to
be only between 38% and 49% [188]. The highest amino acid sequence similarity was found within the ORF1-encoded polyprotein [188]. CTV-ORF3 is located centrally within ORF2, which is different to all other hepeviruses, and the protein encoded by ORF3 shows only 13–16% amino acid sequence similarity to that of HEVgt1 to gt4 [188]. Based on the high diversity of the CTV genome it was discussed, whether the HEV-like virus from fish should be classified into the family *Hepeviridae* or not [84]. Although the virus could be re-isolated from some experimentally CTV infected trout and salmon species, mortality or microscopic pathology was not observed [187]. Molecular epidemiological studies indicated a long-term maintenance of the virus in populations, although a life-long carrier state and vertical transmission have not yet been demonstrated for CTV [188]. The availability of a persistently CTV infected cell line as well as susceptible animal models were prerequisites for the screening of putative antiviral drugs [188]. Recently, salmon embryo cells were used to demonstrate antiviral effects of different substances, such as ribavirin, testosterone and 17β-estradiol [189].
2.6 Human HEV infection

2.6.1 Acute hepatitis E

Hepatitis E affects humans in both industrialized and developing countries worldwide. In industrialized countries sporadic cases of hepatitis E occur associated with ingestion of contaminated animal meats, shellfish, and contact with infected animals [94,190]. Large waterborne outbreaks due to poor sanitation conditions occur in developing countries such as Bangladesh, India, Mexico, China, Egypt and other parts of Africa [190]. HEVgt1 and gt2 strains are limited to the human population, whereas gt3 and gt4 strains are zoonotic and infect humans and other animals. Human to human transmission of HEV is considered rare; however, transmission through blood products by transfusion has been reported [92]. The clinical features of acute autochthonous hepatitis E caused by HEVgt3 and gt4 are indistinguishable from those of hepatitis E in developing countries, except that patients are usually older and mostly male [191]. However, in most cases the source of infection remains commonly unclear [192].

In most patients, hepatitis E causes an asymptomatic and uncomplicated course of disease in which the virus is quickly cleared [18]. Frequent symptoms seen in acute hepatitis E include anorexia, jaundice, darkened urine coloration, hepatomegaly, myalgia, elevated liver enzyme levels in the blood, and occasionally abdominal pain, nausea, vomiting, and fever [59,193]. After an incubation period of two weeks to two months, an acute HEV infection in humans starts with a transient period of viremia and fecal virus shedding, whereby the symptomatic phase associated with elevated liver enzyme levels lasts days to weeks (Figure 2.6). In the majority of patients the disease is self-healing, with symptomatic and biochemical recovery within four to six weeks [59,193]. In humans, the clinical course and pathogenesis of HEV infection can vary substantially between individuals (Figure 2.7). In sporadic, acute hepatitis E cases prolonged fecal virus shedding might be possible [194]. Moderate to severe liver damage including swollen hepatocytes with giant cell formation, lymphocytic portal infiltration, cholangitis, apoptosis of hepatocytes and parenchymal necrosis has been seen in acute autochthonous hepatitis E [195,196]. The severity of HEV infection is considered dose-dependent, and alcohol abuse or concurrent hepatic diseases have been described as

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contributing factors [6,59,193]. It has been shown that hepatitis E patients with underlying chronic liver disease have a poor prognosis, as they frequently develop acute or subacute liver failure [197-199]. Furthermore, in a person voluntarily infected with HEV, viremia was detected before the onset of clinical symptoms, and disappeared from the peripheral blood at the peak of disease. Anti-HEV IgM antibodies appear shortly after the onset of hepatitis [200,201]. They decrease parallel with clinical symptoms, while anti-HEV IgG antibodies normally persist for several years [201,202]. However, not all HEV infected patients had detectable anti-HEV IgM in the first week following symptom onset, and the proportion of IgM-positive cases declined monotonically over the time. Interestingly, most of the initially IgM-negative patients had detectable HEV RNA in feces or serum [203]. In a small cohort study, only six of ten hepatitis E patients with anti-HEV IgM during the acute phase of illness developed detectable anti-HEV IgG [204].

![Figure 2.6 Course of acute hepatitis E virus (HEV) infection.](image)

Acute hepatitis E is characterized by symptoms such as fever, anorexia, vomiting, and jaundice, with onset several weeks after initial exposure. The onset of clinical symptoms coincides with a sharp rise in serum alanine transaminase (ALT) levels. Symptoms may persist for a few weeks to a month or more. ALT levels return to normal during convalescence. HEV RNA may be detected in both serum and stool early in the course of infection, but serum viremia may be difficult to detect by the time cases come to clinical attention. Anti-HEV IgM titers increase rapidly and then wane over the weeks following infection, while anti-HEV IgG antibody titers continue to rise more gradually during the convalescent period and detectable anti-HEV IgG may persist for months to years. Reprinted from Krain et al. 2014 [111].

It was speculated that increases in T helper cells (CD4+CD8-) among patients with hepatitis E may reflect increases in the natural killer cell population, which may in turn produce elevated
levels of INF-γ [205]. In a previous study in humans with acute hepatitis E, patients showed also increased numbers of cytotoxic T cells (CD8+CD4-) and CD4+CD8+ T cells compared to healthy controls [206]. The elevation of CD4+CD25+Foxp3+ and CD4+CD25-Foxp3+ frequencies and the rise in IL-10 suggest that Tregs might be playing also an important role in HEV infection associated with immunosuppressive immune responses [207]. Previously, intrahepatic transcriptome analysis in primates indicated that innate immune responses in HEV infection may play a role as well [77]. Moreover, NK cells may play an important role in HEV infection, and IFN-γ productions by unstimulated PBMCs of hepatitis E patients suggest NK and NKT cells as key players in HEV pathogenesis [111,205,208]. As HEV is presumed to be a non-cytopathogenic virus, immunopathological mechanisms have been proposed as reasons for the liver lesions [209,210]. Patients suffering from fulminant hepatitis E show less marked antiviral cellular reactions but increased humoral responses than patients undergoing mild infections [211]. These findings suggest a correlation between increased IgG production and a poor clinical prognosis, indicating that hepatic injury is mediated by an antibody-dependent enhanced activation of NK or NKT cells [211,212]. In addition, differences in the pathogenicity between HEVg3 and gt4 have been suggested. In a Japanese study, patients infected with HEVg4 had significantly higher liver enzyme levels than those infected with HEVgt3 [213]. A study in France showed that the clinical presentation was more severe in a small group of patients with HEVg4 infections, than in patients with HEVg3 infections [106].

Figure 2.7 Different patterns of hepatitis E virus infection. Reprinted from Kamar et al. 2014 [214].
2.6.2 Chronic hepatitis E

An increasing number of recent studies showed that HEV can cause chronic infection that can rapidly result in cirrhosis, especially in immunosuppressed individuals [110]. Although the majority of chronic HEV cases were diagnosed in the organ transplant patients [110,215], several chronic cases were also seen in patients co-infected by HIV [216] and in patients with hematological neoplasms treated with anticancer chemotherapy [217]. All chronic HEV cases were observed in patients infected by HEVgt3 [110,218]. No case of chronic HEVgt1, gt2 or gt4 infection has been described. All reported chronic HEV infections were autochthonous and were not associated with travel activities. Persisting HEV replication for at least six months is indicative for the diagnosis of chronic hepatitis [219]. However, in the context of organ transplantation it was recently observed that no spontaneous clearance of HEV occurs between three and six months after an acute infection. It can be assumed that chronic HEV infection should be considered when HEV replication persists for more than three months [219]. Chronic active hepatitis characterized by lymphoplasmacellular infiltration, periportal activity and fibrosis was observed in organ-transplanted patients suffering from chronic hepatitis E [220,221]. Interestingly, viremia for over nine months without seroconversion to anti-HEV IgG was observed in this transplant recipient [221]. Kamar et al. suggested that an inadequate antibody response resulted from immunosuppressive therapy [222]. Both viral as well as host factors determine the course of the HEV infection, but detailed mechanisms leading to different clinical outcomes are only partially understood [223]. CD2, CD3 and CD4 positive T cell subsets are significantly decreased in patients who develop chronic hepatitis compared to those with spontaneous HEV clearance [110,224]. The use of a potent immunosuppressant has also been associated with chronic HEV infection [224]. Additionally, HEV-specific T cell proliferative responses are impaired in transplant patients, particularly in those with chronic infection [225]. Moreover, lower serum concentrations of IL-1 receptor antagonist and IL-2 receptor during the acute phase of HEV infection were observed in individuals who developed chronic HEV infection compared to patients with resolving hepatitis [226]. Great quasispecies heterogeneity, a weak inflammatory response, and high serum concentrations of the chemokines involved in leukocyte recruitment to the liver in the acute phase were also associated with persistent HEV infection [226].
2.6.3 **Hepatitis E in pregnancy**

Studies in developing countries reported high mortality rates in HEV infected pregnant females. The mortality rate in hepatitis E patients ranges from 0.5% to 4.0% in immune-competent individuals, and concurrent pregnancy attributed to increases in mortality up to 25% [218]. Pregnant women die of obstetric problems, including hemorrhage or eclampsia, or develop fulminant hepatic failure. Miscarriage and premature birth have also been observed [227]. Up to now, increased mortality in pregnancy has only been associated with HEVgt1 and gt2 [227-229]. The cause of increased maternal mortality in patients with HEV infection is uncertain. During pregnancy the T cell activity is reduced, Th2 cell responses predominate and the antigen presentation in the placenta is down-regulated [227]. Studies in pregnant women with fulminant hepatic failure caused by hepatitis E have shown significant differences in immunological responses [230-234]. Moreover, higher HEV loads were observed in pregnant women compared to non-pregnant women [235,236].

2.6.4 **Extrahepatic disorders**

In addition to the classical hepatic manifestations, HEV can also cause extrahepatic disorders including a range of neurological syndromes, renal injury, pancreatitis, and hematological disorders. Neurological symptoms were described for HEVgt1 and gt3 infections [237]. HEV RNA was detected in the cerebrospinal fluid of patients with chronic HEV infection and neurological symptoms [109]. Evidence of quasispecies evolution associated with neurological symptoms was shown in a kidney transplant recipient infected chronically with HEV. This suggests that HEV-associated neurological injury might be linked to the emergence of neurotropic variants [238]. More recently, cases of Guillain-Barré syndrome associated with HEV infection were described [239,240]. Furthermore, impaired renal function was noted in acute and chronic HEV infections [241,242]. Glomerular disease was observed in immunocompetent [243] and organ transplant patients infected with HEV [242]. The pathophysiological mechanisms of HEV-associated renal injury are uncertain, but cryoglobulinemia may play a role, as cryoglobulinemia has been documented for patients with chronic infection [242]. Furthermore, acute pancreatitis was associated with HEV infection, but only with the HEVgt1 [244-246]. In addition, thrombocytopenia and aplastic anemia have been reported for acute HEV infection [247,248].
2.7 Porcine HEV infection

2.7.1 Natural HEV infection in domestic pigs

In 1997, it was discovered that HEV could not only be found in humans, but also in domestic pigs in farms in the USA [11]. Swines serve as a major reservoir for zoonotic HEVgt3 and gt4 [18], and infection is common and widespread in swine farms worldwide in both developing and industrialized countries [249]. Serological and molecular prevalence studies of HEV in swine revealed variable results in essentially all swine-producing countries. Irrespectively of the human population, swine HEV infection is highly prevalent in pigs [250]. In a study in Canada, HEV RNA was detected in 98.04% of fecal samples and 49.02% of plasma samples [251], while the overall anti-HEV antibody prevalence in pigs was 88.8% in Quebec, 80.1% in Ontario, and 25% in Prince Edward Island [252]. In a nationwide study of 3925 pigs of one to six months of age in Japan, 93% of farms and 57% of swine were positive for anti-HEV IgG, and 84% of pigs were seropositive by six months of age [253]. In The Netherlands, the highest prevalence of HEV RNA in swine at 53% was found on farms housing pigs one to seven months of age [254]. A study in Spain revealed that 98% of swine herds were seropositive since 1985 [255].

Like in humans, the fecal-oral route is considered the main infection route, including direct contact and environmental contaminations responsible for HEV transmission in pigs [256-258]. Urine was identified as a possible source for oral HEV infection as well [259]. Virus transmission via colostrum has been assumed, whereas transplacentar infection is controversially discussed [260-262].

As demonstrated in naturally HEV infected domestic pigs, animals are typically infected at two to four months of age with a transient viremia lasting one to two weeks, and fecal viral shedding lasting three to seven weeks [261]. Effectively 86% of pigs are naturally infected by eighteen weeks of age [251]. Following the decline of maternal antibodies piglets infect themselves with HEV at about two months of age. Seroconversion to anti-HEV IgM, which is associated with the peak of fecal virus shedding, is followed by seroconversion to anti-HEV IgG peaking at four months of age with subsequent clearance of the virus from feces [251,261]. Figure 2.8 depicts an approximation of the natural time course of HEV infection in pigs. However, the age at HEV infection is not strictly dependent upon the proportion of
piglets with maternal antibodies, but is also linked to farm-specific husbandry and hygiene practices [263]. There are several reports on pigs at slaughterhouse carrying HEV [264-267]. Interestingly, the presence of antibodies does not always mean a virus elimination, as anti-HEV antibodies and HEV RNA have been detected simultaneously in sows, suggesting that they act as viral reservoirs [264].

Figure 2.8 Approximation of the natural time course of HEV infection in pigs.
Representation of the kinetics of seroconversion: maternal anti-HEV antibodies (dark blue), IgM anti-HEV (green), IgG anti-HEV (light blue) and fecal excretion of HEV (red) in pigs infected naturally. Reprinted from Pavio et al. 2010 [92].

Whether naturally acquired HEV infections have an effect on the histological status of the liver remains unclear. Following a subclinical course of HEV infection, swines develop only mild microscopic lesions in the liver and associated lymph nodes [11,79]. A prospective study in four naturally HEV infected piglets, the virus of which originated in swine, did not show any apparent gross lesions during necropsy, but characteristic microscopic lesions of hepatitis, lymphoplasmacytic enteritis and interstitial nephritis, respectively [11]. In naturally HEV infected pigs viral antigen was consistently detected in hepatocytes, either diffusely or confined to foci, and positive immunohistochemical signals were also detected in small and large intestine, lymph node, tonsil, spleen, and kidney [268]. However, the presence of HEV RNA in relation to histological alterations of the liver could not be confirmed by another study [264].
2.7.2 **Experimental HEV infection in domestic pigs**

Experimentally infected domestic pigs effectively produce infection with HEVgt3 and gt4. Therefore, this naturally occurring swine model is very useful for the investigation of several aspects of HEV replication, pathogenesis, and cross-species infection [18]. In experimentally HEV infected domestic pigs microscopic lesions include mild to moderate multifocal and periportal lymphoplasmacytic hepatitis, and mild focal hepatocellular necrosis. Moreover, HEV RNA was detectable in feces, liver tissues, and bile [269]. In contact-infected piglets, HEV RNA was detected in feces by one week post infection, with the infectious period estimated as approximately seven weeks [259]. No observable clinical disease or elevation of liver enzyme levels were found in experimentally HEV infected domestic pigs, but viremia, fecal virus shedding and seroconversion could be detected fairly quickly [17,79,269,270]. Comparable to natural conditions, pigs are not susceptible to experimental infections with HEVgt1 or gt2 [17], but viremia and fecal shedding for eight weeks were shown in pigs inoculated with a human HEVgt4 isolate [125]. Experimentally, swines are promptly infected via intravenous inoculation, but the oral route of inoculation is relatively inefficient [93]. The most effective transmission route in experimentally infected pigs has been shown by intravenous inoculation of HEV containing material, such as serum, fecal suspensions, bile or liver homogenate [17,79,259,269,271]. However, intravenously as well as orally infected pigs were able to transmit HEV effectively to contact animals [259,272]. Anyhow, viral RNA in feces and serum was detected earlier and longer in intravenously infected pigs compared to contact-infected animals [259]. Moreover, a human HEV isolate seems to be more virulent in experimentally infected pigs than HEV recovered from swine [269].

As seen in pigs naturally infected with HEV, viral RNA can be found in different tissues of experimentally infected animals [259,270,273]. Moreover, HEV has been detected to a greater extent in pigs infected with a human HEV isolate, and extrahepatic replication sites have been demonstrated [273]. Hepatic lesions such as lymphoplasmacytic infiltration and focal necrosis, however, support the assumption that the liver represents the primary target organ for HEV in pigs [259,269]. Moreover, HEV RNA and viral antigen were mainly found in hepatocytes, Kupffer cells and bile epithelial cells by in-situ-hybridization and immunohistochemistry, whereas lymphatic tissue and the intestinal tract were less frequently tested positive [270,274]. Recently, a study in HEV infected pigs demonstrated that both IFN-
α and Mx protein expression are inversely correlated with the number of HEV infected cells [275]. Thus, the number of HEV infected hepatocytes declined while lymphoplasmacytic hepatitis increased significantly during the experiment [275]. Possibly, IFN-α-induced Mx activity is part of the antiviral response, however, changes in IFN-α and Mx protein expression might be also caused by the decrease of HEV-positive cells in the pigs recovered from infection [275]. More recently, Dong et al. indicated that HEV has developed mechanisms to suppress IFN-α signaling [276].

### 2.7.3 HEV infection in wild boar

In 1999, a first study found anti-HEV antibodies in 17% of investigated free living pigs [15]. Nowadays, the wild boar is assumed to be an important natural reservoir for HEVgt3 and gt4 [104,277-279]. Recent studies in Asia and Europe revealed high HEV seroprevalences and molecular evidence for HEV infection in wild boar [280-287]. Descriptive studies on HEV prevalence in wild boar in Japan vary from 4.5 to 34.3% in anti-HEV seropositivity and 1.1 to 13.3% in HEV RNA detection [253]. In Germany, wild boar is discussed as one of the main sources of human autochthonous infections [288,289], and HEV RNA was reported in sera, bile and liver of wild boar [287,289,290]. HEV isolates obtained from wild boar show great genetic divergence, similar to HEV in humans and domestic pigs [287]. Nevertheless, strains from Europe are all assigned as HEVgt3, and are genetically closely related to human strains circulating in the respective area [291,292].

Severe human HEV infection after ingestion of uncooked liver from wild boar was reported in Japan, whereas foodborne zoonotic transmissions in Europe have been primarily associated with domestic pigs [89,116]. A wild boar derived-HEVgt4 isolate shared 98.6% identity over the entire genome with a human HEV isolate obtained from a patient who developed acute hepatitis after consuming undercooked wild boar meat [104]. Furthermore, individuals with direct contact to pigs are at higher risk of HEV infection and as mentioned before, forestry workers have a higher HEV seroprevalence rate compared to blood donors [163,293,294]. Phylogenetic analyses of Japanese HEV isolates indicated former transmission events from domestic pig to wild boar [295]. Pathological lesions in wild boar have not yet been investigated, but it can be assumed that clinical and pathological effects in HEV infected wild boar are probably similar to those seen in domestic pigs [296].
2.8 Detection of HEV

The commonly used tests for HEV infection in humans and animals include the detection of IgM and IgG anti-HEV antibodies, less frequently anti-HEV IgA, and the detection of HEV RNA. IgM anti-HEV antibodies can be detected during the first few months after HEV infection, whereas IgG anti-HEV antibodies represent either recent or past exposure. HEV infection is usually confirmed by (quantitative) RT-PCR. However, these assays should be standardized to increase reliability. The presence of HEV RNA indicates current infection, be it acute or chronic [297].

Laboratory diagnosis uses serum samples to detect anti-HEV antibodies with western blot assays, indirect enzyme-linked immunosorbent assays (ELISAs) or line assays. For serological tests in domestic pig, meat juice may be a feasible alternative to serum [298]. In humans, anti-HEV IgM levels peak around the time of the ALT peak and may persist for up to five months after the onset of disease [299]. Shortly after the appearance of IgM, IgG antibodies develop and persist throughout the acute and the convalescent phases, remaining high for at least one year after illness recovery. The presence of IgG antibodies is a marker of previous exposure to HEV, but the exact duration of immunity to HEV is not clarified, since reinfection with HEV has been documented [300]. IgA has a similar onset, but although detectable in serum, it is not screened for in most studies [301]. Commercially available immunoassays differ substantially in their sensitivity and specificity for the diagnosis of acute HEV infections [302], with a sensitivity of around 90% and false-positive results varying from 0.3% to 2.5% [302,303]. The variability in HEV genome leads to antigenic variations with important impact on the construction of specific, sensitive and reliable immunoassays [304]. HEVgt1 to gt4 is represented by one serotype, and HEV-specific antibodies appear to be detectable with antigens of all four genotypes. In various assays different antigens were used, even rat HEV antigen [154,305-307]. Broad performance variability among HEV strains with poor sensitivity were seen in assays focusing on pORF3. Conversely, all HEV isolates share some important cross-reactive antigens, especially within the pORF2 being an important key antigen that stimulates the host immune response [100]. HEV recombinant proteins have been used in different formats of diagnostic assays.

For the detection of antibodies against HEV in human serum specimens, a commercially available immunoblot test recomBlot HEV IgG/IgM using overlapping recombinant proteins
covering the entire ORF2, and one recombinant protein covering ORF3 was recently developed (Mikrogen GmbH, Neuried, Germany). This test was adapted for detecting antibodies in swine and used to show HEV-specific seroprevalence of 49.8% among domestic pigs in Germany [114].

Different ELISAs for the detection of anti-HEV antibodies in serum specimens using E. coli-expressed HEV antigens and VLPs expressed in insect cells have been developed [308-314]. Most diagnostic formats are based on the use of host-specific detection reagents, such as anti-human or anti-swine antibody. A host-independent detection is the double-antigen sandwich ELISA [299]. An assay based on this format was recently developed for the detection of anti-HEV in human and animal specimens [315]. Two of the most widespread commercial tests are the MP Diagnostics HEV ELISA kit (MP Biomedicals, Santa Ana, CA, USA; formerly Genelabs Diagnostics, Singapore), utilizing short C-termini of pORF2 and pORF3 of HEVgt1 and gt2, and the Abbott HEV EIA (Abbott Diagnostics, Lake Forest, IL, USA), using the complete pORF3 and a significant portion of pORF2 of gt1. In European surveys the HEV Ab-ELISA kit (Axiom, Buerstadt, Germany) is often used, which is a double-antigen sandwich ELISA based on pORF2 of gt1. The PrioCHECK HEV Ab porcine assay (Prionics, Schlieren-Zurich, Switzerland) is an indirect ELISA for the detection of anti-HEV IgG in domestic pig, and is based on pORF2 and pORF3 from both gt1 and gt3.

In addition, immunochromatographic methods for the detection of serological markers of HEV infections have been developed. A rapid immunochromatographic assay ASSURE™ has been developed by Genelabs Diagnostics, Singapore (nowadays MP Biomedicals, Santa Ana, CA, USA), and evaluated for the detection of IgM anti-HEV in serum specimens from patients with acute hepatitis E infection [316].

To date, detection of HEV RNA by molecular genetic methods is considered the “gold standard” [317]. Detection of RNA is performed by different RT-PCR methods, amplifying genomic fragments in one of the three ORFs [151,287,318-320]. RT-PCR assays published so far are in-house tests characterized by a high degree of performance variability [321]. HEV RNA can be found in blood and feces of patients during the late prodromal phase, and is detectable in the feces for another two weeks [92,322]. The time of viremia is very short;
however, undetectable HEV RNA does not exclude HEV infection. Sequencing of the PCR product allows further determination of the HEV strain.

In immunocompromised patients such as transplant recipients, HEV diagnosis is generally based on the detection of HEV RNA, as testing for antibodies may give false-negative results due to immunosuppression. In this setting, HEV RNA detection and quantification may also have a role in monitoring the clinical response to antiviral therapy [300,323]. In different tissue specimens negative sense HEV RNA, which is an indicator of active viral replication, can be demonstrated by in-situ-hybridization as well [270,273,324].

Recently, the proof of the presence of HEV antigen was introduced as an additional early diagnostic marker [317,325]. However, the application of HEV antigen screening is currently inferior for the early detection of HEV infection due to the decreased sensitivity compared to nucleic acid amplification technology methods [326]. The presence of HEV antigen in different tissues using immunohistochemistry was recently demonstrated in swine [268]. More recently, an immunohistochemical method for the detection of HEV antigen in liver tissue of hepatitis E patients was described representing a valuable tool for the detection of HEV infection in biopsy, autopsy and explant liver tissues [327].
2.9 Prevention and therapy of HEV infection

In contrast to hepatitis B and C, hepatitis E is mostly a self-limited infection, although it has a high mortality in pregnant women and can develop a chronic form in immunocompromised patients. Ribavirin and pegylated INF-α are the only achievable therapies, but both have side effects which are not justifiable for prophylaxis or treatment of mild infections [2,328-330]. As recently shown, HEV inhibits type I interferon induction by ORF1 products in vitro [331]. Anyhow, these drugs are potentially contra-indicated for specific individuals, such as pregnant women, patients with specific organ transplants or with co-morbidities [2].

As up to now only experimental antiviral therapies exist, only the prevention of HEV infections is pivotal. In both industrialized and developing countries, sewage water has been shown to contain infectious HEV strains that are closely related to circulating strains in humans and animals [61,332]. In The Netherlands, HEV RNA of gt3 was detected in river water which most likely originated from sewage [254]. Run-offs from animal facilities have been implicated in human HEV infections with the detection of infectious HEVgt3 in the animal manure and wastewater [254]. HEV infection can be prevented by providing clean drinking water and improving the sanitary infrastructure, especially in developing countries where general standards of hygiene are low, and the morbidity and mortality of HEV and other water-related pathogens are elevated [333]. Inadequate disposal and treatment of sewage and contamination of drinking and irrigation water can lead to epidemics in developing countries [71,334]. Environmental catastrophes and annual flooding are also associated with increased HEV infection rates especially in regions where river, pond, or well water use is prevalent [335]. Recently, a HEV inactivation study indicated that the use of chlorine disinfection is an effective strategy to control HEV waterborne transmission [336]. However, as transmission route for HEVgt3 and gt4 is likely zoonotic other methods of prevention are needed. The meat products from HEV-infected reservoir animal species are capable of transmitting HEV to humans and are a public health concern [334]. Prevention of foodborne HEV transmission relies on avoiding consumption of undercooked animal meat especially when immunocompromised, following good hygiene practices, and being aware of increased risks when traveling to endemic or hyper-endemic regions of the world [94,337]. For example, three cases of hepatitis E in Japan were associated with the consumption of undercooked or raw pork presumably from the same restaurant [117]. Boar meat consumption
was positively associated with HEV infection in a case-control study in Germany [288]. It has been shown that HEV is completely inactivated when heated above 70°C [49]. However, the opinions differ considering lower temperatures for viral inactivation. Some studies revealed that an incubation temperature of 56 to 60 °C for 30 to 60 min may be sufficient [49,338], while others report remaining infectivity of virus incubated at 56°C [209,339]. Moreover, attempts should also focus on the protection of risk groups, such as veterinarians and slaughter house personnel [119,294]. Although rarely, HEV can be transmitted via blood transfusion [340-342], suggesting that screening of donor blood may be warranted especially in endemic areas. In addition, vertical transmission of HEV has been reported [343], indicating that extra caution is required in pregnant women.

Vaccination would be an effective strategy to prevent HEV infection. Recently, such a HEV vaccine based on a recombinant truncated capsid protein was approved in China (Hecolin®) [344]. This vaccine, HEV 239, is a 26-kDa protein encoded by ORF2 of HEVgt1 [345]. The vaccine is expressed in E. coli and occurs in solution as VLPs containing at least two T cell epitopes [346]. In a phase II study conducted among seronegative adults, the vaccine was found to be safe and immunogenic [347]. In a phase III trial the vaccine was well tolerated and protected against hepatitis E, with an efficacy of 100% [344]. This vaccine has been licensed for usage in China, but it is not certain if and when this vaccine will also be licensed for human usage in other countries [214]. Another recombinant vaccine successfully completed phase II clinical trials in Nepal [348], but further development was halted. This vaccine is a recombinant protein encoded by ORF2 of a HEVgt1 strain, expressed in insect cells. Vaccination was well tolerated and highly immunogenic, with efficacy against HEV infection in 95.5% [348]. Such vaccines may be useful as a prophylactic measure in high-risk patients such as immunocompromised patients and pregnant women as well. This would require specific clinical trials to demonstrate protective efficacy of a HEV vaccine in these populations, but such studies have not been reported yet [2].
3. Manuscript I: Natural and experimental hepatitis E virus genotype 3 - infection in European wild boar is transmissible to domestic pigs

Natural and experimental hepatitis E virus genotype 3 - infection in European wild boar is transmissible to domestic pigs

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3.1 Abstract

Hepatitis E virus (HEV) is the causative agent of acute hepatitis E in humans in developing countries, but sporadic and autochthonous cases do also occur in industrialized countries. In Europe, food-borne zoonotic transmission of genotype 3 (gt3) has been associated with domestic pig and wild boar. However, little is known about the course of HEV infection in European wild boar and their role in HEV transmission to domestic pigs. To investigate the transmissibility and pathogenesis of wild boar-derived HEVgt3, we inoculated four wild boar and four miniature pigs intravenously. Using quantitative real-time RT-PCR viral RNA was detected in serum, faces and in liver, spleen and lymph nodes. The antibody response evolved after fourteen days post inoculation. Histopathological findings included mild to moderate lymphoplasmacytic hepatitis which was more prominent in wild boar than in miniature pigs. By immunohistochemical methods, viral antigens were detected mainly in Kupffer cells and liver sinusoidal endothelial cells, partially associated with hepatic lesions, but also in spleen and lymph nodes. While clinical symptoms were subtle and gross pathology was inconspicuous, increased liver enzyme levels in serum indicated hepatocellular injury. As the fecal-oral route is supposed to be the most likely transmission route, we included four contact animals to prove horizontal transmission. Interestingly, HEVgt3-infection was also detected in wild boar and miniature pigs kept in contact to intravenously inoculated wild boar. Given the high virus loads and long duration of viral shedding, wild boar has to be considered as an important HEV reservoir and transmission host in Europe.
4. Manuscript II: Immune response and viral replication in experimentally hepatitis E virus-infected wild boar and domestic pigs are not substantially influenced by dexamethasone-induced immunosuppression

Immune response and viral replication in experimentally hepatitis E virus-infected wild boar and domestic pigs are not substantially influenced by dexamethasone-induced immunosuppression

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4.1 Abstract

As shown recently, wild boar-derived HEV genotype 3 (HEVgt3) is transmissible to domestic pigs and causes a variable degree of hepatic lesions supporting the assumption that liver damage in pigs might be immune-mediated. In swine, detailed studies of cellular immunity to HEV infection are not available yet. Therefore, we have investigated the cellular immune responses to HEVgt3 of intravenously inoculated wild boar and of horizontally infected domestic pigs (contact animals exposed to infectious feces), and compared them to infected wild boar and contact pigs which were immunosuppressed by dexamethasone. Non-infected dexamethasone treated animals served as additional controls for the evaluation of immunosuppressive effects. As determined by flow cytometry there is a strong cellular immune response as revealed by the increases in the numbers of CD8+CD4- T cells, CD4+CD8+ T cells and γδTCR+CD2+CD8+ T cells in the PBMCs in HEV infected wild boar. This effect was less pronounced in the HEV-challenged animals, which had received dexamethasone. However, irrespective of the immunosuppression, comparable liver lesions were found in all infected animals and the dexamethasone treatment had no effect on the viremia levels, humoral immune responses and virus shedding of the HEVgt3 infected animals. Indeed, the infection was spread to all contact pigs independent on their immune status. In conclusion, immunosuppressive effects of dexamethasone were proven, but did not affect substantially the course of hepatitis E in wild and domestic swine.
4.2 Introduction

Hepatitis E virus (HEV), the causative agent of hepatitis E in humans, is the sole member of the genus *Hepevirus* in the family *Hepeviridae*. It is a small, non-enveloped virus with a single-stranded, positive sense RNA genome of approximately 7.2 kb encoding three open reading frames (ORF). In humans, the main transmission pathway of HEV is the fecal-oral route, commonly through contaminated water supplies. Especially in developing countries with poor sanitary conditions, epidemics and small outbreaks of hepatitis E occur [59]. However, increasing cases of sporadic and autochthonous HEV infections are also found in industrialized countries for which the source of infection remains enigmatic most of the times [91,116]. Recently, several HEV-related viruses were identified in other species which significantly broadened the host range and diversity of HEV. A consensus classification system for the family *Hepeviridae* is currently unavailable, but a new taxonomic scheme in which the family is divided into the genera Orthohepeivirus (Orthohepeivirus A with isolates from human, pig, wild boar, deer, mongoose, rabbit and camel; Orthohepeivirus B with isolates from chicken; Orthohepeivirus C with isolates from rat, greater bandicoot, Asian musk shrew, ferret and mink; Orthohepeivirus D with isolates from bat) and Piscihepeivirus has been proposed [19]. Within the mammalian HEV isolates, genotypes (gt) 1 and gt2 are restricted to humans, whereas gt3 and gt4 contain also zoonotic strains. HEV gt3 and gt4 have been found in domestic pigs, wild boar, deer, mongoose, monkeys and rats which therefore represent potential natural reservoirs respectively [94]. In Europe and Asia, food-borne zoonotic transmissions of HEV have been primarily associated with the consumption of contaminated raw or undercooked meat from domestic pigs and wild boar [89,116,288]. Moreover, the direct contact to pigs has been suggested as an additional risk factor for humans for contracting an HEV infection [163,349]. In domestic pigs and wild boar HEVgt3 and 4 infections are fairly common. High antibody prevalence rates and molecular evidence for HEV infection have been found recently, and phylogenetic studies indicate historic transmission events from domestic pig to wild boar [289,295,350]. In consequence, hepatitis E is an emerging zoonosis for which Suidae should be considered as main reservoir [21]. In humans, the clinical course and pathogenesis of HEV infection can vary substantially. As HEV is presumed to be a non-cytopathogenic virus, immunopathological mechanisms have been proposed as reasons for the liver lesions [209,210]. While HEV gt1 and gt2 are causing
acute hepatitis primarily, HEVgt3 can also induce a chronic hepatitis in immunocompromised patients [110]. Both viral as well as host factors determine the course of the HEV infection, but detailed mechanisms leading to different clinical outcomes are not really understood [223]. As recently shown, HEV inhibits type I interferon induction by ORF1 products in-vitro [331]. Moreover, natural and experimental HEVgt3 infection in European wild boar is transmissible to domestic pigs and causes variable degree of hepatic lesions supporting the assumption that liver damage in pigs might also be immune-mediated [351].

Data on the cellular immune response following an HEV infection in humans are sparse, while data on pigs are missing completely. Therefore, we investigated this response following a HEVgt3 infection in wild boar and domestic swine. Moreover, we have assessed whether a dexamethasone treatment, which conveys potent immunosuppressive effects [352], affects the clinical course and pathological outcome of this infection in wild boar. In order to prove horizontal HEVgt3 transmission and determine differences in the susceptibility to HEVgt3 infection dependent on the immune status, dexamethasone treated and non-treated domestic pigs were kept in contact to infectious feces derived from the intravenously inoculated wild boar.
4.3 **Material and methods**

4.3.1 **Inoculum**

The HEV gt3 strain used in this study originated from a liver sample of an experimentally infected wild boar [351]. The liver was frozen immediately and stored at −70 °C. For preparation of the inoculum, the liver was ground in phosphate-buffered saline (PBS) with a mortar and pestle (10%, w/v). The suspension was transferred to a 15 mL tube and mixed for 1 min using a vortex mixer. After centrifugation (20 min at 4000 × g at 4 °C) the supernatant was transferred to a new tube and filtered (0.22 µm MILLEX®GP filter unit, Millipore, Ireland). The suspension was aliquoted in volumes of 2.5 mL and stored at −70 °C. The inoculum contained about 2 × 10^4 HEV genome equivalents per µL RNA.

4.3.2 **Study design**

The experimental setup included 5 experimental groups overall containing 12 European wild boar (*Sus scrofa scrofa*) of 6 month of age and 8 domestic pigs (*S. scrofa domestica*) of 2 months of age. In order to assemble similar weight categories (20 to 30 kg), animals were chosen according to their body weights. Prior to the start of the study all animals were tested to be negative for anti-HEV antibodies in serum and HEV RNA in feces, respectively. Groups 1A and 1B consisted of wild boar (n = 4 each) and were kept in two separated pens. To assess the influence of the immune status on infection dynamics and shedding, animals of group 1B were immunocompromised by dexamethasone injections 1.5 weeks prior and 1.5 weeks after the infection (doses of 0.5 mg / kg dexamethasone per animal at 9 different time points). Wild boar in these groups were inoculated intravenously (2.0 mL liver suspension per animal) via the *Vena cava cranialis*. Uninfected, dexamethasone dosed wild boar (group 1C; n = 4) constituted immunosuppression controls. Biochemical blood parameters, total white blood cell and lymphocyte cell counts were determined in all animals throughout the experiment. Groups 2A and 2B comprised four domestic pigs each, which were housed in two separate pens. Animals in group 2B were immunocompromised with dexamethasone like the wild boar in group 1B. Until necropsy of the wild boar (30 days post inoculation), feces were transferred from group 1A to groups 2A and 2B to facilitate horizontal HEV transmission. Blood and fecal samples were collected at time points 0, 1, 4, 6, 9, 13, 15, 18, 21, 24, 28, 30 days post inoculation (dpi) in group 1A and 1B, respectively at 0, 1, 4, 6, 9, 13, 15, 18, 21, 24,
28, 31, 36, 41 dpi in group 2A and 2B. Aliquots of serum samples were stored at -20°C for antibody detection and at -70°C for RNA extraction. Blood samples containing ethylenediaminetetraacetic acid (EDTA) as anticoagulant were collected for blood cell count and flow cytometry analyses. Fecal samples were diluted in isotonic saline solution (5%, w/v) and stored at -70°C for RNA extraction. The study was terminated after 30 dpi for group 1A, 1B and 1C, and after 41 dpi for group 2A and 2B. For virological and immunohistochemical investigations the following samples were taken at necropsy: liver (4 locations including the right, left, caudate and quadrate lobe), bile and gall bladder, different lymph nodes (including liver, mesenteric, colic, pulmonary, cervical and mandibular lymph nodes), thymus, pylorus, small and large intestine, cecal ingesta, pancreas, kidney, spleen, tonsil, heart, brain, gonads, uterus or prostate gland, vagina or bulbourethral gland, parotid and sublingual gland, tongue and quadriceps femoris muscle. For histological examination one part of each tissue sample was fixed immediately in 4% neutral buffered formalin and for RNA extraction the other part was stored at -70°C. Bile samples and diluted cecal ingesta (5%, w/v in isotonic saline solution) were stored at -70°C for RNA extraction.

All experiments were carried out under high containment conditions (biosafety level 3**) taking into account animal welfare regulations and standards according to EU Directive 2010/63/EU and institutional guidelines. The experimental protocol was reviewed by an independent animal welfare and ethics committee and was approved by the competent authority (State Office for Agriculture, Food Safety and Fisheries of Mecklenburg-Western Pomerania, Rostock, Germany, reference number LALLF M-V/TSD/7221.3-1.1-022/13).

4.3.3 Clinical chemistry

Serum samples were analyzed longitudinally by a spectrophotometric method in an automated analyzer (VetScan Chemistry Analyzer, Abaxis, Union City, USA) using special rotors (VetScan Mammalian Liver Profile reagent rotor, Abaxis) to provide quantitative results for alanine aminotransferase (ALT), albumin (ALB), alkaline phosphatase (ALP), bile acids (BA), total bilirubin (TBIL), total cholesterol (CHOL), gamma-glutamyl transferase (GGT) and blood urea nitrogen (BUN) in serum. Normal enzymatic activity ranges of the biochemical parameters were calculated on the basis of two independent samples taken from the wild boar and domestic swine prior to the infection respectively (n = 24 for wild boar and n = 16 for domestic pigs).
4.3.4 Anti-HEV antibody ELISA

Sera were tested for the presence of anti-HEV IgG-antibodies with the PrioCHECK HEV Ab porcine ELISA kit (Prionics AG, Schlieren-Zurich, Switzerland) according to the manufacturer's instructions. ELISA uses recombinant HEV genotype 1 and 3 antigens for the detection of anti-HEV antibodies (IgG) in porcine serum or meat juice.

4.3.5 RT-qPCR

From all serum samples, fecal and cecal ingesta suspensions, and bile samples a manual extraction of viral RNA was performed using the QIAamp® Viral RNA Mini Kit (QIAGEN GmbH, Hilden, Germany) according to manufacturer's recommendations. From all tissue samples, viral RNA was extracted using the RNeasy Mini Kit (QIAGEN GmbH). An internal control RNA was added for both extraction methods as described previously [353]. HEV RNA was detected by a novel diagnostic quantitative real-time RT-PCR assay (RT-qPCR) using the CFX96™ Real-Time System (Bio-Rad Laboratories GmbH, Munich, Germany). All primer and probes used in this study are listed in Additional file 1. The RT-qPCR was performed using the QuantiTec Probe RT-PCR kit (QIAGEN GmbH) in 25 µL reaction volume with final concentrations of each primer with 0.8 µM, and of the probe with 0.1 µM. A volume of 5 µL RNA was added. Reverse transcription (RT) was carried out at 50°C for 30 min, followed by denaturation/activation at 95°C for 15 min. DNA was amplified immediately with 45 cycles at 95°C (10 sec), 55°C (25 sec) and 72°C (25 sec). The determination of the number of HEV genome equivalents was carried out using a standard curve according to a synthetic external calibrator encompassing the 81 bp sequence of the RT-qPCR amplicon.

4.3.6 Histopathology and immunohistochemistry

For histopathological examinations formalin fixed tissue samples were stained with hematoxylin and eosin (HE) according to standard protocols. For immunohistochemistry (IHC) tissue sections were treated as described before [351]. Viral antigens were detected using a rabbit anti-HEV gt3 serum (rHEVgt3-HIS) in a 1:1000 dilution. Therefore, rabbits were immunized with an Escherichia coli expressed and purified His-tagged C-terminal segment of HEV gt3 capsid protein [163]. A 1:200 diluted commercially available polyclonal rabbit anti-human CD3 antibody (Dako Deutschland GmbH, Hamburg, Germany), which is
also binding to porcine CD3 antigen, was used to characterize the inflammatory response in the liver.

4.3.7 **Differential cell count**

To evaluate changes of cellular composition in the peripheral blood in the course of HEV infection, 150 µL of whole blood were analyzed using an automated XT-2000iV hematology analyzer (Sysmex Corporation, Hyogo, Japan) and the number of leukocytes (WBC), lymphocytes (LYM), monocytes (MON) and neutrophils (NEU) were determined. Reference value ranges for the tested blood parameters in wild boar were calculated for the evaluation of the results. Therefore, blood samples of group 1A, 1B and 1C at 0 dpi (n = 12) and of randomly taken HEV negative wild boar blood samples (n = 12) were subjected to differential cell count for the determination of the mean values and standard deviation for each tested parameter. Reference values for leukocyte distribution in domestic pigs were specified elsewhere [354].

4.3.8 **Flow cytometry**

Peripheral blood mononuclear cells (PBMCs) were subjected to multicolor immunostaining for flow cytometry analysis of porcine cell surface markers using a BD FACSCanto™ flow cytometer (BD Biosciences, Heidelberg, Germany). An overview of primary and secondary antibodies used for flow cytometry staining is given in the Additional file 2. Blood samples containing EDTA as anticoagulant were stained with antibody mix 1. After incubation for 15 min in the dark at 4°C, cells were washed with fluorescence-activated cell sorting (FACS) buffer (0.1% bovine serum albumin, 0.035% sodium bicarbonate and 0.02% sodium azide in Hank's balanced salt solution) and centrifuged for 5 min at 300 × g. Then, antibody mix 2, mix 3 or mix 4 were added and cells were washed and centrifuged. After the last wash step, remaining erythrocytes were lysed by osmosis with erythrocyte lysis buffer (0.83% ammonium chloride, 0.1% potassium hydrogen carbonate, 0.037% tetrasodium ethylenediaminetetraacetate in distilled water) and samples were analyzed. At necropsy liver, spleen and mesenterial lymph node samples were collected for the isolation of mononuclear cells. Harvested tissue samples were torn apart into single cell suspensions using 10 mL PBS (pH 7.4) and were centrifuged at 300 × g for 10 min at 4°C. The pellet was resuspended in FACS buffer and stained as described above. Results from flow cytometry were analyzed
using FlowJo™ software (Treestar, Ashland, USA). Based on γδ T cell receptor (γδTCR), CD3, CD4, CD8, CD2 and CD21 staining characteristics, each subpopulation was then further grouped as follows: γδ T cells (γδTCR+); activated γδ T cells (γδTCR+CD8+); T helper cells (CD4+), activated T helper cells (CD4+CD25high); cytotoxic T lymphocytes (CD8+); T helper/memory cells (CD4+CD8+); naïve B-cells (CD2+CD21+); phenotype of B cells after activation (CD2-CD21+); antibody-forming and/or memory B cells (CD2+CD21−). For further details of tested lymphocyte phenotypes and their functional characteristics see Table 4.1.

4.3.9 Statistical analysis

Significant differences in the mean number of biochemical parameters, differential cell counts and lymphocyte phenotypes were calculated by two way ANOVA with post-hoc-analysis (Bonferroni t-test) using SigmaPlot 11.0 (SystatSoftware Inc., Chicago, USA). Significant differences in the mean number of OD450 values, viral load in serum, feces, bile, cecum ingesta and tissue samples were calculated by one way ANOVA with post-hoc-analysis (Bonferroni t-test) using SigmaPlot 11.0. Significance was determined at P < 0.05. Measured values are reported as mean ± SD.
4.4 Results

To study the involvement of the cellular immune response to a HEV gt3 infection in wild boar and domestic pigs we have conducted the following experiments: group 1A: four intravenously challenged wild boar, group 1B: four immunosuppressed intravenously challenged wild boar, group 1C: four immunosuppressed but unchallenged wild boar, group 2A: four domestic pigs challenged using feces of group 1A and group 2B: four domestic pigs immunosuppressed and challenged using feces of group 1A.

All animals were assessed clinically. Blood and fecal samples were taken regularly in order to determine the biochemical blood parameters, cellular immune cell and antibody responses to HEV, and to reveal the viremia and virus shedding over time. The experiments were eventually terminated by euthanasia after 30 days in wild boar and 41 days in domestic pigs followed by necropsy of the animals. The following samples were tested for HEV RNA by RT-qPCR: liver (4 localizations), gall bladder, bile, liver lymph node, spleen, pancreas kidney, heart muscle, lung, pulmonary lymph node, pylorus, duodenum, proximal and distal jejunum, ileum, ileocecal junction, caecum, cecal ingesta, colic lymph node, ascending and descending colon, rectum, mesenteric lymph node, mandibular lymph node, tonsil, parotid gland, sublingual gland, thymus, tongue, cervical lymph node, ovary / testicle, uterus / bulbourethral gland, vagina / prostate gland, quadriceps femoris muscle and brain. HEV-antigen detection was assessed by immunohistochemistry within the following post mortem tissues: liver (different localizations), gall bladder, liver lymph node, spleen, pancreas, kidney, heart muscle, lung, duodenum, jejunum, ileum, caecum, colic lymph node, colon, rectum, mesenteric lymph node, mandibular lymph node, tonsil, parotid gland, thymus, gonads, quadriceps femoris muscle and brain.

4.4.1 HEV infection in intravenously inoculated wild boar and in dexamethasone-treated wild boar

Biochemical parameters and serology

At the end of experiment elevated ALT and GGT levels in serum were observed in groups 1A and 1B at 28 to 30 dpi (Figure 4.1 A). Other biochemical parameters remained within normal limits. Anti-HEV antibodies were detected first time in group 1A at 6 dpi and in group 1B at 9
dpi. In comparison to group 1A, mean OD450-values in group 1B were slightly increased at 28 to 30 dpi (Figure 4.1 B). In each group, 3 out of 4 wild boar had detectable antibodies at 13 dpi, but only 1 out of 4 in group 1A and 2 out of 4 animals in group 1B were still tested positive at 30 dpi (Table 4.2). No significant influence of the immunosuppressive status (without versus with dexamethasone treatment) on liver enzyme levels and the antibody response to HEV inoculation was provable, but ALT and GGT levels in group 1A were significantly increased compared to the reference value range (Figure 4.1).

**Detection of HEV RNA**

Viral RNA in serum was only found transiently in one wild boar group at 21 dpi (group 1B) respectively at 28 dpi (group 1A), and in both at 30 dpi (Table 4.2). In fecal samples HEV RNA was detected at first in group 1A at 4 dpi and in group 1B at 6 dpi. All wild boar in group 1A shed viral RNA in feces within 28 to 30 dpi, whereas fecal shedding was detectable in all animals of group 1B already at 21 dpi and lasted until the end of experiment (Table 4.3). The time course of HEV RNA detection in serum and feces of wild boar was summarized in Figure 4.1 C. Viral RNA was also detected in bile, small and large intestine, cecal ingesta, liver and gall bladder of all wild boar, whereas in both groups 3 out of 4 liver lymph nodes, in group 1A 3 out of 4 and in group 1B 2 out of 4 spleen samples were tested positive. An overview of the mean HEV excretion including the proportion of positive tested specimens is given in Figure 4.2 A, and the mean viral load in different tissues with the number of positive tested tissue samples is shown in Figure 4.2 B. HEV RNAs were also found in other lymphatic tissues and different intestinal locations of the wild boar (Additional file 3). No significant differences concerning HEV RNA excretion and viral load in tested tissue samples were present between group 1A and 1B (Figure 4.2).

**Liver histopathology and distribution of viral antigens in different tissues**

Mild lymphoplasmacytic infiltrates with single cell necrosis of hepatocytes and Kupffer cell proliferations were found in the livers of all intravenously inoculated wild boar. Partially, hepatic lesions were associated with mild infiltrates of CD3 positive cells, but without marked differences between group 1A and 1B. By IHC, several tissue samples were analyzed for the distribution of viral antigens (Figure 4.8). Detection of viral antigens in tissue samples of wild
boar was not consistent with group allocation. In group 1A and 1B viral antigens were found in liver (3/8), liver lymph nodes (4/7), spleen (1/8) and mesenteric lymph node (1/8). All IHC results of viral antigen detection are summarized in the Additional file 4.

**Cellular immune responses**

Hematological analysis (Figure 4.3) revealed a transient decrease of WBC between 4 and 6 dpi in both wild boar groups which was significant in group 1A compared to the reference value range. In both groups the decrease was followed by an increase of WBC after 13 dpi. WBC counts were much more pronounced in group 1A and significantly distinct between both groups at 13 dpi. Similarly, a slight decrease of LYM at 4 dpi was followed by an increase after 13 dpi in both groups. Lymphocytosis persisted until 30 dpi and LYM counts were significantly distinct between both groups at 13, 21, 28 and 30 dpi. A slight monocytosis was seen in group 1A at 13 to 15 dpi and in group 1B at 9 to 15 dpi, respectively at 21 to 24 dpi. At 4 to 9 dpi a decrease of NEU was observed in both groups followed by an increase in group 1A at 15 dpi.

Parameters indicative for the T cell populations in peripheral blood are summarized in Figure 4.4: All inoculated animals showed a marked elevation of the absolute number of cytotoxic T lymphocytes (CD8+CD4-) starting from 6 dpi to 30 dpi. This increase was significant in group 1A compared to 0 dpi and significantly distinct between both groups at 30 dpi. Only slight changes were observed in both groups. Following the increase of cytotoxic T lymphocytes (CD8+CD4-), an increase of T helper/memory cells (CD4+CD8+) was detectable starting at 15 dpi. This increase was significant in group 1A compared to 0 dpi and significantly distinct between both groups at 30 dpi. The percentage of activated γδ T cells (γδTCR+CD8+) increased in all animals after 13 dpi with a peak level at 18 dpi. In the numbers of γδ T cells (γδTCR+), T helper cells (CD4+CD8-) and activated T helper cells (CD4+CD25high) only slight changes were seen in peripheral blood (data not shown). With regard to the B cell population in peripheral blood (Figure 4.5) the percentage of naïve B cells (CD2+CD21+) and of B cells after activation (CD2-CD21+) declined initially in all inoculated wild boar. Cells representing the phenotype of antibody-forming and/or memory B cells (CD2+CD21-) showed a percentage increase in all inoculated groups with highest changes after 13 to 30 dpi (significant differences in group 1A compared to 0 dpi).
Immune cell phenotypes of separated cells of liver, spleen and mesenterial lymph node were examined after necropsy at 30 dpi (Figure 4.6). Dexamethasone controls (group 1C) were included into the analyses as a comparison group. Lower percentages of T-cells were observed in liver of group 1A and 1B with significant differences between both groups. Moreover, decreased percentages of cytotoxic T lymphocytes (CD8+CD4-), T helper cells (CD4+CD8-) and T helper/memory cells (CD4+CD8+) were observed in group 1A and 1B. Conversely, marked increase in percentages of γδ T cells (γδTCR+) compared to group 1C was seen, but no influence of dexamethasone treatment. In spleen, T cells in group 1B were decreased compared to group 1A and 1C, and an influence of dexamethasone treatment in group 1A and 1B was provable. In contrast to the liver, lower percentages of γδ T cells (γδTCR+) compared to group 1C were seen in spleen, but no influence of dexamethasone treatment. Cytotoxic T lymphocyte (CD8+CD4-) and T-helper/memory cell (CD4+CD8+) percentages were slightly reduced in group 1A and 1B, whereas the percentage of T-helper cells was significantly increased compared to group 1C. No remarkable changes in T cell populations were observed in the mesenterial lymph node, except one significant difference in the percentages of T-helper/memory cells (CD4+CD8+) between group 1B and 1C.

4.4.2 HEV transmission to non-treated domestic pigs and dexamethasone-treated domestic pigs

Biochemical parameters and serology

In group 2A an elevated ALT serum level was observed in the course of experiment significantly increased at 9 to 18, 24, 34 and 41 dpi compared to the reference value range and to group 2B. No significant rise in the ALT serum level was observed in group 2B. Independent on the immunosuppressive status both groups developed a marked elevation of GGT serum levels at 41 dpi. Only 1 out of 4 pigs in group 2A developed anti-HEV antibodies during the observation period, whereas no antibodies were detected in group 2B. Liver enzyme levels and serological data are summarized in the Additional file 5.

Detection of HEV RNA

No viral RNA was detectable in serum samples of group 2A and 2B. HEV RNA in feces was found the first time at 4 dpi in group 2B. In both groups 2 out of 4 animals shed viral RNA via
feces at 41 dpi (Table 4.3). All livers of the domestic pigs were tested positive for HEV RNA, but in group 2B all taken liver lobe samples contained detectable viral RNA. HEV RNA was also detected in all bile samples and gall bladders of the domestic pigs. Intestine and cecal ingesta were tested positive in group 2A and 2B, and spleen only in one animal of group 2B. An overview of the mean HEV excretion including the proportion of positive tested specimens is given in Figure 4.2 A, and the mean viral load in different tissues with the number of positive tested tissue samples is shown in Figure 4.2 B. No significant differences concerning HEV RNA excretion and viral load in tested tissue samples were present between group 2A and 2B, but biliary HEV RNA excretion were slightly increased in group 2B (Figure 4.2). The time course of HEV RNA detection in serum and feces of domestic pigs is summarized in the Additional file 5.

Liver histopathology and distribution of viral antigens in different tissues

Neither histopathological changes specific for viral hepatitis nor an increase of CD3 positive cell infiltrates were seen in the livers of the domestic pigs. By IHC no viral antigens were detectable in domestic pigs, except one positive liver sample in group 2A. All IHC results of viral antigen detection are summarized in the Additional file 4.

Cellular immune responses

Hematological analysis (Additional file 6) revealed no remarkable changes, except an unrelated transient leukocytosis, monocytosis and neutrophilia initially.

Parameters indicative for the T cell populations in peripheral blood are summarized in the Additional file 7: Only in group 2A an increase in the percentages of T helper cells (CD4+CD8-) and γδ T cells (γδTCR+) was observed at 21 respectively 28 dpi to 41 dpi in group 2A. Similar changes in the absolute numbers of T helper cells (CD4+CD8-) and γδ T cells (γδTCR+) were observed, but less pronounced. The percentage of activated γδ T cells (γδTCR+CD8+) decreased in group 2A at 24 to 41 dpi (data not shown). In the numbers of cytotoxic T lymphocytes (CD8+CD4-), T helper/memory cells (CD4+CD8+) and activated T helper cells (CD4+CD25high) animals showed no consistent changes (data not shown). With regard to the B-cell population in peripheral blood (Additional file 8) the percentage of naïve B-cells (CD2+CD21+) and of B-cells after activation (CD2-CD21+) declined in both groups
after 24 dpi. In all inoculated groups, cells representing the phenotype of antibody-forming and/or memory B cells (CD2+CD21-) showed an initial percentage increase from 13 to 18 dpi and another at 28 to 41 dpi.

Immune cell phenotypes of separated cells of liver, spleen and mesenterial lymph node were examined after necropsy at 41 dpi (Figure 4.7). Randomly selected HEV negative domestic pigs (n = 6) served as negative controls. Compared to negative controls significantly higher percentages of T cells and T cell subsets were observed in the liver of group 2A and 2B, but only for T helper cells (CD4+CD8-) an influence of dexamethasone treatment was proven. No remarkable changes in T cell populations were observed in spleen and mesenterial lymph node, except for higher T-helper/memory cell (CD4+CD8+) and γδ T cell (γδTCR+) percentages in the lymph node of group 2A and 1B compared with negative controls.

4.4.3 Monitoring of the immunosuppressive effects in dexamethasone-treated uninfected wild boar

A significant immunosuppressive effect of dexamethasone-treatment was proven in dexamethasone-treated, uninfected wild boar (group 1C). Animals had significant lower total lymphocyte and monocyte counts in peripheral blood after treatment compared to 0 dpi (Figure 4.3). Significant differences were also seen in different lymphocyte subsets in peripheral blood (Figure 4.4 and Figure 4.5). Interestingly, percentages of different T cell subsets in liver and spleen were mostly opposed to HEV inoculated wild boar. Compared to determined reference values no significant changes were seen in serum liver enzyme levels after treatment (Figure 4.1).
4.5 Discussion

In the present study, we investigated the cellular immunity in HEV infected wild boar and domestic pigs as detailed studies on cellular immune responses against HEV in swine are not available yet. Moreover, the effects of immunosuppression induced by the systemic administration of glucocorticoids on the HEVgt3 infection in swine are described. Therefore, comparative analyses of cellular immune responses in peripheral blood and tissue samples, viral loads in different excreta and organ materials, humoral immune responses to HEV infection, histopathological changes and viral antigen distribution in different tissues were performed. Additionally, the immunosuppressive effect of dexamethasone was monitored in uninfected control pigs.

To date, food-borne zoonotic transmission of HEVgt3 in Europe and Asia is primarily associated with domestic pigs and wild boar [89,116], while data on the pathogenesis and transmission of wild boar-derived HEVgt3 in swine are scarce. Especially the role of host factors in porcine HEV infection has not been studied in detail yet. Both human and animal studies have suggested that immune responses, rather than a viral damage to hepatocytes, drive the clinical manifestation of hepatitis E [111,351]. In immunocompromised humans, like patients with a solid-organ transplant, hematologic disorders, and in those who are human immunodeficiency virus-positive, HEV-infections can lead to viral persistence [215]. Multi-faceted interactions between host immune responses and virus diversity seem to be responsible, but the key mechanism leading to a chronic hepatitis E infection is largely unknown. An association between a weak inflammatory response, poor T cell activation and high serum concentrations of chemokines involved in leukocyte recruitment to the liver is assumed to play a role in the development of chronic HEV infections in humans [215].

In the study presented here, we hypothesized that immunosuppression may enhance the susceptibility of pigs to HEV, but also reduces the clinical manifestation due to diminished inflammatory responses possibly leading to viral persistence at the same time. To mimic an immunosuppressed condition, pigs were treated with dexamethasone, which decreases the cytokine production and consequently impairs the immune systems’ activation [355]. Moreover, glucocorticoids have inhibitory effects on T cells and B cells and exert potent suppressive effects on the effector functions of phagocytes [352].
Although the majority of leukocytes will probably have left the blood stream following an HEV infection, changes in the number of the different white blood cell populations can be indicative for the immune response of individuals over time. Taken together, the hematological analysis revealed a leukocytosis, lymphocytosis and monocytosis in all HEV infected wild boar. Moreover, an initial neutropenia was seen. However, changes in differential cell counts were less pronounced in the dexamethasone-treated wild boar group. An increased white blood cell count and a lymphocytosis are frequently found in viral hepatitis [356]. Furthermore, an immunosuppressive effect of dexamethasone-treatment was proven in dexamethasone-treated control pigs as they developed a depletion of white blood cells in peripheral blood. They had significantly lower counts in total lymphocytes and lymphocyte subsets, and monocytes. No particular changes in differential cell populations, apart from a commonly seen initial stress-induced increase in different parameters [354], were observed in any of the domestic pigs after the HEV infection.

In this study, the blood compartment was chosen to investigate the changes of lymphocyte subsets within the course of HEV infection. Of course, the majority of lymphocytes will probably have left the blood stream following an HEV infection, but changes can be indicative for the immune response over time. Porcine lymphocyte phenotypes are well-investigated, but functional analyses of subpopulations are currently not feasible. Lymphocyte subpopulations of pigs have been investigated in a number of studies with the aim to identify correlations between function and the phenotype of these cells. Nevertheless, defined roles of different subsets in the porcine immune system and their functionality are not resolved yet. It has to be taken adequately into account that animals used in this study were of different genetic constitution including distinct haplotypes of swine leukocyte antigens (SLA). The SLA genomic region is extremely polymorphic comprising high numbers of different alleles and plays a crucial role in maintaining overall adaptive immunologic resistance to pathogens [357]. Therefore, the biological diversity between individuals should be appropriately taken into consideration. Using flow cytometry analysis significantly higher numbers of cytotoxic T lymphocytes (CD8+CD4-) and helper/memory cells (CD4+CD8+) in the PBMCs were detected in HEV infected wild boar, less pronounced in the dexamethasone-treated group. A marked cytotoxic T cell response developed after one week post infection and persisted until to the end of experiment. Following the increase of cytotoxic T lymphocytes, an increase of T
helper/memory cells was detectable. In this study, no consistent changes in the percentages of cytotoxic T lymphocytes and T helper/memory cells were observed in peripheral blood of the domestic pigs. Anyhow, in the untreated HEV infected domestic pigs an increase in the percentages of T-helper cells occurred after three weeks. It was speculated that increases in T helper cells (CD4+CD8-) among patients with hepatitis E may reflect increases in the natural killer cell population, which may in turn produce elevated levels of INF-γ [205]. Moreover, only slight changes were seen in the number of T helper cells and γδ T cells (γδTCR+) in PMBCs of HEV infected wild boar. Interestingly, the percentage of activated γδ T cells (γδTCR+CD8+) increased in all HEV infected wild boar. Porcine cytotoxic αβ T cells are a prominent T cell subset during antiviral responses, while porcine αβ T helper cell responses predominantly occur in bacterial and parasitic infections, and γδ T cell responses to viruses have not been reported as frequently as αβ T cell responses [358]. However, accumulating evidence suggests that γδ T cells are components of both innate and adaptive immunity against various viral and bacterial infections, and they are also important in early responses against infections at epithelial surfaces [359-361]. In pigs, responding γδ T cells have been reported in different viral infections [362-365], whereas the responding γδ T cells belonged also to the γδTCR+CD8+ T cell subset [363,364]. Like αβ T cells, these γδ T cells can express CD8α which in swine seems to be correlated with an activation status of T cells, as γδTCR+CD8+ T cell subsets are normally found in the thymus and only after activation in the periphery [366]. It has to be considered that γδ T cells in young animals form a major T cell subpopulation within peripheral blood lymphocytes and the frequency of this population decreases strongly with the age of pigs [367]. Interestingly, γδ T cells in swine are sources of interleukin (IL-) 17 which is a pro-inflammatory cytokine being involved in immunity against viruses [368]. Intrahepatic innate lymphoid cells secrete IL-17 and studies in immunodeficient mice revealed that IL-17 signaling was critical for priming T cell responses in viral hepatitis [369]. Further investigations on this would provide new insights in antiviral immunity during porcine HEV infection. Recently shown, local immune responses by IL-17-secreting γδ T cells serve to contain infections by pathogens to the gut while preventing pathogen dissemination to systemic sites [370]. As HEV is normally fecal-orally transmitted it would be also helpful to investigate intestinal immune responses to HEV infection further on as they could play a critical role in early HEV pathogenesis and in disease’ outcome. Changes in T
cell populations were observed in all HEV infected wild boar, which were much more pronounced in untreated HEV infected wild boar. Therefore dexamethasone seems to inhibit the cellular immune response in HEV infected wild boar, yet without shutting it down completely. T cell mediated adaptive immune responses are important for the elimination of viral infections [356]. In a previous study in humans with acute hepatitis E, patients showed also increased numbers of CD8+CD4- and CD4+CD8+ cells compared to healthy controls [206]. In humans, such double-positive T cells represent a minor subpopulation of T cells with functional characteristics of both CD4+CD8- and CD8+CD4- cells, and carry markers of memory phenotype [371]. Porcine CD4+CD8+ cells exhibit properties of mature antigen-experienced cells, and are inducible by stimulation with recall antigen [372]. The current notion is that both activated and memory T helper cells in swine belong to the CD4+CD8+ population expressing also major histocompatibility complex (MHC) class II antigens, which is not seen in human and murine CD4+CD8+ lymphocytes [373,374]. In many viral infections of pigs CD8+CD4- T-cells are the predominant T cell subpopulation [375-379], but porcine CD4+CD8+ T cell responses have been also reported [363,380-382]. It has been shown that CD2 but not CD21 can be re-expressed on the surface of B cells so that CD21 can be considered as a maturation marker. CD2 on the surface of B cells can be down-regulated by cell-to-cell contact and once recovered, CD2 expression on B cells is re-established [383]. Upon infection, all animals showed a down regulation of CD2+CD21+ cells (phenotype of naïve B-cells) and CD2-CD21+ cells (phenotype of primed and activated B-cells) indicative for B-cell activation. Cells representing the phenotype of antibody-forming and/or memory B cells (CD2+CD21-) showed an increase in all HEV infected pigs. Changes in B lymphocyte subsets were mostly independent on dexamethasone treatment. The increase of antibody-forming and/or memory B cells (CD2+CD21-) probably reflects the chronological events in anti-HEV antibody production as most of the wild boar and one domestic pig developed measureable anti-HEV antibodies within the experiment. Anyhow, it has to be considered that the phenotype CD2+CD21- of non-T cells also included natural killer (NK) cells, but in negligible quantity as their frequency in peripheral blood is very low [366]. In liver and spleen an influence of HEV infection and dexamethasone-treatment on immune cell percentages was also detected. Interestingly, percentages of different T cell subsets in liver and spleen of dexamethasone control group were mostly opposed to those of HEV infected
wild boar groups. As no additional tissue material of HEV negative untreated wild boar was available, results of lymphocyte subsets in tissue of HEV infected wild boar have to be considered carefully and need further investigation. Interestingly, lower percentages of cytotoxic T lymphocytes, T-helper cells and T-helper/memory cells were observed in HEV infected wild boar compared to dexamethasone-treated, uninfected wild boar. Conversely, higher percentages of T cell subsets were observed in the liver of domestic pigs, whereas randomly selected HEV negative domestic pigs served as negative controls. Interestingly, marked increase in the percentage of γδ T cells was observed in the liver of all HEV infected pigs. In contrast to the liver, lower percentages of γδ T cells were seen in spleen of HEV infected wild boar compared to dexamethasone-treated, uninfected wild boar. Additionally, higher T helper/memory cell and γδ T cell percentages were found in mesenterial lymph nodes of the HEV infected domestic pigs. Nevertheless, differences between lymphocyte subsets in tissue and PBMCs of the wild boar and domestic pigs might be due to distinct transmission routes of HEV and intraspecific varieties. Our findings might also imply that T cells detectable in peripheral blood may home into the primary site of infection to function as effector cells in the liver. However, HEV-specific T cell responses have only been studied in hepatitis E patients, but not in pigs to date. In humans, proliferation and cytokine production of CD4+CD8- and CD8+CD4- T cells were studied after stimulation with peptides encoded by HEV-ORF2 and –ORF3 [225]. Nonetheless, studies on innate immune responses to HEV infection are urgently required. Previously, intrahepatic transcriptome analysis in primates indicated that HEV may be susceptible to innate immune responses [77]. Beyond that, differences in the stage of HEV infection and in regulating immune responses cannot be excluded in this study as an early stage of T cell activation in the liver of domestic pigs can be assumed. The liver has unique immune regulatory functions which promote the induction of tolerance rather than responses to antigens encountered locally. Thus, defense against viral infection has to take place in a tolerogenic environment [384]. Presumably, regulating immune responses are playing a particular role within the course of HEV infection as increased frequencies of regulatory T cells (Tregs) have been described for other viral hepatitides [385]. The elevation of CD4+CD25+Foxp3+ and CD4+CD25-Foxp3+ frequencies and the rise in IL-10 suggest that Tregs might be playing an important role in HEV infection associated with immunosuppressive immune responses [207]. Recently, the existence of
Tregs in swine has been demonstrated and it could be shown that porcine Tregs suppress the proliferation of different T cell subsets [386,387]. For a better understanding of HEV pathogenesis in pigs, the functional characterization of porcine Tregs in HEV infection is urgently needed. This would provide new insights into the balance between immunity and tolerance in the liver and how this may influence viral clearance, persistence and virus-induced liver disease. Moreover, NK cells may play an important role in HEV infection and IFN-γ productions by unstimulated PBMCs of hepatitis E patients suggest NK and NK T cells as key players in HEV pathogenesis [111,205,208]. Porcine NK cells have the ability to lyse virus-infected target cells and respond to various regulatory cytokines inducing INF-γ production, as well as the up-regulation of effector/activation molecules [366]. Recently, a novel marker (NKp46) has been described suitable for the discrimination of porcine NK cells with different functional properties which is highly expressed in a subset of CD8 positive liver lymphocytes [388]. Analysis of NK cells in HEV infected swine would provide further information on HEV pathogenesis especially in respect to their potential ability in direct killing of HEV infected cells.

In this study, HEVgt3 infection in pigs induced an elevation of different liver enzymes which was concomitant with enhanced viral replication and anti-HEV immune responses. Moreover, dexamethasone treatment did not significantly influence liver enzyme levels in serum of HEV infected pigs. In dexamethasone-treated, uninfected wild boar no significant changes were seen in serum liver enzyme levels after treatment. Hepatitis E in humans is also characterized by elevated serum levels of ALT and GGT [197]. Clinical HEV infections in pigs based on elevated GGT levels in serum have been reported previously [351]. Nevertheless, variable ALT levels in pigs might be also stress-induced and sex-specific [389].

An efficient HEV replication was shown in all HEV infected wild boar. In both groups comparable viral loads were detected in serum, feces, bile and different tissues. Horizontal intraspecific HEV transmission was proven as fecal HEV RNA excretion was observed in both domestic pig groups, perhaps slightly delayed in dexamethasone-treated animals. Liver and bile samples of all wild boar and mostly all domestic pigs were tested positive for HEV RNA. Mild lymphoplasmacytic and CD3 positive cell infiltrates, and Kupffer cell proliferations were found in the livers of all infected wild boar, but none in domestic pigs. By IHC, viral antigens were found in liver, liver lymph node, mesenteric lymph node and spleen
of the wild boar, but not in domestic pigs. Results indicate that HEVgt3 infection in wild boar is transmissible to domestic pigs and that the liver is the primary location for HEV replication. This is in line with findings obtained by previous studies in intravenously infected pigs [269,351] and naturally infected swine [295]. In contrast to another study [268], viral antigens were not detected in small intestine, large intestine or kidney by immunohistochemical analysis, despite positive signals in RT-qPCR. These findings might be because of differences in the sensitivity between RT-qPCR and immunohistochemistry, but also because of detecting different targets as in the first-mentioned assay viral RNA of HEV-ORF3, and in the latter viral capsid protein encoded by HEV-ORF2 was detected. Nevertheless, contamination of intestinal tissues through HEV-containing ingesta cannot be excluded completely. No significant differences in HEV replication and viral antigen distribution dependent on the immune status were noticed, despite a slight increase of viral loads in dexamethasone-treated domestic pigs. Seroconversion occurred in both wild boar groups independent on their immune status. Possibly, slightly increased antibody levels in dexamethasone-treated wild boar at the end of experiment might be an effect of glucocorticoid administration, but random effects cannot be excluded. Previous studies have indicated that dexamethasone is capable of inducing a shift in the immune response from a T cell helper 1 towards a T cell helper 2 response by influencing the levels of cytokines produced by the lymphocytes [390]. Only one untreated domestic pig developed measureable anti-HEV antibodies within the experiment. It was shown recently that antibody responses in orally infected domestic pigs were less efficient as compared to the intravenous inoculation route [273]. Probably, longer observation periods would have been led to seroconversion in the majority of HEV infected pigs as normally strong anti-HEV antibody responses have been seen during the early course of infection [111].

Our results indicate that HEVgt3 infection in wild boar enhances the cellular and humoral immune responses, surprisingly largely unaffected by a dexamethasone induced immunosuppression. Notably, a significant immunosuppressive effect of dexamethasone was proven in dexamethasone-dosed, uninfected wild boar serving as treatment control. Moreover, HEVgt3 was successfully fecal-orally transmitted to domestic pigs irrespectively of their immune status. Especially an increase of cytotoxic T lymphocytes followed by an increase of T-helper/memory cells and activated γδ T cell subsets was shown in intravenously HEV
infected wild boar. Anyhow, in PMBCs of domestic pigs dominated a T helper response. Moreover, marked increase in percentages of γδ T cells were observed in the liver of all HEVgt3 infected pigs. Additionally, higher T helper/memory cell and γδ T cell percentages were found in mesenterial lymph nodes of the HEVgt3 infected domestic pigs. Of course, differences in cellular immune responses between the wild boar and domestic pigs might be due to distinct transmission routes, stages of infection, intraspecific and individual varieties. No significant differences in viral replication or liver lesions were observed in the HEVgt3 infected groups. Hence, an association between immunosuppression and enhanced susceptibility of pigs to HEVgt3 was not demonstrated. Additionally, no differences in clinical manifestations of hepatitis E due to potentially diminished inflammatory responses in dexamethasone-treated pigs were observed. Possibly, longer observation periods would have indicated viral persistence also in swine. Our findings are in contrast to results obtained in dexamethasone-treated Peste des Petits Ruminants virus (PPRV) infected goats and swine influenza virus infected turkeys. Immunosuppression in turkeys revealed an increase of virus replication, prolonged virus shedding and the possibility of enhancing virus transmission [391]. Contrary to the study presented here, the extent and distribution of PPRV antigen were increased in dexamethasone-treated goats [392]. Supposedly, further HEV infection studies with higher animal numbers are required to define differences in HEV replication more precisely and to exclude statistical outliers. Moreover, longer observation periods would facilitate the possibility of developing viral persistence which may also lead to chronic HEV infection in pigs. Studies in miniature pigs experimentally infected with Leptospira interrogans revealed persistence until the chronic phase, and excretion of leptospires was increased under immunosuppressive conditions, resulting in enhanced horizontal transmissions [393]. However, other methods for immunosuppression have to be tested as a relative resistance to dexamethasone-induced immunosuppression has been shown in long-term treated domestic pigs previously [394]. CD8-depletion in hepatitis B virus infected chimpanzees demonstrated that CD8 positive cells were the main effector cells responsible for viral clearance and disease pathogenesis [395]. In consequence, targeted modifications of the porcine immune system such as the ability to modulate *in vivo* T cell populations of pigs [396] would provide deeper insights into the role of different immune cell subsets and
immune regulation mechanisms in HEV pathogenesis. Exemplary, in vivo depletion of CD8 positive T lymphocytes abrogates protective immunity to African swine fever virus [375].

In conclusion, immunosuppressive effects of dexamethasone were proven, but did not affect substantially the course of HEV infection in wild boar and domestic pigs. However, further research is needed to understand immunopathological processes in porcine HEV infection more precisely.
### 4.6 Tables

Table 4.1 Overview of tested lymphocyte phenotypes and their predicted functionality in swine.

<table>
<thead>
<tr>
<th>Phenotype of tested lymphocyte subpopulation</th>
<th>Shortened nomenclature</th>
<th>Predicted functionality in swine</th>
<th>Literature</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3+γδTCR+</td>
<td>γδ T cells (γδTCR+)</td>
<td>- frequency within PBL: 5–50%, form a major T cell subpopulation within PBLs in young animals (age-dependent decrease)</td>
<td>Hirt et al. 1999 [397], Yang and Parkhouse et al. 1996 [367], Gerner et al. 2009 [366]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- phenotypically heterogeneous</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>- act in innate and adaptive immune responses</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>- cytolytic activity and antigen presentation</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>- source IL-17</td>
<td></td>
</tr>
<tr>
<td>CD3+γδTCR+CD2+CD8+</td>
<td>activated γδ T cells (γδTCR+CD8+)</td>
<td>- scarce in porcine fetuses</td>
<td>Saalmueller et al. 1990 [398], Yang and Parkhouse et al. 1997 [399], Takamatsu et al. 2006 [364], Gerner et al. 2009 [366]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- peripheral γδ T cells subdivide into three subsets based on expression of CD2 and CD8 and differ in their homing characteristics</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>- acquire CD8 upon activation, potentially cytotoxic</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>- present antigen via MHC class II</td>
<td></td>
</tr>
<tr>
<td>CD3+CD4+CD8−</td>
<td>T helper cells (CD4+CD8−)</td>
<td>- T helper cells</td>
<td>Saalmueller et al. 1987 [400], Saalmueller et al. 2002 [373], Kaser et al. 2008 [386], Gerner et al. 2009 [366]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- MHC-class II-restricted</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>- up-regulation of CD8α and MHC-class II upon activation</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>- frequency within PBL: 19–60%</td>
<td></td>
</tr>
<tr>
<td>CD3+CD4+CD8−CD25high</td>
<td>activated T helper cells (CD4+CD25high)</td>
<td>- regulatory T helper cells</td>
<td>Saalmueller et al. 1999 [401], Gerner et al. 2009 [366]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- down-regulate immune responses</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>- frequency within PBL: 1–3%</td>
<td></td>
</tr>
<tr>
<td>CD3+CD4−CD8+</td>
<td>cytotoxic T lymphocytes (CD8+CD4−)</td>
<td>- comprise MHC class-I restricted cytolytic T cells</td>
<td>Saalmueller et al. 1987 [400], Zuckermann et al. 1996 [372], Saalmueller et al. 2002 [373], Gerner et al. 2009 [366]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- frequency within PBL: 8–21%, up to 40%</td>
<td></td>
</tr>
<tr>
<td>CD3+CD4+CD8+</td>
<td>T-helper/memory cells (CD4+CD8+)</td>
<td>- both activated and memory T helper cells in newborn piglets nearly absent, but increases with age</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>- in vitro reactive in primary immune responses and respond to recall antigen in secondary immune response</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>- express MHC class II antigens</td>
<td></td>
</tr>
<tr>
<td>CD3−CD2+CD21+</td>
<td>naïve B cells (CD2+CD21+)</td>
<td>- CD2 and CD21 molecules expressed differentially in mature B cells (divided into four subpopulations that include CD2+CD21+, CD2−CD21+, CD2+CD21− and CD2−CD21− cells)</td>
<td>Sinkora et al. 2009 [402], Sinkora et al. 2012 [403]</td>
</tr>
<tr>
<td>CD3−CD2−CD21+</td>
<td>B cells after activation (CD2−CD21+)</td>
<td>- CD21 can be considered as a maturation marker</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>- CD2 is an adhesion molecule; down-regulated by cell-to-cell contact</td>
<td></td>
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</tbody>
</table>

Lymphocyte subpopulations of pigs have been investigated in a number of studies with the aim to identify correlations between function and the phenotype of these cells. Nevertheless, defined roles of different subsets in the porcine immune system are not resolved yet. MHC = major histocompatibility complex; PBL = peripheral blood lymphocytes; IL = interleukin.
Table 4.2 Detection of HEV RNA and anti-HEV IgG-antibodies in serum of wild boar.

<table>
<thead>
<tr>
<th>Day post inoculation of wild boar</th>
<th>Viral load in serum</th>
<th>Anti-HEV IgG-antibodies</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Group 1A (n=4)</td>
<td>Group 1B (n=4)</td>
</tr>
<tr>
<td></td>
<td>Number of RNA-positive animals</td>
<td>Viral load in positive samples*</td>
</tr>
<tr>
<td>0</td>
<td>0 -</td>
<td>0 -</td>
</tr>
<tr>
<td>4</td>
<td>0 -</td>
<td>0 -</td>
</tr>
<tr>
<td>6</td>
<td>0 -</td>
<td>0 -</td>
</tr>
<tr>
<td>9</td>
<td>0 -</td>
<td>0 -</td>
</tr>
<tr>
<td>13</td>
<td>0 -</td>
<td>0 -</td>
</tr>
<tr>
<td>15</td>
<td>0 -</td>
<td>0 -</td>
</tr>
<tr>
<td>18</td>
<td>0 -</td>
<td>0 -</td>
</tr>
<tr>
<td>21</td>
<td>0 -</td>
<td>1</td>
</tr>
<tr>
<td>24</td>
<td>0 -</td>
<td>0 -</td>
</tr>
<tr>
<td>28</td>
<td>1</td>
<td>14</td>
</tr>
<tr>
<td>30</td>
<td>2</td>
<td>11 / 823</td>
</tr>
</tbody>
</table>

* Viral load in HEV genome equivalents per µl RNA. Group 1A = non-treated wild boar; Group 1B = dexamethasone-treated wild boar.
Table 4.3 Viral loads in fecal samples of wild boar and domestic pigs estimated by RT-qPCR.

<table>
<thead>
<tr>
<th>DPI</th>
<th>Wild boar</th>
<th></th>
<th>Domestic pigs</th>
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</tr>
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<tbody>
<tr>
<td></td>
<td>Group 1A</td>
<td>Group 1B</td>
<td>Group 2A</td>
<td>Group 2B</td>
</tr>
<tr>
<td></td>
<td>WB 47</td>
<td>WB 49</td>
<td>DP 52</td>
<td>DP 53</td>
</tr>
<tr>
<td></td>
<td>WB 50</td>
<td>WB 51</td>
<td>DP 59</td>
<td>DP 64</td>
</tr>
<tr>
<td></td>
<td>WB 44</td>
<td>WB 46</td>
<td>DP 65</td>
<td>DP 70</td>
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<td></td>
<td>WB 48</td>
<td>WB 52</td>
<td>DP 75</td>
<td>DP 97</td>
</tr>
<tr>
<td>0</td>
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<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
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<tr>
<td>18</td>
<td>23</td>
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<td>0</td>
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<tr>
<td>21</td>
<td>166</td>
<td>144</td>
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<td>28</td>
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<td>30/31</td>
<td>439</td>
<td>2725</td>
<td>211</td>
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<td>6</td>
<td>0</td>
</tr>
<tr>
<td>36</td>
<td>-</td>
<td>-</td>
<td>0</td>
<td>15</td>
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<tr>
<td>41</td>
<td>-</td>
<td>-</td>
<td>0</td>
<td>7</td>
</tr>
</tbody>
</table>

Viral copy numbers in fecal samples (1:5 diluted in isotonic saline) were calculated from Ct-values determined by RT-qPCR. Ct-values equal to HEV genome equivalents per µl RNA: Ct 19 – 22 equal to $10^4$, Ct 23 – 26 equal to $10^3$, Ct 27 – 30 equal to $10^2$, Ct 31 – 34 equal to $10^1$, Ct $> 35$ equal to 0; Group 1A and 2A = non-treated; group 1B and 2B = dexamethasone-treated; WB = wild boar; DP = domestic pig; n. d. = viral load was not determined (no sample available); DPI = day post inoculation of wild boar.
4.7 Figures

Figure 4.1 Mean values of liver enzyme levels, antibody responses and HEV RNA loads in serum and feces of wild boar.

Error bars represent ±SD. A. Detection of alanine aminotransferase (ALT) and gamma-glutamyl transferase (GGT) in serum of group 1A, 1B and 1C. Upper reference range limit = grey-dotted line. IU = international units. Two way ANOVA (Bonferroni t-test; P<0.05). The difference in the mean values among group 1A and the uninfected reference group “0 DPI” is greater than would be expected by chance: *c = significant with P<0.05.

B. Detection of anti-HEV IgG-antibodies in serum of group 1A and 1B with mean absorbance (OD450). PrioCHECK® HEV Ab porcine. Cut-off (grey-dashed line): OD450 > 0.6. One way ANOVA (Bonferroni t-test; P<0.05). C. HEV RNA in serum and feces of group 1A and 1B estimated by RT-qPCR. One way ANOVA (Bonferroni t-test; P<0.05). DPI = day post inoculation of group 1A and 1B.
Figure 4.2 Viral loads in different excreta and tissue samples of wild boar and domestic pigs.

A. Mean excreted HEV genome equivalents in bile, small and large intestine, ingesta (of cecum) and feces with positive standard deviation (only reactive samples considered). The proportion of positive tissue samples is included in each bar column (n positive sample / n total sample). One way ANOVA (Bonferroni t-test; P<0.05); n.s. = not significant P>0.05. B. Mean HEV genome equivalents in liver, gall bladder, liver lymph node and spleen with positive standard deviation (only reactive samples considered). The proportion of positive tissue samples is included in each bar column (n positive sample / n total sample). One way ANOVA (Bonferroni t-test; P<0.05); n.s. = not significant P>0.05. DPI = day post inoculation of group 1A and 1B.
Figure 4.3 Differential cell counts in peripheral blood of wild boar.  
The mean number (in K/µL) of leukocytes (WBC), lymphocytes (LYM), monocytes (MON) and neutrophils (NEU) were determined using an automated XT-2000iV hematology analyzer. Error bars represent ±SD. Reference value ranges (grey-dotted line) for the tested blood parameters in wild boar were calculated for the evaluation of the results. Two way ANOVA (Bonferroni t-test; P<0.05). The difference in the mean values among group 1A and 1B is greater than would be expected by chance: *a = significant with P<0.05. The difference in the mean values among group1C and the uninfected reference group “0 DPI” is greater than would be expected by chance: *b = significant with P<0.05. The difference in the mean values among group1A and the uninfected reference group “0 DPI” is greater than would be expected by chance: *c = significant with P<0.05. DPI = day post inoculation of group 1A and 1B.
Figure 4.4 T-cell related responses upon infection in blood lymphocytes of wild boar.
The number of T cell subpopulations of blood lymphocytes is given: cytotoxic T lymphocytes (CD8+CD4-), T helper/memory cells (CD4+CD8+) and activated γδ T cells (γδTCR+CD8+). Two way ANOVA (Bonferroni t-test; P<0.05). The difference in the mean values among group 1A and 1B is greater than would be expected by chance: *a = significant with P<0.05. The difference in the mean values among group 1C and the uninfected reference group “0 DPI” is greater than would be expected by chance: *b = significant with P<0.05. The difference in the mean values among group 1A and the uninfected reference group “0 DPI” is greater than would be expected by chance: *c = significant with P<0.05. DPI = day post inoculation of group 1A and 1B.
Figure 4.5 B-cell related responses upon infection in blood lymphocytes of wild boar.
Blood lymphocytes were immune-stained to determine the frequency of different B cell subpopulations by FACS analysis: naïve B cells (CD2+CD21+), activated B cells upon antigen contact (CD2-CD21+) and antibody-forming and/or memory B cells (CD2+CD21-). Two way ANOVA (Bonferroni t-test; P<0.05). The difference in the mean values among group 1C and the uninfected reference group “0 DPI” is greater than would be expected by chance: *b = significant with P<0.05. The difference in the mean values among group 1A and the uninfected reference group “0 DPI” is greater than would be expected by chance: *c = significant with P<0.05. DPI = day post inoculation of group 1A and 1B.
Figure 4.6 T-cell related responses upon infection in liver, spleen and mesenterial lymph node of wild boar.

Immune cell phenotypes of separated cells of liver, spleen and mesenterial lymph node were examined after necropsy: T cells (CD3+), cytotoxic T lymphocytes (CD8+CD4-), T helper cells (CD4+CD8-), T helper/memory cells (CD4+CD8+) and γδ T cells (γδTCR+). A. T cell related responses in wild boar. Two way ANOVA (Bonferroni t-test; P<0.05). The difference in the mean values among group 1A and 1B is greater than would be expected by chance: *AB = significant with P<0.05. The difference in the mean values among group 1A and 1C is greater than would be expected by chance: *AC = significant with P<0.05. The difference in the mean values among group 1B and 1C is greater than would be expected by chance: *BC = significant with P<0.05. The difference in the mean values among group 1A, 1B and 1C is greater than would be expected by chance: *ABC = significant with P<0.05.
Figure 4.7 T-cell related responses upon infection in liver, spleen and mesenterial lymph node of domestic pigs.

Immune cell phenotypes of separated cells of liver, spleen and mesenterial lymph node were examined after necropsy: T cells (CD3+), cytotoxic T lymphocytes (CD8+CD4-), T helper cells (CD4+CD8-), T helper/memory cells (CD4+CD8+) and γδ T cells (γδTCR+). A. T cell related responses in wild boar. Randomly selected HEV negative domestic pigs (n = 6) served as negative controls. Two way ANOVA (Bonferroni t-test; P<0.05). The difference in the mean values among group 2A and 2B is greater than would be expected by chance: *AB = significant with P<0.05. The difference in the mean values among group 2A and 2C is greater than would be expected by chance: *AC = significant with P<0.05. The difference in the mean values among group 2B and control is greater than would be expected by chance: *BC = significant with P<0.05. The difference in the mean values among group 2A, 2B and control is greater than would be expected by chance: *ABC = significant with P<0.05. Control = negative controls.
Figure 4.8 Detection of viral antigens in the liver, liver lymph node and spleen of wild boar by immunohistochemistry.
A - C: Diffuse distribution of viral antigens within a liver lobule (group 1B). D - E: Viral antigens in the cortical zone (central area of the node) and in secondary follicles of a liver lymph node (group 1A). F: Immunolabelling of viral antigens in the germinal center of a lymphoid follicle in spleen (group 1B). All scale bars represent 100 µm.
5. Manuscript III: Evidence of persistent hepatitis E virus infection in European wild boar naturally infected with genotype 3 and transmissibility to domestic pigs

Evidence of persistent hepatitis E virus infection in European wild boar naturally infected with genotype 3 and transmissibility to domestic pigs

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5.1 Abstract

Hepatitis E virus (HEV) causes acute hepatitis E in humans in developing countries, but sporadic and autochthonous cases do also occur in industrialized nations. In Europe, food-borne zoonotic transmission of genotype 3 (gt3) have been associated with the consumption of raw and undercooked products from domestic pig and wild boar. As shown recently, naturally acquired HEVgt3 replicates efficiently in experimentally infected wild boar and is transmissible from wild boar to domestic pigs. Generally, following an acute infection swine suffer from a transient febrile illness and viremia in connection with fecal virus shedding. However, little is known about sub-acute or persistent HEV infections in swine, and how and where HEV survives the immune response. In this paper we describe the incidental finding of a persistent HEVgt3 infection in two naturally infected European wild boar which were raised and housed at FLI over years. The wild boar displayed fecal HEV RNA excretion and viremia over nearly the whole observation period of more than five months. The animal had mounted a substantial antibody response, yet without initial clearance of the virus by the immune system. Further analysis indicated a subclinical course of HEV with no evidence of chronic hepatitis. Additionally, we could demonstrate that this persistent wild boar infection was still transmissible to domestic pigs, which were housed together with this animal. Sentinel pigs developed fecal virus shedding accompanied by seroconversion. Wild boar should therefore be considered as natural reservoir and transmission host for HEVgt3 in Europe.
5.2 Introduction

Hepatitis E virus (HEV) is a non-enveloped, positive-stranded RNA virus, which is the causative agent of hepatitis E in humans [8]. The virus is member of the genus *Hepeivirus* in the family *Hepeviridae*. The genome contains three open reading frames (ORF), whereas ORF1 codes for a polyprotein that is post-translationally processed into non-structural proteins including a methyltransferase, papain-like protease, helicase and RNA-dependent RNA polymerase. ORF2 encodes the viral capsid protein, and ORF3 codes for a small phosphoprotein [25]. HEV consists of four major mammalian genotypes (gt): while gt1 and gt2 are exclusively found in humans, gt3 and gt4 are zoonotic and commonly infect both humans as well as other mammals like pigs, wild boar, deer and other. According to a new taxonomic scheme human and porcine HEV are assigned to the species *Orthohepevirus A*. Other genotypes were classified into species *Orthohepevirus B* with isolates from chicken, *Orthohepevirus C* with isolates from rat, ferret, mink and others as well as *Orthohepevirus D* with isolates from bat [19]. HEV is transmitted via the fecal-oral route, usually via the consumption of contaminated water or food. Gt1 and gt2 are responsible for the majority of HEV infections in humans in endemic areas of Asia, Africa and Mexico. In contrast, HEVgt3 and gt4 have been identified with increasing frequency in human sporadic and autochthonous cases in Europe, the USA, China and Japan, and are associated with zoonotic transmissions [21,192]. Sources were mainly contaminated raw or undercooked meat derived from domestic pigs and wild boar, as well as deer [11,404,405]. This was confirmed by the molecular characterization of HEV sequences recovered from food products and from affected patients [116]. In Europe main reservoirs of HEV are pigs and wild boar as revealed by high anti-HEV antibody seroprevalence rates in farmed pigs and wild boar populations [406]. Furthermore phylogenetic studies also indicate HEV transmissions from domestic pig to wild boar [289,295,350]. In humans HEV causes acute hepatitis including jaundice, fever, malaise, abdominal pain and vomiting lasting 2 to 18 weeks (on average 4 weeks). Chronic infections are observed in immunosuppressed patients as well as in patients co-infected with HIV [237]. In pigs, HEV infections induce a temporary viremia lasting 1–2 weeks which is accompanied by virus shedding via feces for over 3–7 weeks. Subclinical infections have been associated only with mild to moderate hepatitis [407]. As shown recently, experimental HEV infections in wild boar can induce hepatic lesions [351]. Experimentally infected animals mounting
early and robust anti-HEV antibody responses were able to clear the virus within one month, while animals with insufficient antibody responses developed prolonged HEV infections [351]. Both viral as well as host factors seem to determine the course of HEV infection, but the details are only partially understood [223]. In summary, while acute HEV infections in swine have been studied in more detail [259,269,351], little is known about persistent HEV infections leading to chronic hepatitis E.

To shed light into the still unknown pathogenesis of chronic infected animals we studied here naturally HEVgt3 infected European wild boar (Sus scrofa scrofa) over a time period of six months. In doing so we also were able to demonstrate that excreted HEV easily infects domestic pigs (S. scrofa domestica).
5.3 Materials and methods

5.3.1 Experimental design

Two wild boar of nine months of age and two domestic pigs of four months of age were used in the experiment. The animals utilized in the study were obtained from local farmers in Mecklenburg-Western Pomerania, Germany. Animals were fed with commercial pig feed and had access to water *ad libitum*. Measurement of the body weight and collection of blood and fecal samples were done weekly, whereas rectal temperatures were determined daily. Fever was defined as a body temperature ≥ 40.0 °C for at least two consecutive days. Aliquots of serum samples were stored at −20 °C for antibody detection, and at −70 °C for RNA extraction. Fecal samples were diluted in isotonic saline solution (10%, w/v) and stored at −70 °C for RNA extraction. The experiment was finished after 25 weeks. At necropsy, tissue samples (liver, liver lymph node, mesenteric and mandibular lymph nodes, gall bladder, small and large intestine, pancreas, kidney, spleen, tonsil, heart, brain and quadriceps femoris muscle) were collected for virological, histopathological and immunohistochemical investigations. One part of each tissue sample was fixed immediately in 4% neutral buffered formalin for histological examination and the other part was stored at −70 °C for RNA extraction. All experiments were carried out under biosafety level 3** taking into account animal welfare regulations and standards according to EU Directive 2010/63/EU and institutional guidelines. The experimental protocol was reviewed by an independent animal welfare and ethics committee and was approved by the competent authority of Mecklenburg-Western Pomerania (State Office for Agriculture, Food Safety and Fisheries of Mecklenburg-Western Pomerania, Rostock, Germany, reference number LALLF M-V/TSD/7221.3-1.1-022/12).

5.3.2 RT-qPCR and phylogenetical analysis

Viral RNA from serum samples and fecal suspension was extracted using the QIAamp® Viral RNA Kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer’s instructions. Viral RNA from tissue samples was extracted using the RNeasy Mini Kit (QIAGEN GmbH). All samples to be tested by quantitative real-time reverse transcriptase PCR (qRT-PCR) were spiked with an internal control RNA and analyzed with HEV-specific primers and a probe, which targets the ORF3 region as described in elsewhere [351]. The assay was carried out
using the Quantitect probe RT-PCR kit (QIAGEN GmbH). HEV copy number was calculated using a standard curve based on a synthetic external calibrator encompassing the 81 bp sequence of the RT-qPCR amplicon [351]. Phylogenetic analysis is based on a 349 nt sequence of the hypervariable region according to Vina-Rodriguez et al. 2015 (submitted).

5.3.3 Anti-HEV antibody ELISA

Sera were tested for the presence of anti-HEV antibodies with a species independent HEV-Ab ELISA kit (Axiom, Buerstadt, Germany) according to the manufacturer’s instructions. The ELISA uses recombinant HEV antigens for the detection of total anti-HEV antibodies (IgA, IgM, and IgG) in serum or plasma. Values of the optical density at 450 nm (OD450) equal to or greater than 1 are prescribed as seropositive.

5.3.4 Histopathology and immunohistochemistry

For histopathological examinations formalin fixed liver samples were stained with hematoxylin and eosin (HE) according to standard protocols. For immunohistochemistry (IHC) tissue sections were treated as described elsewhere [351]. Therefore, 3 µm sections were cut, de-paraffinised and rehydrated. The pretreatment included a blocking step for the endogenous peroxidase using 3% H2O2/methanol for 30 min, followed by an antigen retrieval step in the microwave for 10 min at 600 W. Viral antigens were detected using a rabbit anti-HEVgt3 serum (rHEVgt3-HIS) in a 1:1000 dilution. Therefore, rabbits were immunized with an Escherichia coli expressed and purified His-tagged C-terminal segment of HEV gt3 capsid protein [163]. The slides were incubated with biotinylated goat anti-rabbit immunoglobulin (Vector Laboratories, LINARIS, Dossenheim, Germany) and an avidin/biotinylated enzyme complex (VECTASTAIN®ABC Reagent, Vector Laboratories, Burlingame, United States of America) followed by visualisation with 3,3-Diaminobenzidine (DAB, Sigma-Aldrich Chemie GmbH, Steinheim, Germany).
5.4 Results

Infection monitoring of animals from a wild boar colony kept in outdoor pens at the FLI revealed two animals (WB05 and WB09) which were positive for HEV RNA in feces. Surprisingly these animals were also viremic while simultaneously carrying anti-HEV antibodies. Viral RNA levels in blood and feces as well as their antibody status were therefore monitored over the next 6 months (Figure 5.1). Initially, viral loads in feces of both wild boar exceeded 60000 copies per µl, while RNA genome copies in serum were at about 52 and 320 copies per µl. Phylogenetic analysis of partial sequences recovered from feces assigned the isolates to genotype 3, subtype 3i, which is a common sub-genotype in the German swine population (Figure 5.2, modified according to Vina-Rodriguez, 2015). In WB05, viral RNA was concomitantly found in serum and feces for 14 weeks before penned up. Viral shedding in feces lasted for about 16 weeks. Surprisingly, after an absence of detectable viral genome copies for almost 8 weeks, HEV RNA in serum reappeared one single time 23 weeks post the initial start of the analysis. In WB09, HEV RNA was detected until week 16 and stayed negative until the end of the observation period. In both wild boar antibody titers remained at high levels throughout the time independently from the presence or absence of virus shedding/viremia. Antibody levels increased four weeks after HEV naïve domestic pigs were housed together with the wild boar and started to shed virus themselves (see below).

A transmission experiment was initiated 14 weeks after first detection of HEV in wild boar: two domestic pigs (DP17 and DP21) were stabled together with both HEV persistently infected and virus shedding wild boar under high containment conditions. Prior to the start of the transmission experiment domestic pigs were tested to be negative for anti-HEV antibodies in serum and did not carry HEV RNA in feces and serum, respectively. Two weeks after the co-housing of the animals HEV RNA was first time detected in feces of both domestic pigs, which seroconverted another two weeks after the initial contact to the wild boar (Figure 5.1 A). Interestingly, anti-HEV antibodies persisted only in DP21 until to the end of experiment. In contrast serum antibody levels of DP17 dropped back after four weeks post exposure reaching eventually the base line again (Figure 5.1 B).

Neither wild boar nor domestic pigs showed any clinical symptoms indicative for viral hepatitis or abnormalities regarding body temperature and body weight (see Additional file 9). In liver tissues, clear histopathological alterations typical for acute or chronic viral hepatitis
were not seen and no viral antigens were detectable (Figure 5.3).

None of the tissue samples taken at necropsy (liver, liver lymph node, mesenteric and mandibular lymph nodes, gall bladder, small and large intestine, pancreas, kidney, spleen, tonsil, heart, brain and quadriceps femoris muscle) were tested positive for HEV RNA.
5.5 Discussion

In Europe, food-borne zoonotic transmission of HEVgt3 has been mainly associated with pigs and wild boar [329]. As shown recently, naturally required HEVgt3 replicates efficiently in experimentally infected wild boar and is transmissible from wild boar to domestic pigs [351]. In humans HEV gt1 and gt2 are known to cause acute hepatitis in contrast to gt3 that was identified as a novel causative agent of chronic hepatitis in immunocompromised patients only [223]. Chronic HEV infection in humans is characterized by elevated liver enzyme levels and detectable HEV RNA in the serum and/or stool for at least six months [408]. While a similar chronic liver pathology, including a chronic inflammatory cell infiltration, portal fibrosis and HEV shedding can be induced in experimentally rabbit HEV infected rabbits as well [149], such cases were never reported to date in swine.

In this study, we examined the naturally HEVgt3 infections in two European wild boar for up to six months, and found that a persistent HEV infection, as characterized by long-term viremia and fecal shedding, can prevail despite the presence of anti-HEV antibodies. Moreover, we could show that wild boar transmitted the HEV infection to domestic pigs which were co-housed with them. Normally, an enhancement of the adaptive cellular immune response might prevent persistent HEV infections [225] and HEV should be cleared by high titters of neutralizing anti-HEV antibodies [348]. However, growing evidence indicates that particular non-enveloped viruses can survive in the blood stream when enveloped in host-derived cell membranes -- so-called “membrane-hijacking” mechanism [23]. Halac et al. observed also in an immunosuppressed liver transplant recipient viremic episodes despite the presence of anti-HEV IgG and IgM antibodies in serum [409]. Moreover, chronic hepatitis E in humans can be associated with impaired HEV-specific T-cell responses [225]. In addition, the activation of the interferon system and a viral evolution may be associated with severity or chronicity of hepatitis E [223]. The reasons for the development of chronic HEV infections are unclear, but host factors like the age at exposure and the presence of co-infections have been discussed to modulate the clinical outcome of HEV infections [272,410]. To date, chronic hepatitis E cases caused by HEVgt3 infections have been described in immunosuppressed patients only [223].

As for humans the underlying cause of the HEV persistence in wild boar remains largely unresolved. There may be HEV genotype effects as the here recovered HEVgt3 subtype 3i
was also associated with chronic hepatitis in kidney transplant recipients in France [411]. In humans, studies of circulating HEV subtypes have identified several variants with increased or decreased virulence [412,413], but more detailed studies are lacking.

None of the collected tissue samples in this study were tested positive for HEV RNA or viral antigens. Extra-hepatic replication sites have been reported [273], and HEV RNA was detected in the brain of intravenously inoculated wild boar and SPF rabbits with prolonged viral shedding [149,351]. In humans, neurological disorders are potential extra-hepatic manifestations of HEV infections [109]. In fact it cannot be ruled out that an earlier time point of necropsy of the wild boar would have resulted in positive HEV RNA in this tissue as well. In this study, clinical and pathological examinations were unsuggestive of viral hepatitis and no signs for chronic hepatitis were seen in the persistently infected wild boar livers. This is in contrast to several studies in domestic pigs using intravenous or contact transmission of pig-derived HEV and showing histopathological signs of acute or subacute hepatitis, but no clinical symptoms [11,259,269,414].

The transmission of HEV from wild boar to two domestic was associated with virus shedding two weeks after the first contact and subsequent seroconversion, which resembles the natural course of HEV infection. These results supported earlier studies showing that the fecal-oral transmission of HEV is considered to be the main transmission route among pigs [259,271].

Interestingly, anti-HEV antibodies persisted only in one domestic pig while in the second animal a rapid decrease within five weeks was observed. The reason for this individual variability remains unclear but similar results were described in humans as well. Humeral antibody responses can vary also substantially among HEV infected patients as shown by individuals displaying no or only transient IgM antibodies, despite of HEV viremia and RNA shedding in their feces [203].

In conclusion, wild boar can be subclinically persistently HEV infected over months in the presence of anti-HEV antibodies. However, although wild boar can develop a liver pathology following experimental infection [415], no clear histopathological alterations and immunohistochemical indications for chronic viral hepatitis were observed when the animals were eventually sacrificed three months after the cessation of viremia and virus shedding, respectively. Additionally it has been shown that HEV transmitted from these wild boar to
domestic pigs via natural routes. Therefore wild boar should be considered as a steady reservoir and transmission host of HEVgt3 in Europe. Pathomechanisms for the development of persistent HEV infections in pigs should be further investigated as this may lead to a *bona fide* animal model for chronic hepatitis E.
5.6 Figures

Figure 5.1 (A) Course of HEV viral load in wild boar and domestic pigs. (B) Course of antibody response to HEV in serum.

Viral amount was indicated as copies/µl RNA. Values of the optical density at 450 nm (OD450) equal to or greater than 1 are prescribed as seropositive (cut-off). (+) indicate virus positive samples (serum/feces), (-) indicates virus negative sample (serum/feces); wild boar (WB); domestic pig (DP); ↑= start of transmission experiment (week 14).
Figure 5.2 Phylogenetic analysis of HEV isolates obtained from naturally infected wild boar used in this study based on a 349 nt sequence of the hypervariable region (HVR).

The sequences obtained in this study are indicated by filled circles. Genetic distances are calculated according to the Maximum Likelihood method.
Figure 5.3 Histopathology and immunohistochemistry of the liver.
(A - B) Hepatic lobules of a wild boar (left: WB05) and domestic pig (right: DP21) with mild hyperemia of the sinusoids. (C - D) No detection of viral antigens within the liver lobules of a wild boar (left: WB09) and domestic pig (right: DP17); wild boar (WB); domestic pig (DP); All scale bars represent 100 µm.
6. Manuscript IV: Experimental infection of different mouse strains, Wistar rats and rabbits with wild boar-derived hepatitis E virus genotype 3

Experimental infection of different mouse lines, Wistar rats and rabbits with wild boar-derived hepatitis E virus genotype 3

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6.1 Abstract

Hepatitis E virus (HEV) is the causative agent of acute hepatitis E in humans in developing countries, but sporadic and autochthonous cases do also occur in industrialized countries. In Europe, food-borne zoonotic transmission of genotype (gt) 3 has been associated with domestic pig and wild boar. Potentially zoonotic strains have been also detected in rats and genetic analyses of rabbit HEV indicate close relationship with HEVgt3 strains. Natural HEV infection in mice has not been demonstrated yet, but gt4 was successfully transmitted to nude mice. We have shown recently that HEVgt3 of wild boar origin (wbHEVgt3) is transmissible to domestic pigs. Currently, experimental approaches on wbHEVgt3 infections in rodents and rabbits are missing. Therefore, we experimentally inoculated C57BL/6, IFNRI -/-, CD4 -/-, CD8 -/- and Balb/c nu/nu mice, Wistar rats and European rabbits with wbHEVgt3 and monitored viral replication and humoral immune responses. These challenge experiments revealed that even the immunocompromised mice were not susceptible to wbHEVgt3 infection. However, HEV RNA and anti-HEV antibodies were demonstrated in rats and rabbits. Interestingly, dexamethasone treatment in rats did not enhance their susceptibility to HEV infection. In rabbits, viral replication was more efficient and an immunization with recombinant HEV capsid protein protected against viral shedding with feces. In conclusion, the rabbit model for HEVgt3 infection may serve as a suitable alternative to the non-human primate and swine models, and as an appropriate basis for vaccine evaluation studies.
6.2 Introduction

Hepatitis E virus (HEV) is the causative agent of hepatitis E in humans and the sole member of the genus *Hepevirus* in the family *Hepeviridae*. It is a small, non-enveloped virus with a single-stranded RNA genome of positive polarity [8,59]. In many developing countries where sanitary conditions are suboptimal, hepatitis E is an important public health problem, with the virus being primarily transmitted via the fecal-oral route through contaminated food or water [25]. However, emerging cases of sporadic and autochthonous hepatitis E also occur in industrialized countries, including Japan and European countries [90,91,116]. Recently, several HEV-related viruses were identified and significantly broadened the host range and diversity of HEV. A consensus classification system for the family Hepeviridae is currently unavailable, but a new taxonomic scheme in which the family is divided into the genera Orthohepevirus (Orthohepevirus A with isolates from human, pig, wild boar, deer, mongoose, rabbit and camel, Orthohepevirus B with isolates from chicken, Orthohepevirus C with isolates from rat, greater bandicoot, Asian musk shrew, ferret and mink and Orthohepevirus D with isolates from bat) and Piscihepevirus has been proposed [19]. Within the mammalian HEV isolates infecting humans, genotypes (gt) 1 and gt2 are restricted to humans, whereas gt3 and gt4 are zoonotic strains and have been molecularly detected in domestic pig, wild boar, deer, mongoose, monkey and rat [94]. In Europe and Asia, food-borne zoonotic transmissions of HEV have been primarily associated with domestic pigs and wild boar as one of the main sources of human autochthonous infections [89,116,288]. Additionally to the consumption of contaminated raw or undercooked meat, direct contact to pigs has to be considered as an additional risk factor for HEV infection [163,349]. At first, HEV was known to cause only acute hepatitis, but lately HEVgt3 was also identified as a novel causative agent of chronic hepatitis in immunocompromised patients [110]. Pigs being the natural host species for HEVgt3 and 4 can be used as a homologous animal model system for HEV studies [416]. Laboratory mice and rats have been also explored as potential animal models for HEV. Although an earlier study indicated susceptibility of laboratory rats to gt1 infection [80], recent trials to infect laboratory rats with gt1 or 2 were unsuccessful [81,82]. Moreover, experiments to infect laboratory rats with gt3 were also not successful [81,82,126]. Injection of transcripts of a gt4 cDNA into the liver of rats led to transient seroconversion [127]. This genotype was also shown to be infectious for Balb/c nude mice [128]. Another study in
C57BL/6 mice demonstrated that animals intravenously inoculated with gt1, 3 and 4 were not susceptible to HEV [83]. Newly developed swine HEV virus-like-particles have the capacity to induce antigen-specific antibody and INF-\(\gamma\) production in immunized mice [417]. Furthermore, experimental infection of rabbits with gt3 and 4 strains resulted in seroconversion, but virus shedding was dependent on the strain used [129]. Experimental inoculation of rabbits with rabbit HEV led to seroconversion, fecal virus shedding, viremia and elevated liver enzyme levels [129,148]. Pigs intravenously inoculated with rabbit HEV strains developed transient viremia and sporadic virus shedding, thus indicating a zoonotic potential of the virus [140].

Currently, experimental approaches studying wild boar-derived HEVgt3 (wbHEVgt3) infection in rodents and rabbits are missing. Recently shown, natural and experimental HEVgt3 infection in European wild boar is transmissible to domestic pigs causing a variable degree of hepatic lesions [351]. As HEV has the ability to cross species barriers, it is important to identify the possibility of cross-species transmission between wild boar and rodents, respectively rabbits. Therefore, we experimentally inoculated Wistar rats, C57BL/6, IFNRI -/-, CD4 -/-, CD8 -/- and Balb/c nu/nu mice, and European rabbits with wbHEVgt3. To determine their susceptibility to HEV infection originated in European wild boar, viral replication and humoral immune responses were monitored. In particular the availability of an appropriate mouse model, like type I interferon receptor knock-out mice, would provide new insights into HEV pathogenesis and in host’s defense mechanisms to viral infection. Dexamethasone, like any typical glucocorticoid, is a potent immunosuppressive agent which exerts multiple effects on immune cell functions [352]. Hence, we also assessed whether dexamethasone treatment in rats has an effect upon the susceptibility to wbHEVgt3 infection. Additionally, the protective ability of a HEV vaccine candidate in wbHEVgt3 inoculated rabbits was evaluated exemplarily.
6.3  Material and methods

6.3.1  Inocula

The HEVgt3 strain (wbHEVgt3) used in this study originated from a liver sample of a naturally infected wild boar hunted in Northern Germany (Mecklenburg-Western Pomerania) in 2010. The liver was frozen immediately at −20 °C and stored at −70 °C. For preparation of the inoculum, the liver was ground in phosphate-buffered saline (PBS) with a mortar and pestle (10%, w/v). The suspension was transferred to a 15 mL tube and mixed for 1 min using a vortex mixer. After centrifugation (20 min at 4000 × g at 4 °C) the supernatant was transferred to a new tube and filtered (0.22 µm MILLEX®GP filter unit, Millipore, Ireland). The suspension was aliquoted in volumes of 2.5 mL and stored at −70 °C. The inoculum contained about 2.0 × 10⁴ HEV RNA copies per µL RNA. Previously, the infectivity of this HEVgt3 strain was demonstrated in intravenously inoculated wild boar and domestic pigs [351]. Additionally, bile and feces of aforementioned intravenously infected wild boar were used for the inoculation of different mouse strains. Therefore, bile was diluted in PBS (20%, w/v) and sterile-filtered (0.22 µm MILLEX®GP filter unit), and aliquoted in volumes of 2.5 mL and stored at −70 °C. The inoculum contained about 1.7 × 10⁴ HEV RNA copies per µL RNA. Feces were suspended in PBS at a proportion of 20% (w/v). The fecal suspension was transferred to a 15 mL tube and mixed for 1 min using a vortex mixer. After centrifugation (20 min at 4000 × g at 4 °C) the supernatant was transferred to a new tube and filtered (0.22 µm MILLEX®GP filter unit). The suspension was aliquoted in volumes of 2.5 mL and stored at −70 °C. The inoculum contained about 1.0 × 10¹ HEV RNA copies per µL RNA. All control animals received sterile-filtered PBS (0.22 µm MILLEX®GP filter unit).

6.3.2  Experimental design

The experiments were approved by the competent authority of the Federal State of Mecklenburg-Western Pomerania, Germany, on the basis of national and European legislation, namely the EU council directive 86/609/EEC for the protection of animals used for experiments (LALLF M-V/TSD/7221.3-2.1.-014/10). Prior to the start of the experiments all animals were tested to be negative for anti-HEV antibodies in serum and HEV RNA in feces, respectively. Following an initial clinical examination all animals were allowed to get accustomed to the new surroundings for approximately 1 to 2 weeks prior to the initiation of
experiments. All animals, except the BALB/c nu/nu mice, were fed with commercial feed and had access to water *ad libitum*. The experiments were carried out under biosafety level 3** conditions. The isolator-maintained BALB/c nu/nu mice were kept in a specific-pathogen-free (SPF) area including autoclaved feed and drinking water. All animals were checked for clinical signs every day during the entire period of the experiment including the measurement of the body weight. Additionally, rectal temperatures were measured in Wistar rats and rabbits.

6.3.2.1 **Inoculation of mice with wbHEVgt3**

The C57BL/6, IFNRI -/-, CD4 -/- and CD8 -/- mice used in this study were bred in the SPF breeding unit of the Friedrich-Loeffler-Institute, Insel Riems, Germany. The Balb/c nu/nu mice (homozygous, inbred) were obtained from Charles River Laboratories, Sulzfeld, Germany. Inoculations were performed either by the injection of material into the lateral tail vein (*Vena coccyygea lateralis*) and/or by receiving material orally by gavage. Mice receiving PBS served as negative controls and mice co-habited with inoculated mice served as indicators of contact infection. Blood and fecal samples were collected at different time points within the experiment and at necropsy. Aliquots of serum samples were stored at −20 °C for antibody detection. Fecal samples were diluted in isotonic saline solution (10%, w/v) and stored at −70 °C for RNA extraction. Mice were euthanized by exsanguination under anesthesia and samples were collected from their liver, gall bladder, small and large intestine, kidney, spleen, heart, brain and quadriceps femoris muscle. Aliquots of all tissue samples were also stored at −70 °C for RNA extraction. An overview of the experimental setup is given in Table 6.1.

6.3.2.2 **Inoculation of Wistar rats with wbHEVgt3**

Trials were carried out in female Wistar rats (Wistar RecHan™, Harlan Laboratories, The Netherlands). An overview of the animal experiments is shown in Table 6.2. In the first experiment, 8 rats were inoculated intravenously into the lateral tail vein (*Vena coccyygea lateralis*) receiving 0.25 mL liver suspension containing wbHEVgt3 and accordingly, 8 rats serving as negative controls received 0.25 mL PBS. One additional rat was co-habited with the rats receiving wbHEVgt3. At days 4, 7, 14 and 21 post inoculation 2 rats of each intravenously inoculated group were necropsied. The contact rat was euthanized at day 32. In the second experiment, 8 rats received simultaneously 0.25 mL liver suspension intravenously
and orally, respectively. Accordingly, 4 negative control rats received PBS. To assess the influence of the immune status on infection dynamics and shedding within the second experiment, all rats were pre-treated subcutaneously with dexamethasone (0.15 mg/kg, Voren® Suspension®, Boehringer Ingelheim Vetmedica, Germany) at -7, -4 and -1 dpi. 2 intravenously inoculated rats and 1 control rat were necropsied at 4, 7, 14 and 21 dpi. Collection of blood and fecal samples were done at different time points within the experiment and at necropsy. Aliquots of serum samples were stored at −20 °C for antibody detection. Fecal samples were diluted in isotonic saline solution (10%, w/v) and stored at −70 °C for RNA extraction. Rats were euthanized by exsanguination under anesthesia and samples were collected from their liver, small and large intestine, pancreas, kidney, spleen, heart, brain, thymus, ovaries, uterus, parotid gland, lung and quadriceps femoris muscle. Each tissue sample was stored at −70 °C for RNA extraction.

6.3.2.3 Inoculation of European rabbits with wbHEVgt3

Trials were carried out in adult rabbits obtained from the quarantine and breeding facility of the Friedrich-Loeffler-Institute, Insel Riems, Germany. An overview of the animal experiments is given in Table 6.3. In the first experiment, 1 rabbit was inoculated intravenously into the ear vein (Vena auricularis) receiving 1.0 mL liver suspension containing wbHEVgt3 and accordingly, 1 rabbit received 1.0 mL PBS as negative control. In the second experiment, 1 rabbit received also 1.0 mL liver suspension and 1 negative control rabbit received 1.0 mL PBS. Additionally, 2 rabbits were immunized with an Escherichia coli expressed and purified His-tagged C-terminal segment of HEVgt3 capsid protein prior to the start of the experiment [163] and inoculated intravenously receiving 1.0 mL liver suspension containing wbHEVgt3. Collection of blood and fecal samples were done at different time points within the experiment and at necropsy (first experiment at 45 dpi and second experiment at 46 dpi). Aliquots of serum samples were stored at −20 °C for antibody detection. Fecal samples were diluted in isotonic saline solution (10%, w/v) and stored at −70 °C for RNA extraction. Rabbits were euthanized by exsanguination under anesthesia and samples were collected from their liver, gall bladder, small and large intestine, mesenterial and mandibular lymph nodes, thymus, kidney, spleen, heart, lung, brain, parotid gland and quadriceps femoris muscle. Each tissue sample was stored at −70 °C for RNA extraction.
6.3.3 **Anti-HEV antibody ELISA**

Sera were tested for the presence of total anti-HEV antibodies with a species independent HEV-Ab ELISA kit (Axiom, Buerstadt, Germany) according to the manufacturer’s instructions. The ELISA uses recombinant HEV gt1 antigens for the detection of anti-HEV antibodies in serum or plasma. Values of the optical density at 450 nm (OD450) equal to or greater than 1 are prescribed as seropositive.

6.3.4 **RNA detection**

Manual extraction of viral RNA from all serum samples and fecal suspensions was performed using the QIAamp® Viral RNA Mini Kit (QIAGEN GmbH, Hilden, Germany) according to manufacturer’s recommendations. From all tissue samples, viral RNA was extracted using the RNeasy Mini Kit (QIAGEN GmbH). For both extraction methods, an internal control RNA (IC2) was added as described previously [353]. HEV RNA was detected by a novel diagnostic quantitative real-time RT-PCR assay (RT-qPCR) using the CFX96™ Real-Time System (Bio-Rad Laboratories GmbH, Munich, Germany). All primer and probes used in this study are listed in the Additional file 1. The RT-qPCR was performed using the QuantiTec Probe RT-PCR kit (QIAGEN GmbH) in 25 µL reaction volume with final concentrations of each primer with 0.8 µM, and of the probe with 0.1 µM. A volume of 5 µL RNA was added. Reverse transcription (RT) was carried out at 50 °C for 30 min, followed by denaturation/activation at 95 °C for 15 min. DNA was amplified immediately with 45 cycles at 95 °C (10 s), 55 °C (25 s) and 72 °C (25 s). The determination of the HEV copy number was carried out using a standard curve according to a synthetic external calibrator encompassing the 81 bp sequence of the RT-qPCR amplicon.
6.4 Results

Wild-type and genetically immunocompromised mice, Wistar rats and European rabbits were inoculated experimentally with wbHEVgt3 to determine their susceptibility to HEV infection. Animals were checked for clinical parameters, humoral immune responses and viral replication in blood and fecal samples collected at different time points within the experiment and in tissue samples taken at necropsy.

6.4.1 WbHEVgt3 in mice

Challenge experiments were carried out with C57BL/6 (n = 6), IFNRI \(-/-\) (n = 8), CD4 \(-/-\) (n = 6) and CD8 \(-/-\) (n = 6) and Balb/c nu/nu mice (n=20; homozygous, inbred) either by intravenous and/or peroral inoculation. For contact-exposure mice were co-habited with inoculated mice. Surprisingly there was no evidence of a clinical disease in any of the wbHEVgt3 challenged wild-type or immunocompromised mouse lines. The body weights remained within normal limits. In none of the mouse strains HEV RNA was detected in feces or tissue samples. Moreover, no seroconversion was observed in any of the animals. An overview of the results is shown in Table 6.1.

6.4.2 WbHEVgt3 in Wistar rats

Two different trials were carried out in Wistar rats. In a first experiment, 8 rats were challenged with wbHEVgt3 intravenously, while another 8 rats were mock controls and one co-habited rat was a contact exposure control. In a second experiment 8 dexamethasone-treated rats (plus 4 mock controls) were simultaneously inoculated intravenously and orally to assess the influence of the immune status on infection dynamics and shedding. Again in none of the challenged rats clinical symptoms were observed, and their body weights and rectal temperatures remained within normal limits. It should be mentioned that slight initial body weights decreases were observed in the dexamethasone-treated rats prior to the inoculation, which normalized during the challenge experiment. In the first experiment anti-HEV antibodies were seen in 2 out of 8 intravenously inoculated rats after 17 respectively 21 dpi and HEV RNA was detectable in 1 out of 8 rats at 7 dpi. No viral RNA was found in tissue of intravenously inoculated rats. The contact animal did not seroconvert within the experiment and viral RNA was only found in the liver. In the second experiment both feces nor tissue samples were tested positive for HEV RNA, and none of the animals showed detectable anti-
HEV antibodies in serum. Moreover, negative controls remained seronegative within the experiment and viral RNA was not detected in any of the tissues and feces of the negative controls. An overview of all results is shown in Table 6.2.

6.4.3 WbHEVgt3 in European rabbits

Two different approaches were followed with rabbits. In the first experiment, 1 rabbit was inoculated intravenously with wbHEVgt3 and another rabbit served as negative control. The same experimental setup was used in the second experiment, i.e. 1 rabbit was challenged intravenously and 1 rabbit served as mock control. However, 2 more rabbits were immunized with a recombinant C-terminal fragment of HEVgt3 capsid protein prior to the wbHEVgt3 challenge. As for the mice and rats there was no evidence of clinical disease detected in any of the rabbits. The body weights and rectal temperatures remained within normal limits. In the first experiment, the intravenously inoculated rabbit seroconverted within 28 dpi. Viral RNA was detected in feces from 3 to 14 dpi, but not in tissue samples. In the second experiment, anti-HEV antibodies were seen in the intravenously inoculated rabbit after 39 dpi and a booster effect was observed in the immunized inoculated rabbits at 14 to 25 dpi. In the intravenously inoculated rabbit, fecal RNA excretion was detected from 5 to 39 dpi, but not in the immunized rabbits (Figure 6.1). Moreover, HEV RNA was found in liver and gall bladder of the intravenously inoculated rabbit. In the immunized rabbits no viral RNA was detectable in any tissue sample. The mock controls were negative for anti-HEV antibodies or HEV RNA respectively. An overview of the results is given in Table 6.3.
6.5 Discussion

Up to date experimental studies on a wbHEVgt3 infection in rodents and rabbits have not been carried out. Therefore, we experimentally inoculated C57BL/6, IFNRI -/-, CD4 -/-, CD8 -/- and Balb/c nu/nu mice, Wistar rats and European rabbits with wbHEVgt3 and monitored the viral replication and humoral immune responses. We tested also, whether a dexamethasone treatment in rats has an effect on their wbHEVgt3 susceptibility. Additionally, the protective ability of a HEV vaccine candidate in HEV inoculated rabbits was assessed in a proof of principle approach.

Several types of animal models for HEV infection have been described previously [416]. In general, non-human-primates are the best known model animals as they can be infected with a variety of HEV genotypes. Pigs have been successfully infected with HEVgt3 and 4. As shown recently a wbHEVgt3 infection in European wild boar is transmissible to domestic pigs causing a variable degree of hepatic lesions [351]. However, primate and swine HEV infection models are quite complex and expensive so that a small animal model for HEVgt3 infection would be desirable. Moreover, a productive infection of immunodeficient mice, like type I interferon receptor or CD8-molecule knock-out mice would also provide new insights into host’s defense mechanisms to HEV infection. T-cell mediated adaptive immune responses are important for the elimination of viral infections [356]. In a previous study in humans with hepatitis E, patients showed increased numbers of CD8+ and CD4+CD8+ cells compared to healthy controls [206]. As recently shown, HEV inhibits type I interferon induction by ORF1 products in-vitro [331]. Laboratory mice and rats, and European rabbits have been explored as potential animal models for HEV [80,82,128,129,148], but it remained to be determined whether this also applies for wbHEVgt3 infections.

In the study presented here, none of the mouse lines were susceptible to wbHEVgt3 infection, but HEV RNA and anti-HEV antibodies were demonstrated in rats and rabbits. It has been reported that male Balb/c nude mice can be infected and produce anti-HEV IgG when challenged with a HEVgt4 isolate derived from a domestic pig [128]. Unfortunately, it remains unclear which kind of zygosity the Balb/c nude mice in the aforementioned study had, as we used homozygous mice. Contrary to heterozygotes, homozygous Balb/c nude mice lack a functional thymus and are unable to produce T cells. The nude allele on chromosome 11 is an autosomal recessive mutation and the heterozygotes do not show partial expression of
the nude phenotype [418]. Generally, IgG responses to viruses are assumed to be T-cell dependent, but polyomavirus infection of T-cell-deficient mice was also shown to elicit protective T-cell-independent antiviral IgM and IgG responses [419]. Although it is quite improbable, the discrepancy in the nude mice results in this (resistant) and the former (susceptible) study may have arisen from gender effects (female versus male). In accordance with the here described results Li et al. also failed to infect C57BL/6 mice with HEVgt3, as well as with gt1 and gt4 isolates [83].

Recently, HEVgt3 strains were obtained from different species of wild-caught rats in the United States [123]. In experimental challenge studies, controversial data have been obtained for the susceptibility of rats to primate- and suid-derived HEV isolates. Wistar rats could be experimentally infected with a human HEV isolate (genotype not known) in earlier studies [80]. Contrary, Wistar rats were resistant to intravenously inoculated HEVgt1 originated from a cynomolgus monkey, HEVgt3 collected from a domestic pig, and to a wild boar-derived HEVgt4 isolate in a more recent study [82]. Similar to homologous challenge studies in rats using rat HEV [82], we were also able to detect HEV RNA and anti-HEV antibodies in intravenously inoculated rats, but inconsistently. Interestingly, dexamethasone treatment in rats did not enhance the susceptibility to HEV infection, to the contrary; neither seroconversion nor viral RNA was detectable in the treated rats. Similarly, Li et al. found no evidence that nude rats are susceptible to infection with HEVgt3 [126], albeit enhanced viral replication of rat HEV was seen in nude rats [82].

Intravenously infected rabbits seroconverted within 4 to 5 weeks and a booster effect was seen in immunized animals 2 weeks post inoculation indicative for antigen-specific memory B cells. In rabbits, viral replication was therefore more efficient and immunization with a recombinant capsid protein derivative protected against viral shedding with feces. Our findings are in accordance to another study in HEVgt3 infected rabbits [129]; beside the protective ability of recombinant HEV proteins and the detection of seroconversion, we were also able to demonstrate fecal viral shedding and HEV RNA in liver and gall bladder. As a human HEVgt3 strain was used in the study mentioned before, it cannot be excluded that rabbits might be more susceptible to HEVgt3 of wild boar origin. Nevertheless, rabbits could be experimentally infected with human HEVgt4 originated from patients with acute hepatitis E [129,148]. Interestingly, HEV sequences of a human strain in France and rabbit strains were
closely related sharing a 93-nucleotide insertion [139]. Recently shown, rabbit HEV is able to infect domestic pigs, but rat HEV failed to infect pigs [140]. A study in China found no evidence of natural cross-species infection with rabbit HEV [136]. Rabbit HEV is a distant member of HEVgt3 and studies indicated that rabbit HEV belongs to the same serotype as human HEV [147], but the antigenically relationship between rabbit and wbHEVgt3 is unclear. Therefore, it would be also interesting to determine whether immunization with recombinant HEVgt3 capsid protein protects rabbits against rabbit HEV infection. Moreover, further studies including histopathological and immunological analyses, and the determination of liver enzyme levels would provide more information on viral pathogenesis and clinical significance of wbHEVgt3 infection in rabbits.

In conclusion, no signs of viral replication were seen in different mouse strains inoculated with wbHEVgt3 and resistance to HEVgt3 in mice can be assumed. Accordingly, natural HEV infection in mice has not been demonstrated yet. In contrast, HEV RNA and anti-HEV antibodies were demonstrated in rats and rabbits. Interestingly, wbHEVgt3 infection was not demonstrated in dexamethasone treated rats. In rabbits, viral replication was more efficient and immunization protected against viral shedding with feces. The rabbit model for wbHEVgt3 infection may serve as a suitable alternative to the non-human primate and swine models, and as an appropriate basis for vaccine evaluation studies.
### 6.6 Tables

#### Table 6.1 WbHEVgt3 challenge of wild-type and immunocompromised mouse lines.

<table>
<thead>
<tr>
<th>Mouse line</th>
<th>Age and sex</th>
<th>Inoculum</th>
<th>Inoculation route</th>
<th>Animal number</th>
<th>Observation period</th>
<th>Sampling time points</th>
<th>Anti-HEV antibodies OD₄₅₀ ≥ 1.0</th>
<th>RNA in feces Cₜ &lt; 34</th>
<th>RNA in liver Cₜ &lt; 34</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6</td>
<td>8 weeks, male</td>
<td>wbHEVgt3 (liver)</td>
<td>oral (250 µl) and intravenous (250 µl)</td>
<td>2</td>
<td>21 days (necropsy at 21 dpi)</td>
<td>feces at 0, 1, 4, 7, 10, 12, 14, 17, 19, 21 dpi and serum at 0, 4, 7, 10, 17, 21 dpi</td>
<td>0 / 2</td>
<td>0 / 2</td>
<td>0 / 2</td>
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<tr>
<td></td>
<td></td>
<td>wbHEVgt3 (feces)</td>
<td>oral (250 µl) and intravenous (250 µl)</td>
<td>2</td>
<td>21 days (necropsy at 21 dpi)</td>
<td>feces at 0, 1, 4, 7, 10, 12, 14, 17, 19, 21 dpi and serum at 0, 4, 7, 10, 17, 21 dpi</td>
<td>0 / 2</td>
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<tr>
<td></td>
<td></td>
<td>PBS</td>
<td>oral (250 µl) and intravenous (250 µl)</td>
<td>2</td>
<td>21 days (necropsy at 21 dpi)</td>
<td>feces at 0, 1, 4, 7, 10, 12, 14, 17, 19, 21 dpi and serum at 0, 4, 7, 10, 17, 21 dpi</td>
<td>0 / 2</td>
<td>0 / 2</td>
<td>0 / 2</td>
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<td>IFNRI⁻/⁻ (exp 1)</td>
<td>8-12 weeks</td>
<td>wbHEVgt3 (liver)</td>
<td>oral (250 µl)</td>
<td>3</td>
<td>14 days (necropsy at 7 and 14 dpi)</td>
<td>feces and serum at 0, 3, 7, 14 dpi</td>
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<td>0 / 3</td>
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<tr>
<td></td>
<td></td>
<td>PBS</td>
<td>oral (250 µl)</td>
<td>1</td>
<td>14 days (necropsy at 7 and 14 dpi)</td>
<td>feces and serum at 0, 3, 7, 14 dpi</td>
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<td>IFNRI⁻/⁻ (exp 2)</td>
<td>8-12 weeks</td>
<td>wbHEVgt3 (liver)</td>
<td>intravenous (250 µl)</td>
<td>3</td>
<td>9 days (necropsy at 9 dpi)</td>
<td>feces and serum at 0, 2, 6, 9 dpi</td>
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<td></td>
<td>PBS</td>
<td>intravenous (250 µl)</td>
<td>1</td>
<td>9 days (necropsy at 9 dpi)</td>
<td>feces and serum at 0, 2, 6, 9 dpi</td>
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<td>CD4⁻/⁻</td>
<td>10 weeks, male</td>
<td>wbHEVgt3 (liver)</td>
<td>oral (250 µl) and intravenous (250 µl)</td>
<td>2</td>
<td>21 days (necropsy at 21 dpi)</td>
<td>feces at 0, 1, 4, 7, 10, 12, 14, 17, 19, 21 dpi and serum at 0, 4, 7, 10, 17, 21 dpi</td>
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<td></td>
<td></td>
<td>wbHEVgt3 (feces)</td>
<td>oral (250 µl) and intravenous (250 µl)</td>
<td>2</td>
<td>21 days (necropsy at 21 dpi)</td>
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<td>PBS</td>
<td>oral (250 µl) and intravenous (250 µl)</td>
<td>2</td>
<td>21 days (necropsy at 21 dpi)</td>
<td>feces at 0, 1, 4, 7, 10, 12, 14, 17, 19, 21 dpi and serum at 0, 4, 7, 10, 17, 21 dpi</td>
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<tr>
<td>CD8⁻/⁻</td>
<td>8 weeks, male</td>
<td>wbHEVgt3 (liver)</td>
<td>oral (250 µl) and intravenous (250 µl)</td>
<td>2</td>
<td>21 days (necropsy at 21 dpi)</td>
<td>feces at 0, 1, 4, 7, 10, 12, 14, 17, 19, 21 dpi and serum at 0, 4, 7, 10, 17, 21 dpi</td>
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<tr>
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<td></td>
<td>wbHEVgt3 (feces)</td>
<td>oral (250 µl) and intravenous (250 µl)</td>
<td>2</td>
<td>21 days (necropsy at 21 dpi)</td>
<td>feces at 0, 1, 4, 7, 10, 12, 14, 17, 19, 21 dpi and serum at 0, 4, 7, 10, 17, 21 dpi</td>
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<td>PBS</td>
<td>oral (250 µl) and intravenous (250 µl)</td>
<td>2</td>
<td>21 days (necropsy at 21 dpi)</td>
<td>feces at 0, 1, 4, 7, 10, 12, 14, 17, 19, 21 dpi and serum at 0, 4, 7, 10, 17, 21 dpi</td>
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<td>BA/129/cnu</td>
<td>6 weeks, female</td>
<td>wbHEVgt3 (liver)</td>
<td>intravenous (80 µl)</td>
<td>4 + 1 contact animal</td>
<td>28 days (necropsy at 7, 14, 21 and 28 dpi)</td>
<td>feces at 0, 1, 2, 4, 7, 10, 12, 14, 16, 18, 21, 23, 25, 28 dpi and serum at 0, 1, 2, 4, 7, 10, 12, 14, 16, 18, 21, 23, 25, 28 dpi</td>
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<td>intravenous (80 µl)</td>
<td>4 + 1 contact animal</td>
<td>28 days (necropsy at 7, 14, 21 and 28 dpi)</td>
<td>feces at 0, 1, 2, 4, 7, 10, 12, 14, 16, 18, 21, 23, 25, 28 dpi and serum at 0, 1, 2, 4, 7, 10, 12, 14, 16, 18, 21, 23, 25, 28 dpi</td>
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<td></td>
<td>wbHEVgt3 (bile)</td>
<td>intravenous (80 µl)</td>
<td>4 + 1 contact animal</td>
<td>28 days (necropsy at 7, 14, 21 and 28 dpi)</td>
<td>feces at 0, 1, 2, 4, 7, 10, 12, 14, 16, 18, 21, 23, 25, 28 dpi and serum at 0, 1, 2, 4, 7, 10, 12, 14, 16, 18, 21, 23, 25, 28 dpi</td>
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<td>intravenous (80 µl)</td>
<td>4 + 1 contact animal</td>
<td>28 days (necropsy at 7, 14, 21 and 28 dpi)</td>
<td>feces at 0, 1, 2, 4, 7, 10, 12, 14, 16, 18, 21, 23, 25, 28 dpi and serum at 0, 1, 2, 4, 7, 10, 12, 14, 16, 18, 21, 23, 25, 28 dpi</td>
<td>0 / 5</td>
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dpi = day post inoculation; exp = experiment.
Table 6.2 WbHEVgt3 challenge of Wistar rats.

<table>
<thead>
<tr>
<th>Exp.</th>
<th>Age and sex</th>
<th>Inoculum</th>
<th>Inoculation route</th>
<th>Pre-treatment</th>
<th>No.</th>
<th>Observation period</th>
<th>Sampling time points</th>
<th>Anti-HEV antibodies OD450 ≥ 1.0</th>
<th>RNA in feces Ct &lt; 34</th>
<th>RNA in liver Ct &lt; 34</th>
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</thead>
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<tr>
<td>1</td>
<td>8-12 weeks, female</td>
<td>wbHEVgt3 (liver)</td>
<td>intravenous (250 µl)</td>
<td>none</td>
<td>8</td>
<td>32 days (2 animals each per group per necropsy at 4, 7, 14, 21 dpi and 1 contact animal at 32 dpi)</td>
<td>feces at 0, 2, 4, 7, 10, 14, 17, 21, 25, 28, 32 dpi and serum at 0, 4, 7, 14, 21, 32 dpi</td>
<td>2 / 8 (^a)</td>
<td>1 / 8 (^b)</td>
<td>0 / 8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PBS</td>
<td>intravenous (250 µl)</td>
<td>none</td>
<td>8</td>
<td></td>
<td>0 / 8</td>
<td>0 / 8</td>
<td>0 / 8</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>wbHEVgt3 (liver)</td>
<td>contact</td>
<td>none</td>
<td>1</td>
<td></td>
<td>0 / 1</td>
<td>0 / 1</td>
<td>1 / 1</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) at 17 dpi and 21 dpi; \(^b\) at 7 dpi; Exp. = experiment; No. = animal number; s.c. = subcutaneously; dpi = day post inoculation.

Table 6.3 WbHEVgt3 challenge of rabbits.

<table>
<thead>
<tr>
<th>Exp.</th>
<th>Age in months</th>
<th>Inoculum</th>
<th>Inoculation route</th>
<th>No.</th>
<th>Pre-treatment</th>
<th>Observation period</th>
<th>Sampling time points</th>
<th>Anti-HEV antibodies OD450 ≥ 1.0</th>
<th>RNA in feces Ct &lt; 34</th>
<th>RNA in tissue Ct &lt; 34</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6</td>
<td>wbHEVgt3 (liver)</td>
<td>intravenous (1000 µl)</td>
<td>1</td>
<td>none</td>
<td>45 days (feces and serum at 0, 1, 3, 5, 7, 11, 14, 18, 21, 25, 28, 32, 35, 39, 42, 45 dpi)</td>
<td>28 – 45 dpi</td>
<td>3 - 14 dpi</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>PBS</td>
<td>intravenous (1000 µl)</td>
<td>1</td>
<td>none</td>
<td></td>
<td>none</td>
<td>none</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>wbHEVgt3 (liver)</td>
<td>intravenous (1000 µl)</td>
<td>1</td>
<td>none</td>
<td>46 days (feces at 0, 1, 3, 5, 7, 11, 14, 18, 21, 25, 28, 31, 34, 39, 42, 45, 46 dpi)</td>
<td>39 - 46 dpi</td>
<td>5 - 39 dpi</td>
<td>liver, gall bladder</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>wbHEVgt3 (liver)</td>
<td>intravenous (1000 µl)</td>
<td>2</td>
<td>immunized(^a)</td>
<td></td>
<td>2 / 2 (^b)</td>
<td>none</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>PBS</td>
<td>intravenous (1000 µl)</td>
<td>1</td>
<td>none</td>
<td></td>
<td>none</td>
<td>none</td>
<td>None</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) immunization with a C-terminal segment of HEVgt3 capsid protein; \(^b\) with booster effect at 14 - 25 dpi; Exp. = experiment; No. = animal number; dpi = day post inoculation.
6.7 Figures

Serology and RNA detection in feces

Figure 6.1 Serology and HEV RNA detection in feces of rabbits. Antibody responses to HEV in serum of inoculated rabbits measured by a double-antigen sandwich ELISA and fecal excretion of viral RNA quantified by RT-qPCR. wbHEVgt3 = one rabbit inoculated with HEVgt3 obtained from a wild boar liver; wbHEVgt3_immun = two rabbits immunized with a C-terminal segment of HEVgt3 capsid protein and inoculated with HEVgt3 obtained from a wild boar liver; control = one rabbit inoculated with PBS. OD450-values ≥ 1 are prescribed as seropositive. DPI = day post inoculation.
7. **General discussion**

Severe human HEV infection after ingestion of uncooked liver from wild boar was reported in Japan, whereas foodborne zoonotic transmissions in Europe were primarily associated with domestic pigs [89,116]. Individuals with direct contact to pigs are at higher risk of HEV infection and forestry workers have a higher HEV seroprevalence rate compared to blood donors [163,293,294]. Recent studies in Asia and Europe revealed high HEV seroprevalences and molecular evidence for HEV infection in wild boar [280-287]. In Germany, the wild boar is considered as one of the main sources of human autochthonous infections [288,289]. Moreover, phylogenetic analyses of Japanese HEV isolates indicated former transmission events from domestic pig to wild boar [295]. Until now several studies in domestic pigs were performed by intravenous or contact transmission of domestic pig-derived HEV [79,259,260,269,271,272]. Thereby, histopathological signs of hepatitis, but no clinical symptoms were described [11,269,414]. Conversely, to date little is known about the course of HEV infection in European wild boar and their role in HEV transmission to domestic pigs. Experimental challenge studies have not yet been carried out. Moreover, data on the cellular immune response following an HEV infection in humans are sparse, while data on pigs are completely missing. Until now several studies focused on acute HEV infections in swine [259,269], but little is known about persistent HEV infections in swine potentially leading to chronic hepatitis E. As HEV has the ability to cross species barriers, it is important to identify the possibility of cross-species transmission between wild boar and rodents, respectively rabbits. Laboratory mice, rats and rabbits were explored as potential animal models for HEV [80,82,128,129,148], but it remains to be determined whether they can be used as a suitable model for wild boar-derived HEVgt3 infection studies. Understanding HEV interspecies transmission is needed to implement effective prevention and control measures.

Therefore, the aim of this thesis was to investigate the pathogenesis of a wild boar-derived HEVgt3 strain in European wild boar and to assess possible horizontal transmissions among wild boar and to domestic pigs. We investigated cellular immune responses following a HEVgt3 infection in wild boar and domestic pigs. Additionally we assessed, whether a dexamethasone treatment, which leads to potent immunosuppressive effects, affects the clinical course and pathological outcome of HEV infection in experimentally infected wild
boar. To determine differences in the susceptibility to HEVgt3 infection depending on the immune status, dexamethasone treated and non-treated domestic pigs were kept in contact to infectious feces derived from the intravenously inoculated wild boar. We also examined the course of hepatitis E in naturally gt3 infected European wild boar up to six months to obtain an initial impression of the potential existence of porcine chronic hepatitis E. In addition, the possibility of HEV transmission from naturally infected wild boar to domestic pigs when they were kept in direct contact was investigated. To determine the susceptibility of different small mammalian species to HEV infection originated in European wild boar, viral replication and humoral immune responses were studied in several mouse strains, Wistar rats and European rabbits. Moreover we hypothesize that immunosuppression may lead to viral persistence in swines as well, but simultaneously reduces the manifestation of liver disease due to diminished inflammatory responses.
7.1 Pathogenesis of HEV in European wild boar

To date, the foodborne zoonotic transmission of HEVgt3 in Europe is primarily associated with domestic pigs [116], while data on the pathogenicity of HEV in wild boar and their role in HEV transmission to domestic pigs are missing. HEV prevalence studies in hunted wild boar and serological studies in humans with documented contact with wild boar suggest zoonotic transmissions [163,283,285-287,293]. Until now several studies in experimentally HEV infected domestic pigs were performed [79,259,260,269,271,272], but none involving the wild boar. Manuscript I presents an infection experiment of wild boar inoculated with a HEVgt3 strain originated from a wild boar hunted in Mecklenburg Western-Pomerania, Germany. To obtain an initial impression of horizontal HEV transmission among wild boar, a contact animal was included into the study. Detailed studies of cellular immunity to HEV infection in wild boar are not yet available. Therefore, manuscript II addresses cellular immune responses to HEVgt3 and the effect of dexamethasone treatment on HEV replication in experimentally infected wild boar. Non-infected dexamethasone treated wild boar served as controls for the evaluation of immunosuppressive effects. Until now several studies focused on acute HEV infections in the swine [259,269]. Manuscript I and II deal with the early course of HEV infection in wild boar as well, but little is known about persistent HEV infections in swines which could potentially lead to chronic hepatitis E. Therefore, manuscript III addresses the course of hepatitis E in naturally gt3 infected European wild boar up to six months. Additionally, the possibility of HEV transmission from persistently HEV infected wild boar to domestic pigs was investigated.

The results presented in manuscript I and II demonstrate that the experimental inoculation of wild boar leads to an effective HEV replication with substantial virus shedding. HEV RNA was detected in serum, feces and different tissues of all intravenously inoculated wild boar. Following intravenous challenge of wild boar, HEV infection was successfully transmitted to the contact wild boar. This contact animal infection resembled the natural course of the disease and confirmed that HEV rapidly spreads to other pigs. The fecal-oral transmission of HEV is considered to be the main transmission route among pigs [420]. Studies in contact infected pigs estimated a basic reproduction ratio for HEV transmission among pigs at 8.8, showing the potential of HEV to cause epidemics in pig populations [256].
As presented in manuscript I and II, seroconversion occurred in the experimentally infected wild boar, but not consistently. In intravenously inoculated wild boar anti-HEV antibodies were detected at the earliest point two weeks post inoculation which is in line with previously performed infection studies in domestic pigs [17,269]. The observation of slightly increased antibody levels in dexamethasone-treated wild boar at the end of experiment might be an effect of glucocorticoid administration, but random effects cannot be excluded. Previous studies indicated that dexamethasone is capable of inducing a shift in the immune response from a Th1 towards a Th2 cell response by influencing the levels of cytokines produced by the lymphocytes [390]. In humans, the clinical course of HEV infection can vary substantially between different individuals, and chronic cases of hepatitis E were described in immunosuppressed patients [223]. Our results indicated different patterns within the course of HEV infection in wild boar as well. Animals with early anti-HEV seroconversion were able to clear the virus, while animals with lacking antibody responses suffered from prolonged HEV persistence until the end of the experiment. Our findings support the hypothesis that adaptive immune responses are essential to control HEV infection [225].

In humans, hepatitis E is characterized by symptoms such as fever, anorexia, vomiting and jaundice which correlate with rising serum liver enzyme levels [421]. The studies presented in manuscript I and II addressed also the clinical outcome of HEV infection in wild boar. A clinical course of HEV infection was proven in experimentally HEV infected wild boar based on elevated serum levels of GGT, ALT and BA. The elevation of different liver enzymes was associated with enhanced viral replication and anti-HEV immune responses. Former studies in domestic pigs described only subclinical HEV infections [259,269,272]. Increased GGT levels have also been reported for experimentally HEV infected non-human primates [422], but not described for pigs before. Our results support laboratory findings in humans with HEV infection which are similar to other forms of viral hepatitis, and characterized by elevated serum levels of ALT and GGT as well [197]. Moreover, dexamethasone treatment did not significantly influence liver enzyme levels in serum.

Our histopathological findings for hepatic lesions varied for wild boar ranging from diffuse moderate lesions with swelling, vacuolation and single cell necrosis of hepatocytes, to multifocal more severe hepatocellular degenerations. In previous studies microscopic liver lesions with multifocal lymphoplasmacytic viral hepatitis were observed in both
experimentally [259,269] and naturally [11] HEV infected domestic pigs. Swelling of hepatocytes with vacuolation of the cytoplasm was also seen in acute liver injury of HEV infected domestic pigs [259]. Possibly, a weak cytotoxic response leads to viral persistence, yet without obvious liver damage, whereas a sufficient immune response may cause an effective HEV clearance that is, however, accompanied by a variable degree of hepatic damage. As presented in manuscript II, the degree of detected liver lesions in intravenously inoculated wild boar was not influenced by the dexamethasone treatment.

Viral antigens and highest viral loads were mainly found in liver samples confirming that the liver is the primary location of HEV replication also in wild boar. Extra-hepatic replication sites have previously been reported [273] and in this study, HEV RNA and viral antigens were observed in spleen and different lymph nodes. In humans, neurotropic HEVgt3 variants are under discussion and HEV RNA was recently detected in the cerebrospinal fluid of chronic HEV infected patients with neurological symptoms [109]. Interestingly, HEV RNA was also detected in the brain of two intravenously inoculated wild boar. In HEV infected animals and humans only few immunohistochemical investigations on viral antigen distribution have as yet been published [268,274,327,423]. We demonstrate here the first immunohistochemical studies in HEV infected wild boar. We were able to detect viral antigens mainly in Kupffer cells and LSEC, partially associated with hepatic lesions and infiltrates of CD3 positive cells. It was shown that HEV replicates in hepatocytes and in extra-hepatic tissues such as small intestine, colon, spleen, bile duct and lymph nodes [273,324]. A virus proliferation in Kupffer cells and liver sinusoidal endothelial cells is possible, if not essential. Since these cells have antigen presenting functions [424], they may also play a role in the host defense mechanisms and immunopathogenesis of HEV infection. In wild boar livers, we observed different patterns of hepatic lesions and HEV antigen distribution. HEV antigens were either diffusely distributed without association to liver lesions or associated with hepatocellular degeneration. Previous immunohistochemical studies in the liver of acute HEV infected humans revealed that infiltrates consisted mainly of CD3 positive T cells containing predominantly cytotoxic CD8 positive cells [425]. Interestingly, CD3 positive T cell infiltrations within liver lesions were also observed in this study. As viral antigens were found in the spleen, and hepatic and mandibular lymph nodes as well, lymphatic tissues might represent extra-hepatic HEV replication sites. Contrary to reports in previously described
HEV infected gerbils [130], no viral antigens were detected by immunohistochemistry in the intestine of wild boar. These findings might be explained by differences in the sensitivity between RT-qPCR and immunohistochemistry. Otherwise, these discrepancies in both assays might also be due to the fact that different targets were detected, namely in the formerly mentioned assay viral RNA targeting HEV-ORF3, and in the latter viral capsid protein encoded by HEV-ORF2.

Cellular immune responses and the role of host factors in porcine HEV infection have not yet been studied in detail. Both human and animal studies have suggested that immune responses, rather than viral damage to hepatocytes, drive the clinical manifestation of hepatitis E [111,351]. In immunocompromised humans, such as patients with a solid-organ transplant, hematologic tumors, or in those who are human immunodeficiency virus-positive, HEV infections can lead to viral persistence [215]. Multi-faceted interactions between host immune responses and virus diversity seem to be responsible, but the key mechanism leading to a chronic hepatitis E infection is largely unknown. Hence, manuscript II addresses on the one hand the cellular immunity in HEV infected wild boar, and on the other hand the effects of immunosuppression induced by systemic administration of glucocorticoids on the pathogenesis of HEVgt3 infection. Dexamethasone, a potent glucocorticoid, decreases the cytokine production and consequently impairs the immune systems’ activation [355]. Moreover, glucocorticoids have inhibitory effects on T and B cells, and exert potent suppressive effects on the effector functions of phagocytes [352]. We hypothesized that immunosuppression may enhance the susceptibility of wild boar to HEV, but also reduces the clinical manifestation due to diminished inflammatory responses possibly leading to viral persistence at the same time. In this study, the blood compartment was chosen to investigate the changes of leukocytes during the course of HEV infection. Of course, the majority of immune cells will probably have left the blood stream following an HEV infection, but changes of the blood cells can still be indicative for the immune response over time. Lymphocyte subpopulations of pigs have been investigated in a number of studies with the aim to identify correlations between function and the phenotype of these cells. Anyhow, detailed functional analyses of subpopulations are currently not feasible. Therefore, defined roles of different subsets in the porcine immune system and their functionality are not yet resolved. It has to be taken into account that animals used in this study were of different
general discussion including distinct SLA haplotypes. The SLA genomic region is extremely polymorphic comprising high numbers of different alleles and plays a crucial role in maintaining overall adaptive immunologic resistance to pathogens [357]. Therefore, the potential biological diversity between individuals should be taken into consideration. Differential cell counts revealed a leukocytosis, lymphocytosis and monocytosis in all HEV infected wild boar. However, changes in differential cell counts were less pronounced in the dexamethasone-treated wild boar group. Our findings are in accordance with other studies investigating viral hepatitis in which increased white blood cell counts and a lymphocytosis were frequently found [356]. Furthermore, an immunosuppressive effect of dexamethasone-treatment was proven in dexamethasone-treated control pigs as they developed a depletion of white blood cells in the peripheral blood. Changes in T cell populations were observed in all HEV infected wild boar, which were much more pronounced in the non-treated HEV infected wild boar. Therefore dexamethasone seems to weaken cellular immune responses in HEV infected wild boar, yet without shutting it down completely. T cell mediated adaptive immune responses are important for the elimination of viral infections [356]. Significantly higher numbers of cytotoxic T lymphocytes (CD8+CD4-) and helper/memory cells (CD4+CD8+) in the PBMCs were detected in HEV infected wild boar, less pronounced in the dexamethasone-treated group. A marked cytotoxic T cell response developed one week post infection and persisted until to the end of the experiment. Following the increase of cytotoxic T lymphocytes, an increase of T helper/memory cells was detectable. In a previous study in humans with acute hepatitis E, patients showed also increased numbers of CD8+CD4- and CD4+CD8+ cells compared to healthy controls [206]. In many viral infections of pigs CD8+CD4- T cells are the predominant T cell subpopulation [375-379], but porcine CD4+CD8+ T cell responses have also been reported [363,380-382]. Moreover, the percentage of activated γδ T cells (γδTCR+CD8+) increased in all HEV infected wild boar. Accumulating evidence suggests that γδ T cells are components of both innate and adaptive immunity against various viral and bacterial infections, and they are also important in early responses against infections at epithelial surfaces [359-361]. In pigs, responding γδ T cells have been reported in different viral infections [362-365], whereas the responding γδ T cells belonged also to the γδTCR+CD8+ T cell subset [363,364]. Like αβ T cells, these γδ T cells can express CD8α which in swine seems to be correlated with an activation status of T cells,
as γδ TCR+CD8+ T cell subsets are normally found in the thymus and only after activation in the periphery [366]. It was shown that CD2 but not CD21 can be re-expressed on the surface of B cells so that CD21 can be considered as a maturation marker. CD2 on the surface of B cells can be down-regulated by cell-to-cell contact and once recovered, CD2 expression on B cells is re-established [383]. Upon infection, all wild boar showed a down regulation of CD2+CD21+ cells (phenotype of naïve B cells) and CD2-CD21+ cells (phenotype of primed and activated B cells) indicative for B cell activation. Cells representing the phenotype of antibody-forming and/or memory B cells (CD2+CD21-) showed an increase in all HEV infected pigs. Changes in B lymphocyte subsets were mostly not affected by dexamethasone treatment. The increase of antibody-forming and/or memory B cells (CD2+CD21-) probably reflects the chronological events in anti-HEV antibody production. Anyhow, it has to be considered that the phenotype CD2+CD21- of non-T cells also included NK cells, but in negligible quantity as their frequency in peripheral blood is very low [366]. In the liver and spleen an influence of HEV infection and dexamethasone-treatment on immune cell percentages was also detected. Interestingly, percentages of different T cell subsets in the liver and spleen of the dexamethasone control group were mostly on the other end of the scale compared to those of HEV infected wild boar groups. As no additional tissue material of HEV negative untreated wild boar was available, the results of lymphocyte subsets in the tissue of HEV infected wild boar should be interpreted with care and need further investigation. Interestingly, a marked increase in the percentage of γδ T cells was observed in the liver of all HEV infected wild boar. In contrast to the liver, lower percentages of γδ T cells were seen in the spleen of HEV infected wild boar compared to dexamethasone-treated, uninfected wild boar. Our findings might also suggest that T cells detectable in peripheral blood may migrate into the primary site of infection to function as effector cells in the liver. However, HEV-specific T cell responses have up to now only been studied in hepatitis E patients, but not in pigs. In humans, proliferation and cytokine production of CD4+CD8- and CD8+CD4- T cells were studied after stimulation with peptides encoded by HEV-ORF2 and –ORF3 [225].

In the beginning, HEV was known to cause only acute hepatitis, but lately HEVgt3 was also identified as a novel causative agent of chronic hepatitis in immunocompromised patients [223]. Recent studies in humans were able to associate the activation of the interferon system and viral evolution with severity or chronicity of hepatitis E [223]. As recently shown, HEV
inhibits type I interferon induction by ORF1 products in-vitro [331]. Studies in humans also revealed that chronic hepatitis E might be associated with impaired HEV-specific T cell responses and enhancing adaptive cellular immunity against HEV might prevent persistent HEV infections [225]. In swine and other animals, various factors have been discussed as possible modulators of the clinical outcome of HEV infection [272,410], such as virus titer, ratio of infectious to defective particles, route of infection and host factors like the immune status, age of exposure and the presence of co-infections.

As shown in manuscript I and II, naturally required HEVgt3 replicates effectively in experimentally infected wild boar and is transmissible from wild boar to domestic pigs. To obtain an initial impression of the potential existence of chronic hepatitis E in swine, manuscript III addresses the course of hepatitis E in naturally gt3 infected European wild boar up to six months. Chronic HEV infection in humans was defined as the presence of persistently elevated liver enzyme levels and detectable HEV RNA in the serum and/or stool for at least six months [408]. Evidence of a chronic course was also observed in experimentally rabbit HEV infected SPF rabbits, as persistent fecal shedding and elevated liver enzymes were noted for more than six months after infection [149]. However, persistent HEV infections in swine were not yet documented. Our results indicate persistent HEV infection also in wild boar which showed intermittent fecal HEV RNA excretion and viremia over 4 months. Moreover, infectivity was proven, as natural gt3 infection in wild boar was transmissible to domestic pigs three months after the first detection of viral RNA. In our study, the infectious dose of the contact animals remained unknown, but fecal HEV RNA excretion of the wild boar was very low compared to the results in transmission studies described in manuscript I and II. Interestingly, naturally infected wild boar were positive for both HEV RNA and anti-HEV antibodies in serum. Persistent fecal virus shedding together with high anti-HEV antibody levels was also observed in experimentally infected rabbits [149]. Usually, HEV should be cleared by high titers of neutralizing anti-HEV antibodies [348]. However, growing evidence indicates that some non-enveloped viruses, like the HAV, circulate in the blood of infected individuals and are enveloped in host-derived membranes that provide protection from neutralizing antibodies [23]. Potentially, this membrane-hijacking contributes to the persistence of HEV in its hosts as well. Viremic episodes in an immunosuppressed liver transplant recipient was recently shown [409] in spite of the presence
of anti-HEV antibodies in the serum. In contrast to the results presented in manuscript I and II, none of the collected tissue samples in naturally infected wild boar were tested positive for HEV RNA or viral antigens. In fact, it could be that an earlier time point of necropsy would have resulted in positive RNA or antigen detection in tissue. Possibly, high viral loads initially detected in serum samples of naturally infected wild boar were indicative of an extrahepatic manifestation of HEV infection. In contrast to histopathological findings presented in manuscript I and II, no signs of hepatitis were seen in livers of naturally infected wild boar. In persistently HEV infected SPF rabbits chronic inflammatory cell infiltrations and portal fibrosis were observed in the liver tissue [149]. A clinical course of hepatitis E based on elevated liver enzyme levels in serum was proven in experimentally infected wild boar as described in manuscript I and II. Possibly, retrospective analysis on this issue would provide further information on clinical parameters also in naturally infected wild boar. However, as liver enzyme activities decrease within extended storage periods, respective laboratory results have to be interpreted with caution. In accordance to the findings presented in manuscript I and II, clinical investigations based on the measurements of body weight and rectal temperature were also inconspicuous in naturally infected wild boar. However, the underlying cause of this chronic trend in naturally HEV infected wild boar remained unclear. Phylogenetic analysis of HEV isolates obtained from the naturally infected wild boar revealed an infection with HEVgt3 subtype 3i (HEVgt3i). Interestingly, HEVgt3 subtype 3i was also associated with chronic hepatitis in kidney transplant recipients in France [411]. There is evidence that differences in circulating virus isolates may influence the pattern and severity of illness. Studies of different HEV strains identified several variants with altered virulence [412,413]. Recently, an integration of a nucleotide sequence from human host RNA into the HEV genome of a patient with chronic hepatitis E was shown [426]. Moreover, this gt3 strain has replicated more effectively in cell culture than other tested HEV isolates [55].

Taken together, our data underline the importance of wild boar as HEV reservoir hosts and their relevance in the transmission of HEVgt3 to domestic pigs. Histopathological analyses in wild boar showed mild to moderate intralobular lymphoplasmacytic or lymphohistiocytic infiltrates with variable degree of hepatocellular degeneration. Furthermore, in our studies we found Kupffer cells, liver sinusoidal endothelial cells and extra-hepatic lymphatic cells as potential virus replication sites. Some of the wild boar used in our studies carried also
nematodes and showed mild gastrointestinal symptoms possibly caused by other swine-affecting infectious agents or also by stress or a modified feeding regime. Anyhow, clinical and pathological examinations showed no indication of other infections and liver homogenates given to the experimental animals were sterile filtered to prevent parasitic and bacterial superinfections. However, future studies should clarify the impact of co-infections on the HEV pathogenesis. Our results indicate that HEVgt3 infection in wild boar enhances the cellular and humoral immune responses, surprisingly largely unaffected by a dexamethasone induced immunosuppression. Especially an increase of cytotoxic T lymphocytes followed by an increase of T helper/memory cells and activated γδ T cell subsets was shown in intravenously HEV infected wild boar. Moreover, marked increase in percentages of γδ T cells were observed in the liver. Anyhow, no significant differences in clinical manifestations, viral replication, and liver lesions were observed between dexamethasone-treated and untreated wild boar. Our findings are in contrast to results obtained in dexamethasone-treated Peste des Petits Ruminants virus (PPRV)-infected goats and swine influenza virus-infected turkeys. Immunosuppression in turkeys revealed an increase of virus replication, prolonged virus shedding and the possibility of enhancing virus transmission [391]. In contrast to the study presented here, the extent and distribution of PPRV antigen were increased in dexamethasone-treated goats [392]. Supposedly, further HEV infection studies with higher animal numbers are required to define differences in HEV replication more precisely and to exclude statistical outliers. Moreover, longer observation periods would facilitate the possibility of developing viral persistence which may also lead to chronic HEV infection in pigs. Studies in miniature pigs experimentally infected with Leptospira interrogans revealed persistence until the chronic phase, and excretion of leptospires was increased under immunosuppressive conditions, resulting in enhanced horizontal transmissions [393]. However, other methods for immunosuppression have to be tested, because a relative resistance to dexamethasone-induced immunosuppression was previously demonstrated [394] in domestic pigs treated over a prolonged time. CD8-depletion in HBV infected chimpanzees demonstrated that CD8 positive cells were the main effector cells responsible for viral clearance and disease pathogenesis [395]. Targeted modifications of the porcine immune system such as the ability to modulate in vivo T cell populations of pigs [396] would provide deeper insights into the role of different immune cell subsets and
immune regulation mechanisms in HEV pathogenesis. For example, in vivo depletion of CD8 positive T lymphocytes abrogates protective immunity to African swine fever virus [375]. In conclusion, immunosuppressive effects of dexamethasone were proven, but did not affect substantially the course of HEV infection in wild boar. However, further research is needed to understand immunopathological processes in porcine HEV infection more precisely. Interestingly, our results indicate persistent HEV infection in naturally infected wild boar despite the presence of anti-HEV antibodies, but histopathology revealed no evidence of chronic active hepatitis in wild boar. Infectivity was also proven, because natural HEVgt3 infection in wild boar was transmissible to domestic pigs. Pathomechanisms for the development of persistent HEV infections in wild boar should further be assessed, as this may provide an animal model for the chronic HEV infection in humans. Possibly, profound phylogenetic analyses of circulating subtypes in swine will also identify HEV variants with increased virulence in pigs. Based on the observation of the long duration of viral shedding, especially in persistently infected animals, wild boar has to be considered as an important reservoir and transmission host of HEVgt3 in Europe. Since large amounts of virus particles were excreted in feces of wild boar, droppings can contaminate the environment and pose a particular risk to susceptible species.
7.2 Transmission of wild boar-derived HEV to domestic pigs

Several HEV transmission studies in domestic pigs were performed [79,259,271,272]. Conversely, little is known about HEV transmission from European wild boar to domestic pigs to date. Phylogenetic analyses of Japanese HEV isolates indicated past transmission events from domestic pig to wild boar [295]. The study presented in manuscript I addresses the transmission and pathogenesis of wild boar-derived HEVgt3 in intravenously and contact-infected miniature pigs, whereas manuscripts II and III deal with horizontal transmission experiments in common domestic pig breeds. Miniature pigs have been used in several fields of biomedical research [427], but HEV infection studies have never been carried out in this pig breed. Compared to common domesticated swine breeds, the miniature pig offers several breeding and handling advantages. Manuscript II addresses the cellular immunity in fecal- orally HEV infected domestic pigs. Moreover, the effects of immunosuppression induced by the administration of glucocorticoids on HEVgt3 infection are described. We hypothesized that immunosuppression may enhance the susceptibility of domestic pigs to HEV. Therefore, comparative analyses of cellular immune responses in peripheral blood and tissue samples, viral loads in different excreta and organ materials, humoral immune responses to HEV infection, histopathological changes and viral antigen distribution in different tissues were performed. Contrary to the studies presented in manuscripts I and II, manuscript III addresses the horizontal transmission of HEV from naturally HEVgt3 infected wild boar to domestic pigs via direct contact.

The experimental inoculation of miniature pigs presented in manuscript I revealed an effective HEV replication with substantial virus shedding as shown by the detection of HEV RNA in serum, feces and different tissues. Following an intravenous challenge of wild boar, HEV infection was successfully fecal-orally transmitted to miniature pigs. As presented in manuscript II, horizontal HEV transmission between intravenously inoculated wild boar and domestic pigs was proven as well, as fecal HEV RNA excretion was observed, somewhat delayed in dexamethasone-treated animals. No significant differences in HEV replication depending on the immune status were noticed, despite a slight increase of viral loads in dexamethasone-treated domestic pigs. Moreover, an early virus replication was observed in the liver of one miniature pig already one day post inoculation. Our findings confirm that the liver is the primary location of HEV replication. The duration of fecal HEV shedding in most
intravenously inoculated miniature pigs was similar, but higher viral loads were found in feces of wild boar. Seroconversion occurred in intravenously inoculated miniature pigs after two weeks which is in line with previous studies in domestic pigs [17,269]. The reason for the lack of an antibody response in two contact miniature pigs, despite elevated liver enzyme levels and fecal virus shedding, remains unclear. Most probably the duration of the experiment was not long enough or the HEV infection was not systemic, as described before [271]. In the study presented in manuscript II, only one untreated domestic pig developed measurable anti-HEV antibodies within the experiment. Probably, longer observation periods would have led to seroconversion in the majority of HEV infected pigs, as normally strong anti-HEV antibody responses were seen during the early course of infection [111]. It was demonstrated previously that domestic pigs could be infected orally. Nevertheless, not each contact pig was infected and the antibody response was less effective as compared to the intravenous inoculation route [273]. Single doses given by the intravenous route were demonstrated to cause HEV infection in pigs more reliably [118,420]. The oral route of infection is effective only when HEV is given in multiple doses, what implies a greater efficiency of transmission by a repeated ingestion of inoculum [271]. In the experiments described in manuscripts I and II, the domestic pigs were only exposed to collected feces of HEV infected wild boar and the infectious dose of the contact animals remains unknown. As HEV RNA was detected in urine of experimentally infected domestic pigs [259], HEV might have been also transmitted via urine. Therefore, it can be assumed that animals with direct contact to intravenously inoculated wild boar might be exposed to a higher infectious dose because of permanent contact to excreta. As described in manuscript III, seroconversion in domestic pigs occurred two weeks after the first detection of fecal RNA excretion which is in line with other HEV transmission studies in pigs [259]. Anyhow, none of the collected tissue samples were tested positive for HEV RNA or viral antigens. Interestingly, anti-HEV antibodies persisted only in one domestic pig until the end of the experiment. In agreement with the results presented in manuscript I and II, anti-HEV responses in contact infected domestic pigs were less marked than in intravenously inoculated pigs. Certainly, the exposition to infectious HEV particles was lower in this study, as fecal shedding in naturally infected wild boar was less effective compared to intravenously inoculated wild boar as described in manuscript I and II. Bouwknegt et al. suggested recently also a dose-dependent
response in HEV infected pigs [428]. Our findings support the hypothesis that adaptive immune responses are important to control HEV infection [225], even if not consistently. Finally, the persistence of high anti-HEV antibody levels in domestic pigs seemed not to be essential for the control of HEV infection. Based on a study in non-human primates, HEV seems to be more susceptible to innate immunity than HCV [77], even though HEV has also developed mechanisms to suppress IFN-α signaling [276]. Furthermore, recombinant HEV antigens corresponding to the capsid protein of the native virus were used in this study to detect all classes of antibodies to HEV in serum. The role of detecting the anti-HEV IgA in conjunction with anti-HEV IgM in the diagnosis of acute HEV infection was explored in several studies [314,429]. Probably, the time course of antibody levels observed in the current experiment was also influenced by HEV-specific IgA in serum, as IgA could be detected in the serum of patients with hepatitis E [301,430]. In pigs, the intestinal wall contains the majority of all IgA secreting plasma cells of the body [431], and the intestine is the major source of the IgA present in the porcine blood [432]. Nevertheless, variability in the sensitivity and specificity of HEV assays often complicates the interpretation of serological data [299].

In domestic pigs, only subclinical HEV infections were described [259,269,272] and no deviations in the levels of liver enzymes could be detected in the serum of experimentally infected animals [17,79,269]. In the studies described in manuscript I and II, a clinical course of HEV infection could be proven in domestic pigs, based on elevated GGT levels in serum. Increased GGT levels have also been reported for experimentally HEV infected non-human primates [422], but not for domestic pigs before. As presented in manuscript II, dexamethasone treatment did not significantly influence liver enzyme levels in serum of fecal-orally HEV infected domestic pigs. The course of HEV infection in domestic pigs, which were in direct contact with naturally HEV infected wild boar, was asymptomatic, but biochemical analyses of serum liver enzyme levels have not yet been performed.

Multifocal lymphoplasmacytic viral hepatitis was previously observed in both experimentally [259,269] and naturally [11] HEV infected domestic pigs. Moreover, swelling of hepatocytes with vacuolation of the cytoplasm was seen in acute HEV infection of domestic pigs [259]. Our histopathological findings for hepatic lesions in the intravenously infected miniature pigs were in concordance with the above mentioned published data. All contact animals showed a
randomly distributed multifocal mild lymphohistiocytic infiltration in the liver associated with single cell necrosis. Only two immunohistochemical studies in HEV infected domestic pigs have been published by Chinese researchers before [268,274]. In our study, viral antigens were only found in the liver of intravenously inoculated miniature pigs, but not in contact miniature pigs. In the contact miniature pigs, viral antigens were demonstrated exclusively in the follicles of the mandibular lymph nodes. Previous studies in domestic pigs suggested that HEV replicates in hepatocytes and in extra-hepatic tissues such as small intestine, colon, spleen, bile duct and lymph nodes [273,324]. As described in manuscript II, no liver lesions indicative for viral hepatitis were seen in fecal-orally infected domestic pigs, despite the presence of viral RNA in liver tissue. Viral antigens were detected solely in one domestic pig. Pathohistological analyses were also inconspicuous in domestic pigs as presented in manuscript III. Increased susceptibility of miniature pigs for wild boar-derived HEVgt3 cannot be excluded.

No particular changes in differential blood cell populations, apart from a commonly seen initial stress-induced increase in different parameters [354], were observed in any of the domestic pigs presented in manuscript II. Contrary to changes observed in wild boar, no consistent changes in the percentages of cytotoxic T lymphocytes and T helper/memory cells were observed in peripheral blood of the domestic pigs. Anyhow, in the untreated HEV infected domestic pigs an increase in the percentages of T helper cells occurred after three weeks. It was speculated that increases in T helper cells (CD4+CD8-) among patients with hepatitis E may reflect increases in the natural killer cell population, which may in turn produce elevated levels of INF-\(\gamma\) [39]. Upon infection, all animals showed a down regulation of CD2+CD21+ cells (phenotype of naïve B cells) and CD2-CD21+ cells (phenotype of primed and activated B cells) indicative for B cell activation. Cells representing the phenotype of antibody-forming and/or memory B cells (CD2+CD21-) showed an increase in all HEV infected domestic pigs. Changes in B lymphocyte subsets were mostly independent from dexamethasone treatment. Conversely to intravenously inoculated wild boar and negative control pigs, higher percentages of all tested T cell subsets were observed in the liver of domestic pigs, especially marked in the percentage of \(\gamma\delta\) T cells. Additionally, higher T helper/memory cell and \(\gamma\delta\) T cell percentages were found in mesenterial lymph nodes of the HEV infected domestic pigs. Differences in cellular immune responses between the wild boar
and domestic pigs might be due to distinct transmission routes, and intraspecific and individual varieties. Beyond that, differences in the stage of HEV infection and in regulating immune responses cannot be excluded in this study, as an early stage of T cell activation in the liver of domestic pigs can be assumed.

Taken together, our data demonstrated that miniature pigs are susceptible to wild boar-derived HEVgt3 transmission, either by direct contact or the intravenous route, and confirmed that the miniature pig is a suitable model for HEV infection. Moreover, HEVgt3 was successfully fecal-orally transmitted to common domestic pigs irrespectively of their immune status. Hence, an association between immunosuppression and enhanced susceptibility of pigs to HEVgt3 could not yet be proven. Marked increase in percentages of γδ T cells were observed in the liver of fecal-orally infected domestic pigs. Additionally, higher T helper/memory cell and γδ T cell percentages were found in the mesenterial lymph nodes. Anyhow, in PMBCs of domestic pigs dominated a T helper response. Infectivity was also proven as natural HEVgt3 infection in wild boar was transmissible to domestic pigs, but less effective compared to horizontal HEV transmission from experimentally infected wild boar. Actually, in most industrialized countries the HEV infected population of domestic swine is far larger than those of the wild boar. Accordingly, wild boar and other wildlife can be at infection risk either indirectly by using pig manure as fertilizer on agricultural land or directly by contact to domestic pigs kept on open land.
7.3 **Immunopathogenesis of HEV also in swine?**

Following virus infections host cell injury may be mediated either by a direct effect of the infectious agent or indirectly through the antiviral host response, or a combination of both. There is a general consensus that virus-specific CD8 positive T cells most likely play an important role in viral clearance by noncytolytic and cytotoxic effector functions as soon as viral antigens are presented by MHC class I alleles. The dominant role of virus-specific CD8 positive T cells is supported by experimental depletion studies of CD8 positive T cells that delay viral clearance of hepatotropic viruses in non-human primates [395,433]. Anyhow, help from CD4 positive T cells is required to prime and maintain strong and protective CD8 positive T cell responses. Nevertheless, the mechanism which contributes to the failure of virus-specific CD8 positive T cell responses and to the persistence of hepatotropic infections is still not clearly understood. Possible mechanisms discussed as reasons for virus-specific T cell failure include the emergence of viral escape mutants, insufficient help of CD4 positive T cells, or direct suppression by cytokines or Tregs [385]. Recently, innate immune responses involving NK cells which account for the majority of innate immune cells in the liver, and nonconventional T cells were also brought up as potential mechanism. Moreover, liver cell populations such as LSECs and Kupffer cells have scavenger functions and are crucial in the uptake of blood-borne molecules and transcytosis to hepatocytes, probably facilitating liver cell targeting of hepatotropic viruses [434]. The hepatic sinusoids are the main place where immune cells from peripheral blood first interact with liver cell populations. Immune cells from peripheral blood function directly as effector cells, or migrate into the liver parenchyma [435]. In HBV infected chimpanzees the onset of viral clearance is closely related with the appearance of IFN-γ-producing CD8 positive T cells in the liver that precedes the peak of liver damage [436]. Interestingly, non-cytolytic antiviral effects have been also described primarily mediated by IFN-γ. During the acute phase of HCV infection in non-human primates, viral clearance occurred in the absence of elevated liver enzyme levels with only mild histopathologically verifiable liver cell damage, but with detectable IFN-γ messenger RNA in hepatic tissue [437].

As HEV itself appears to be non-cytopathic [218], an immunopathogenesis is assumed for hepatitis E in humans [223]. Both human and animal studies have suggested that immune responses, rather than viral damage to hepatocytes, drive the clinical manifestation of hepatitis
Multi-faceted interactions between host immune responses and virus diversity seem to be responsible, but the key mechanism leading to a chronic hepatitis E infection is largely unknown. An association between a weak inflammatory response, poor T cell activation and high serum concentrations of chemokines involved in leukocyte recruitment to the liver is assumed to play a key role in the development of chronic HEV infections in humans [215]. Interestingly, our results indicate the presence of persistent HEV infections also in swine, but the underlying pathomechanisms which may lead to chronic hepatitis E in pigs has still to be elucidated. Especially the role of host and viral factors in porcine HEV infection has not been examined in depth yet. Bearing in mind that the porcine immune system has several distinctive properties in its structure and physiology, immunopathogenetical events in porcine HEV infection can be assumed to be somehow similar to known mechanisms in human hepatitis E. For the usage as a model for research in humans, the fact that the pig is a monogastric omnivorous animal represents an advantage, although the porcine ileum Peyer's patch has no direct anatomical equivalent in man [438]. Contrary to human lymph nodes, the pig lymph nodes, both peripheral and mucosa-associated, have a specific structure that is called inverted. As it is usual in many species, the immigration of lymphocytes into the lymph node tissue takes place either by afferent lymph vessels or by high endothelial venules [439]. After travelling through the parenchyma of the lymph node, the porcine lymphocytes exit the node again via the high endothelial venules, and not via efferent lymph vessels, as seen in most other species [440]. Porcine lymphocyte phenotypes are well-investigated, but detailed functional analyses of subpopulations are currently not available. Therefore, the definite roles of different subsets in the porcine immune system and their functionality are not yet resolved. We hypothesize that immunosuppression may lead to viral persistence in the swine as well, but simultaneously reduces the manifestation of liver disease due to diminished inflammatory responses. A weak cytotoxic response possibly leads to viral persistence, yet without obvious liver damage, whereas a sufficient immune response may cause an effective HEV clearance that is, however, accompanied by a variable degree of hepatic damage. Based on our findings, and other published studies in HEV infected humans and animals, the predicted courses of either acute self-limited or persistent HEV infection in the swine are compiled in Figure 7.1.
Figure 7.1 The predicted course of HEV infection in swine.
A. Predicted course of acute self-limited HEV infection in swine. B. Predicted course of persistent HEV infection in swine. "+" = positive for HEV RNA; "-" = negative for HEV RNA.
Our studies confirmed that the liver is the primary location for viral replication also in the swine, but extrahepatic antigens were detected in lymphatic tissue as well. In the studies presented here, two different patterns within the course of HEV infection were observed in experimentally infected pigs: animals with early anti-HEV seroconversion were able to clear the virus, while animals with lacking antibody responses suffered from prolonged HEV persistence until the end of the experiment. Moreover, HEV antigens were either diffusely distributed without obvious association with liver lesions or associated with hepatocellular degeneration. Our findings support the hypothesis that adaptive immune responses are essential to control HEV infection [225]. In addition, we found persistent HEV infections in naturally infected wild boar despite the presence of anti-HEV antibodies suggesting chronic hepatitis E also in pigs, but histopathology revealed no evidence of an active chronic hepatitis. In humans, the course of HEV infection can vary substantially between different individuals and chronic hepatitis E cases have been described in immunosuppressed patients [223].

Previous immunohistochemical studies in the liver of acute HEV infected humans revealed that infiltrates consisted mainly of CD3 positive T cells. These infiltrates contained predominantly cytotoxic CD8 positive cells probably playing an important role in HEV induced liver injury [425]. Interestingly, CD3 positive T cell infiltrations within liver lesions were also observed in our studies. The consistent coincidence of inflammatory infiltrates, hepatocellular degenerations and viral antigens supports the assumption that liver damage in pigs might be immune-mediated as well. The hepatic microenvironment and certain liver cell populations actively modulate local immune responses in the liver and thus determine the outcome of hepatic immune responses. Interestingly, we were able to detect viral antigens mainly in Kupffer cells and LSECs, partially associated with hepatic lesions and infiltrates of CD3 positive cells. Since Kupffer cells and LSECs have antigen presenting functions [424], they may also play a role in the host defense mechanisms and immunopathogenesis of HEV infection. LSECs induce regulatory CD4 positive T cells [441], and cross-present soluble antigens to CD8 positive T cells [442]. Such cross-presentation recruits naïve T cells in an antigen-specific manner to the liver [443]. Antiviral CD8 positive T cell immunity has been shown to be improved by cross-priming through LSECs that circumvents viral immune escape at the level of MHC I-restricted antigen presentation during the initial phase of immune response [444]. CD8 positive T cells activated by cross-presentation, also known as
non-canonical CD8 positive T cell effector function, release large amounts of TNF which induce cell death selectively in virus-infected cells [445]. In chronic infections, the benefit of attenuated virus-specific immune response may result in the prevention of liver immunopathology. In humans infected with HBV and HCV, virus-specific CD8 positive cells fail to clear viruses from the liver and viral persistence occurs, but the mechanisms are not completely understood [385]. Several studies have shown that virus-specific CD8 positive T cells derived from the liver and peripheral blood exhibit reduced ability to proliferate or secrete antiviral cytokines such as INF-γ [446-448]. It can be assumed that the exhaustion of CD8 positive T cell functions in the liver play a crucial role in the development of HEV persistence as well. Ongoing antigen recognition, lack of CD4 positive T cell help, direct suppression by Tregs or inhibitory cytokines such as IL-10 or TGF-β potentially contribute to virus-specific CD8 positive T cell exhaustion [385]. Additionally, nonconventional T cells in the liver have been reported being involved in liver pathology. At the site of infection in the liver, a large proportion of infiltrating CD8 positive T cells may not be antigen specific [385]. Furthermore, NKT cells, CD161 positive CD8 positive T cells and γδ T cells are also enriched in the liver and may also contribute to antiviral activity [449-451]. A central role of CD4 positive T cells in viral hepatitis was proven in depletion studies. For HBV infection it could be shown, that depletion of CD4 positive T cells before inoculation of a normally rapidly controlled inoculum precluded T cell priming, and that this caused persistent infection with minimal immunopathology [452]. Furthermore, Gerlach et al. suggested that a virus-specific CD4 positive/Th1 T cell response which eliminates the virus during the acute phase of disease has to be maintained permanently to achieve long-term control of the virus [453]. It was speculated that increases in T helper cells among patients with hepatitis E may reflect increases in the natural killer cell population, which may in turn produce elevated levels of INF-γ [205].

Porcine cytotoxic αβ T cells are a prominent T cell subset during antiviral responses, while porcine αβ T helper cell responses predominantly occur in bacterial and parasitic infections. Responses of γδ T cells to viruses have not been reported as frequently as αβ T cell responses [358]. However, accumulating evidence suggests that γδ T cells are components of both innate and adaptive immunity against various viral and bacterial infections, and they are also important in early responses against infections at epithelial surfaces [359-361]. Our findings
in HEV infected pigs support the hypothesis that adaptive immune responses are important to control HEV infection as well [225]. Differential cell counts revealed a leukocytosis, lymphocytosis and monocytosis in peripheral blood of HEV infected wild boar. Moreover marked CD3 positive T cell responses, including especially CD8 positive T cells (CD8+CD4), developed one week post infection and persisted until to the end of the experiment. Following the increase of CD8 positive T cells, an increase of T helper/memory cells (CD4+CD8+) was detectable. Contrary, T helper cell (CD4+CD8-) responses dominated in peripheral blood of fecal-orally infected domestic pigs. However, differences between wild boar and domestic pigs might be primarily due to distinct transmission routes of HEV. Interestingly, the percentage of activated γδ T cells (γδTCR+CD8+) increased in all HEV infected wild boar. In pigs, responding γδ T cells were reported in different viral infections [362-365], whereas the responding γδ T cells belonged also to the γδTCR+CD8+ T cell subset [363,364]. Like αβ T cells, these γδ T cells can express CD8α which in swine seems to be correlated with an activation status of T cells, as γδTCR+CD8+ T cell subsets are normally found in the thymus and only after activation in the periphery [366]. It has to be considered that γδ T cells in young animals form a major T cell subpopulation within peripheral blood lymphocytes and the frequency of this population decreases strongly with the age of pigs [367]. Interestingly, γδ T cells in swine are sources of IL-17 which is a pro-inflammatory cytokine being involved in immunity against viruses [368]. Intrahepatic innate lymphoid cells secrete IL-17 and studies in immunodeficient mice revealed that IL-17 signaling was critical for priming T cell responses in viral hepatitis [369]. Further investigations on this could provide new insights in antiviral immunity during porcine HEV infection. Recently it was shown that local immune responses by IL-17-secreting γδ T cells can serve to contain infections by pathogens to the gut while preventing pathogen dissemination to systemic sites [370]. In a previous study in humans with acute hepatitis E, patients showed also increased numbers of CD8+CD4- and CD4+CD8+ cells compared to healthy controls [206]. In humans, such double-positive T cells represent a minor subpopulation of T cells with functional characteristics of both CD4+CD8- and CD8+CD4- cells, and carry markers of memory phenotype [371]. Porcine CD4+CD8+ cells exhibit properties of mature antigen-experienced cells, and are inducible by stimulation with recall antigens [372]. The current notion is that both activated and memory T helper cells in swine belong to the CD4+CD8+ population expressing also MHC II antigens, which is not
seen in human and murine CD4+CD8+ lymphocytes [373,374]. Interestingly, marked increase in the percentage of γδ T cells was observed in the liver of all HEV infected pigs. In contrast to the liver, lower percentages of γδ T cells were seen in spleen. Our findings might also suggest that T cells detectable in peripheral blood may migrate into the primary site of infection to function as effector cells in the liver. However, to date HEV-specific T cell responses have only been studied in hepatitis E patients, but not in pigs. In humans, proliferation and cytokine production of CD4+CD8- and CD8+CD4- T cells were studied after stimulation with peptides encoded by HEV-ORF2 and –ORF3 [225]. Nonetheless, studies on innate immune responses to HEV infection are urgently required. Recently, a study in HEV infected pigs demonstrated that both IFN-α and Mx protein expression are inversely correlated with the number of HEV-infected cells [275]. Thus, the number of HEV infected hepatocytes declined while lymphoplasmacytic hepatitis increased significantly during the experiment [275]. Possibly, IFN-α-induced Mx activity is part of the antiviral response, however, changes in IFN-α and Mx protein expression might be also caused by the decrease of HEV-positive cells in the pigs recovered from infection [275]. Based on a study in non-human primates, HEV seems to be more susceptible to innate immunity than HCV [77], even though HEV has also developed mechanisms to suppress IFN-α signaling [276]. Kakimi et al. suggested analogously that, if activated, the innate immune response like the adaptive immune response has the potential to control viral replication during natural HBV infection [451].

The liver has unique immune regulatory functions, which promote the induction of tolerance rather than responses to antigens which are encountered locally. Thus, defense against viral infection has to take place in a tolerogenic environment [384]. Presumably, regulating immune responses are playing a particular role within the course of HEV infection, as increased frequencies of Tregs have been described for other viral hepatitides [385]. As Tregs can suppress the activation, proliferation, differentiation, and effector functions of many cell types, including CD4 and CD8 positive T cells, B cells, NK cells, NKT cells and dendritic cells, they might be critical in the outcome of hepatitis virus infections [454]. The elevation of CD4+CD25+Foxp3+ and CD4+CD25-Foxp3+ frequencies and the rise in IL-10 suggest that Tregs might play an important role in HEV infection associated with immunosuppressive immune responses [207]. Manigold et al. suggested as well, that Treg cell responses may be
both either beneficial or baneful to cells infected with HBV and HCV, by either limiting liver immunopathology or suppressing protective T cell responses [454]. Recently, marked Treg cell activity was present in patients with chronic HCV infection, which may also contribute to weak HCV-specific CD8 positive T cell responses and viral persistence [455]. Knolle et al. indicated, that a major role of Tregs may be to limit liver immunopathology in the chronic phase of infection [385]. Recently, the existence of Tregs in swine has been demonstrated and it could be shown that porcine Tregs suppress the proliferation of different T cell subsets [386,387]. For a better understanding of HEV pathogenesis in pigs, the functional characterization of porcine Tregs in HEV infection is needed. This would provide new insights into the balance between immunity and tolerance in the liver, and how this may influence viral clearance, persistence and virus-induced liver disease.

Furthermore, NK cells play an important role in the control of viral infections, as they have direct cytolytic or noncytolytic, and regulatory effects [385]. In human livers, they present the majority of innate immune cells [456]. Interestingly, the IFN-γ production by unstimulated PBMCs of hepatitis E patients suggests NK and NKT cells as key players in HEV pathogenesis [111,205,208]. Porcine NK cells have the ability to lyse virus-infected target cells and respond to various regulatory cytokines inducing INF-γ production, as well as the up-regulation of effector/activation molecules [366]. Recently, a novel marker (NKp46) has been described as suitable for the discrimination of porcine NK cells with different functional properties, which is highly expressed in a subset of CD8 positive liver lymphocytes [388]. Analysis of NK cells in HEV infected swine would provide further information on HEV pathogenesis especially in respect to their potential ability in direct killing of HEV infected cells.

Host neutralizing humoral immune responses play also a relevant role in the outcome of viral hepatitis [457,458]. Upon infection, all pigs showed a down regulation of CD2+CD21+ cells (phenotype of naïve B cells) and CD2-CD21+ cells (phenotype of primed and activated B cells) indicative for B cell activation. Cells representing the phenotype of antibody-forming and/or memory B cells (CD2+CD21-) showed an increase in all HEV infected pigs. The increase of antibody-forming and/or memory B cells (CD2+CD21-) probably reflects the chronological events in anti-HEV antibody production. Anyhow, it has to be considered that the phenotype CD2+CD21- of non-T cells also includes NK cells, but in negligible quantity in
the peripheral blood [366]. Nevertheless, the persistence of high anti-HEV antibody levels in domestic pigs seemed not to be essential for the control of HEV infection in our studies. In human hepatitis E, not all HEV infected patients had detectable anti-HEV IgM in the first week following the onset of symptoms, and the proportion of IgM-positive cases declined monotonically over the time. Interestingly, most of the initially IgM-negative patients had detectable HEV RNA in feces or serum [203]. In a small cohort study, only six of ten hepatitis E patients with anti-HEV IgM during the acute phase of illness developed detectable anti-HEV IgG [204]. Our data suggest that antibody titers can vary substantially among HEV infected pigs as well.

In swine factors like virus titer, ratio of infectious to defective particles, route of infection and host factors like the immune status, age of exposure and the presence of co-infections were discussed to modulate the outcome of HEV infection [283,284]. Some of the pigs used in our studies carried also nematodes (Ascaris suum). The modulation of the host immune system, including cells of the innate immune system and different T cell subsets, by nematode infection has recently been shown [459,460]. Therefore, a focus of current scientific interest is the question which cells, receptors and signaling pathways of the immune system are targeted. Interestingly, very effective regulatory T cells were induced in the development of a nematode infection, and consequently strong Th2 cell responses and immunopathology in the host were suppressed [461,462]. However, future studies should clarify the impact of co-infections with nematodes on the HEV pathogenesis.

In recent years, knowledge about the pathogenesis in human and porcine hepatitis E has rapidly increased, but little information is available about the specific role of different liver cell and immune cell populations, and molecular mechanisms in liver damage. Complex host-virus interactions are taking place in a virus-infected liver microenvironment, and thus different cell types and soluble factors contribute to the elimination of viral infections in the liver. Some similarities can be drawn between human and porcine hepatitis E, but considering immunopathogenetical events in swine, particularities of the porcine immune system have to be taken into account. Based on our results and other published studies in HEV infected individuals, the predicted course of hepatitis E in pigs and the role of porcine immune responses in HEV pathogenesis are depicted in Figure 7.2. In experimentally and naturally infected swine subclinical HEV infections accompanied by mild alterations of the liver were
most often described. In this study, a clinical course of hepatitis E in pigs has been also demonstrated based on changes in liver enzyme levels and lymphocyte populations, especially pronounced in intravenously infected wild boar. Different patterns within the course of HEV infection were observed in experimentally infected pigs, as animals with early anti-HEV seroconversion were able to clear the virus, while animals with lacking antibody responses suffered from prolonged HEV persistence. Interestingly, our results indicate persistent HEV infection in naturally infected wild boar as well, but histopathology revealed no evidence of chronic active hepatitis in wild boar. Presumably, acute HEV infections in pigs may also lead to viral persistence under certain conditions. Histopathological analyses revealed mild to moderate intralobular lymphoplasmacytic or lymphohistiocytic infiltrates, predominantly consisting of CD3 positive cells, with variable degree of hepatocellular degeneration. Furthermore, our studies revealed Kupffer cells, LSECs and extra-hepatic lymphatic cells as important cell populations possibly being involved in HEV pathogenesis. Anyhow, a role as potential virus replication sites cannot be excluded. Taken together, our findings support the hypothesis that adaptive immune responses are essential to control HEV infection also in swine, but innate immune responses might be critical likewise. The consistent coincidence of inflammatory infiltrates, hepatocellular degenerations and viral antigens supports the assumption that liver damage in pigs might be immune-mediated as well. Possibly, targeted modifications of the porcine immune system such as the ability to modulate in vivo T cell populations of pigs would provide deeper insights into the role of different immune cell subsets and immune regulation mechanisms in porcine HEV pathogenesis.
Figure 7.2 The predicted course of hepatitis E in swine and the potential role of porcine immune responses in HEV pathogenesis.
7.4 Small animal models for wild boar-derived HEV infection

Manuscript I and II present an efficient viral replication of a wild boar-derived HEVgt3 strain in experimentally HEV infected wild boar and domestic pigs. The availability of a suitable small animal for hepatitis E would provide considerable advantages in studying HEV biology, especially in respect to the investigation of immunopathogenetical mechanisms, and to evaluate novel therapeutics and vaccines. The ideal model would be one that adequately represents most aspects of human and porcine hepatitis E, is affordable, easily available, and reproducible. The availability of many different lines of mice and rats is a big advantage of the rodent models in biomedical research. In addition, the genetically modified mouse lines can be used to study a specific molecule in the organism using gene knock-out approaches. In contrast to rodents where the animals are kept under highly standardized conditions, many pig experiments are carried out in outbred pigs reared in conventional farms. Anyhow, wild boar are extremely limited in their availability and proper handling is difficult. Moreover, infection studies in pigs under high containment conditions are expensive. As a consequence, many results have to be generated in experiments with low numbers of animals. Aside from that, the biological diversity in domestic pigs and wild boar should be appropriately taken into consideration. Pigs used in our studies were of different genetic constitution including distinct SLA haplotypes. In outbred pigs, the SLA genomic region is extremely polymorphic comprising high numbers of different alleles. Different strains of miniature pigs, each homozygous for a different allele of the MHC locus, have been developed [463], but their availability is limited. Porcine lymphocyte phenotypes are well-investigated, but detailed functional analyses of subpopulations are currently not available. Contrarily, functional analyses in common laboratory animals such as mice, rats or rabbits are well-established.

Experimental studies investigating wild boar-derived HEVgt3 infection in rodents and rabbits were missing. Therefore, manuscript IV addresses HEV infection studies in C57BL/6, IFNRI -/-, CD4 -/-, CD8 -/- and Balb/c nu/nu mice, Wistar rats and European rabbits. Viral replication and humoral immune responses were monitored to investigate their susceptibility to wild boar-derived HEVgt3. We tested also if dexamethasone treatment in rats increases the susceptibility to HEVgt3. Additionally, the protective ability of a HEV vaccine candidate in HEVgt3 inoculated rabbits was examined in a proof of principle approach. Several types of animal models for HEV infection were described previously [416]. In general, non-human
primates are the best known models as they can be infected with a variety of HEV genotypes. Moreover, pigs have also been successfully infected with HEVgt3 and gt4. However, primate and swine HEV infection models are quite complex and expensive, so that a small animal model for HEVgt3 infection would be desirable. Moreover, a productive infection of immunodeficient mice, like type I interferon receptor or CD8-molecule knock-out mice, would also provide new insights into host’s defense mechanisms to HEV infection. Laboratory mice and rats, and European rabbits have been explored as potential animal models for HEV [80,82,128,129,148], but it remains to be determined whether they can be used as a reproducible HEV infection model for HEVgt3 obtained from European wild boar. As presented in manuscript IV, none of the mouse strains were susceptible to wild boar-derived HEVgt3 infection, but HEV RNA and anti-HEV antibodies were demonstrated in rats and rabbits. It has been reported that male Balb/c nude mice were infected with a HEVgt4 strain collected from a domestic pig and were tested positive for anti-HEV IgG [128]. Unfortunately, it remains unclear which kind of zygosity the Balb/c nude mice in the aforementioned study had, as we used homozygous mice. Contrary to heterozygotes, homozygous Balb/c nude mice lack a functional thymus and are unable to produce T cells. The nude allele on chromosome 11 is an autosomal recessive mutation and the heterozygotes do not show partial expression of the nude phenotype [418]. Generally, IgG responses to viruses are assumed to be T cell dependent, but polyomavirus infection of T cell deficient mice was shown to elicit protective, T cell-independent antiviral IgM and IgG responses [419]. Although it is quite improbable, the discrepancy in the nude mice results in our and the former study, resistant versus susceptible to HEV, may have arisen from gender effects. In accordance with the here described results Li et al. also failed to infect C57BL/6 mice with HEVgt3, as well as with gt1 and gt4 isolates [83].

Recently, strains of HEVgt3 were obtained from different species of wild-caught rats in the United States [123]. In a former study, rats were infectable with a human HEV isolate, but the genotype was not reported [80]. A more recent study demonstrated that Wistar rats were not susceptible to intravenously inoculated HEVgt1 (originated from a cynomolgus monkey), HEVgt3 (collected from a domestic pig), and HEVgt4 (wild boar-derived isolate) [82]. In our study, HEV RNA and anti-HEV antibodies were detectable in Wistar rats, but not consistently. Similar to a homologous challenge study in rats using rat HEV [82], we were
able to detect HEV RNA and anti-HEV antibodies in intravenously inoculated rats, but inconsistently. Interestingly, dexamethasone treatment in rats did not enhance the susceptibility to HEV infection, on the contrary; neither seroconversion nor viral RNA was detectable in the treated rats. Accordingly, Li et al. found no evidence that nude rats are susceptible to infection with HEVgt3 [126]. In a homologous challenge study using rat HEV, enhanced viral replication was seen in nude rats [82].

Intravenously infected rabbits seroconverted within four to five weeks and a booster effect was seen in immunized animals two weeks post inoculation indicative for antigen-specific memory B cells. In rabbits, viral replication was therefore more efficient and immunization with a recombinant capsid protein derivative protected against viral shedding with feces. Our findings are in agreement with another study in HEVgt3 infected rabbits [129]. However, aside from the protective ability of recombinant HEV proteins and the detection of seroconversion, we were also able to demonstrate fecal viral shedding and HEV RNA in liver and gall bladder in non-vaccinated animals. As a human HEVgt3 isolate was used in the study mentioned before, it cannot be excluded that rabbits might be more susceptible to HEVgt3 of wild boar origin. Nevertheless, rabbits could be experimentally infected with human HEVgt4 which originated from patients with acute hepatitis E [129,148]. Interestingly, HEV sequences of a human isolate in France and rabbit strains were closely related sharing a 93-nucleotide insertion [139]. As recently shown, rabbit HEV is able to infect domestic pigs, but rat HEV failed to infect pigs [140]. In a study in China, no evidence of natural cross-species infection with rabbit HEV was found [136]. Rabbit HEV is a distant member of HEVgt3 and studies indicated that rabbit HEV belongs to the same serotype as human HEV [147], but the antigenically relationship between rabbit and wild boar-derived HEVgt3 is unclear. Therefore, it would be interesting to determine whether immunization with recombinant HEVgt3 capsid protein protects rabbits against rabbit HEV infection. Moreover, further studies including histopathological and immunological analyses, and the determination of liver enzyme levels would provide more information on viral pathogenesis and clinical significance of wild boar-derived HEVgt3 infection in rabbits, and their suitability as an infection model for HEV.

Taken together, our data underline the importance of wild boar as HEV reservoir hosts and their relevance in the transmission of HEVgt3 to domestic pigs. Because of the limited availability of suitable in vitro models for HEV infection and high costs when using porcine
HEV infection models, the establishment of an appropriate small animal model would provide an exceptional advantage in studying HEV pathogenesis. Especially the availability of a molecularly defined mouse model would facilitate profound studies of pathophysiological mechanism in HEV infection. We present here a promising small animal model for HEVgt3 in European rabbits, whereas viral replication in Wistar rats was less effective. Unfortunately, the different mouse lines tested were not susceptible to wild boar-derived HEVgt3, and resistance to HEVgt3 can be assumed. Accordingly, natural HEV infection in mice has not been demonstrated yet. In contrast, HEV RNA and anti-HEV antibodies were demonstrated in rats and rabbits. Interestingly, wild boar-derived HEVgt3 infection was not demonstrated in dexamethasone treated rats. In rabbits, viral replication was more efficient and immunization protected against viral shedding with feces. The rabbit model for wild boar-derived HEVgt3 infection may serve as a suitable alternative to the non-human primate and swine models, and as an appropriate basis for vaccine evaluation studies. Possibly, the establishment of a transgenic animal model would be also useful to examine potential pathogenic effects of HEV structural proteins on liver cells. One disadvantage of using transgenic models to study the potential pathogenesis of HEV proteins is the fact that the animals are tolerant to the transgenic protein, and thus, the role of the immune response to HEV proteins cannot be evaluated. To determine the role of the cellular immune response in the development of hepatitis, other potentially HEV susceptible knock-out mouse lines, nude rats or T cell depleted rabbits would be useful. However, xenograft models potentially display an appropriate small animal model for HEV as well. Xenograft models for studying HCV have been developed and are now being used to evaluate HCV biology and anti-HCV therapies [464]. Those models rely on transplantation of human hepatocytes into mice and subsequent repopulation of the mouse liver. Probably, xenograft models can also be applied correspondingly using porcine hepatocytes.
8. Summary
Josephine Schlosser

Transmission and pathogenesis of hepatitis E virus infection in European wild boar and domestic pigs, and the establishment of a small animal model for hepatitis E

Hepatitis E virus (HEV) is the causative agent of acute hepatitis E in humans, which occurs mostly in developing countries, but sporadic and autochthonous cases do also occur in industrialized countries. To date, four major mammalian genotypes (HEVgt1 to gt4) have been identified. While HEVgt1 and gt2 are exclusively found in humans, HEVgt3 and gt4 are zoonotic. Sources are mainly contaminated raw or undercooked meat derived from domestic pigs and wild boar, representing the main HEV reservoir hosts. Cross-species infections with HEVgt3 were demonstrated experimentally. However, not all sources of human infections were identified thus far and in many cases, the origin of HEV infection remains unknown. Understanding HEV interspecies and intraspecies transmission is needed to implement efficient prevention and control measures. In Europe, foodborne zoonotic transmission of HEVgt3 is primarily associated with domestic pigs, but the wild boar is also discussed as one of the main source of human autochthonous infections. However, little is known about the course of HEVgt3 infection in European wild boar and their role in HEV transmission to domestic pigs, and other species.

Therefore, one objective of this thesis was to provide insights into the HEV pathogenesis in European wild boar and to characterize the transmissibility of wild boar-derived HEVgt3 infection to domestic pigs. Furthermore, the following questions were also addressed: The cellular and humoral immune responses following a HEVgt3 infection were determined. It was assessed, whether a dexamethasone treatment, which causes potent immunosuppressive effects, affects the course of HEV infection in experimentally infected swine. To obtain an initial impression of the potential existence of porcine chronic hepatitis E, the course of HEV infection in naturally infected European wild boar was investigated up to six months. The infectivity of naturally acquired persistent HEV infection in wild boar was assessed in domestic pigs. Suitable small animal models for porcine HEV infection were investigated.

In this study, the clinical course of porcine HEV infection was examined based on changes in liver enzyme levels. Different patterns within the course of HEV infection were observed in
experimentally infected pigs, as animals with early anti-HEV seroconversion were able to clear the virus, while animals lacking antibody responses showed prolonged HEV replication. In experimentally infected pigs, liver histopathology was characterized by mild to moderate inflammatory infiltrates, predominantly consisting of CD3 positive cells, with variable degree of hepatocellular degeneration. Furthermore, studies showed that Kupffer cells, liver sinusoidal endothelial cells and extra-hepatic lymphatic cells are important cell populations possibly involved in HEV pathogenesis. Following up on this, their role as potential virus replication sites cannot be excluded. Our results indicate that experimental HEV infection enhances cellular immune responses in swine, surprisingly largely unaffected by a dexamethasone induced immunosuppression. An increase of CD8+CD4- and CD4+CD8+ T cells, and activated γδ T cell subsets was shown in PMBCs of intravenously HEV infected wild boar. Anyhow, in PMBCs of domestic pigs dominated a CD4+CD8- T cell response. Moreover, marked increase in the percentages of γδ T cells were observed in the liver of HEV infected wild and domestic swine. Interestingly, the results indicate persistent HEV infection also in naturally infected wild boar despite the presence of anti-HEV antibodies. Furthermore, the present study showed that European rabbits seem to be a promising small animal model for wild boar-derived HEVgt3, whereas viral replication in Wistar rats was less effective. The protective ability of a HEV vaccine candidate in HEVgt3 inoculated rabbits was shown. Different tested mouse lines were not susceptible to wild boar-derived HEVgt3, and resistance to HEVgt3 could be assumed.

Taken together, the present study underlines the importance of European wild boar (Sus scrofa scrofa) as HEV reservoir hosts and their relevance in the transmission of HEVgt3 to domestic pigs (S. scrofa domestica), European rabbits (Oryctolagus cuniculus) and rats of the species Rattus norvegicus. Since large amounts of virus particles are excreted in feces of wild boar, droppings can contaminate the environment and pose a particular risk to susceptible species. The rabbit model for wild boar-derived HEVgt3 infection may serve as a suitable alternative to the non-human primate and swine models, and as an appropriate basis for the development and evaluation of novel vaccines.

With respect to HEV perpetuation in its reservoirs and possible public health risk, especially regarding swine as a main source of human autochthonous infection, this study contributes to understand the dynamics and biology of this zoonotic disease.
9. Zusammenfassung

Josephine Schlosser

Übertragung und Pathogenese der Hepatitis E Virus-Infektion in Wild- und Hausschweinen, sowie die Etablierung eines geeigneten Kleintiermodells für Hepatitis E


Anhand veränderter Leberenzymwerte konnte ein klinischer Verlauf der HEV-Infektion im Wildschwein nachgewiesen werden. Dies gelang ebenfalls nach experimenteller Übertragung einer HEVgt3-Infektion vom Wildschwein auf das Hausschwein. Außerdem zeigten sich verschiedene Verlaufsformen der HEV-Infektion in experimentell infizierten Schweinen:
Zusammenfassung

Infektionsmodelle in Primaten und Schweinen könnte ebenfalls das Hauskaninchen-Modell für eine vom Wildschwein stammende HEVgt3-Infektion in Frage kommen, und zukünftig als geeignete Basis für die Entwicklung und Evaluierung von Vakzinen dienen. Hinsichtlich des Fortbestehens der HEV-Infektionen in seinen Wirten und den damit verbundenen Risiken für die öffentliche Gesundheit, insbesondere bezüglich des Schweines als Hauptquelle autochthner Infektionen, trägt diese Studie dazu bei, die Dynamiken und Biologie dieses zoonotischen Erregers besser zu verstehen.
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11. Appendix

Additional file 1
Primers and probe used in this study.

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</tr>
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ORF = open reading frame.
### Additional file 2
Primary and secondary antibodies used for flow cytometry staining.

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<th>Antibody</th>
<th>Isotype</th>
<th>Labeling</th>
<th>Clone</th>
<th>Source</th>
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<td></td>
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## Additional file 3

### Viral load in different tissue samples of wild boar (WB) and domestic pigs (DP) estimated by RT-qPCR.

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<th>HEV genome equivalents per µl RNA in tissue</th>
<th>Wild boar</th>
<th>Domestic pigs</th>
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<tr>
<td></td>
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</tr>
<tr>
<td>WB 47</td>
<td>WB 49</td>
<td>WB 51</td>
</tr>
<tr>
<td>WB 44</td>
<td>WB 46</td>
<td>WB 48</td>
</tr>
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<td>WB 52</td>
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<tr>
<td>right hep lb</td>
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<td>left hep lb</td>
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</tr>
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<tr>
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<tr>
<td>vagina/pr gland</td>
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<td>0</td>
</tr>
<tr>
<td>qf muscle brain</td>
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Viral copy numbers in tissues were calculated from Ct-values determined by RT-qPCR. Ct-values equal to HEV genome equivalents per µl RNA : Ct 19 – 22 equal to 10^4, Ct 23 – 26 equal to 10^3, Ct 27 – 30 equal to 10^2, Ct 31 – 34 equal to 10^1, Ct > 35 equal to 0; Group 1A and 2A = non-treated; Group 1B and 2B = dexamethasone-treated; n. d. = viral load was not determined (no sample available); hep = hepatic; lb = lobe; ln = lymph node; prox = proximal; dist = distal; ic = ileocecal; asc = ascends; desc = descends; gl = gland; bug = bulbourethral gland; pr = prostate; qf = quadriceps femoris.
Additional file 4
HEV antigen detection within post mortem tissues of wild boar and domestic pigs assessed by immunohistochemistry.

<table>
<thead>
<tr>
<th>Viral antigen staining in tissue</th>
<th>Wild boar</th>
<th>Domestic pigs</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Group 1A</td>
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<tr>
<td></td>
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<td>liver</td>
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<tr>
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<tr>
<td>lung</td>
<td>0</td>
<td>0</td>
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<tr>
<td>duodenum</td>
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<td>jejunum</td>
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<td>mand ln</td>
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<td>brain</td>
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</table>

The viral antigen staining was graded as follows: 0 = negative antigen staining and 1 = positive antigen staining; Group 1A and 2A = non-treated; Group 1B and 2B = dexamethasone-treated; n. d. = not determined (no sample available). \(^a\) For details see Figure 4.8 A – C; \(^b\) For details see Figure 4.8 D; \(^c\) For details see Figure 4.8 E; \(^d\) For details see Figure 4.8 F; ln = lymph node; mesent = mesenterial; mand = mandibular; gl = gland; qf = quadriceps femoris.
Appendix

Additional file 5
Mean values of liver enzyme levels, antibody responses and HEV RNA loads in serum and feces of domestic pigs. Error bars represent ±SD. One way ANOVA (Bonferroni t-test; P<0.05). The difference in the mean values among group 2A and 2B is greater than would be expected by chance: *a = significant with P<0.05. The difference in the mean values among group 2A and the uninfected reference group “0 DPI” is greater than would be expected by chance: *b = significant with P<0.05. DPI = day post inoculation of group 1A and 1B. A.) Detection of alanine aminotransferase (ALT) and gamma-glutamyl transferase (GGT) in serum of group 2A and 2B. Upper reference range limit = grey-dotted line. IU = international units. B.) Detection of anti-HEV IgG-antibodies in serum of group 2A and 2B with mean absorbance (OD450). PrioCHECK® HEV Ab porcine. Cut-off (grey-dashed line): OD450 > 0.6. C.) HEV RNA in serum and feces of group 2A and 2B estimated by RT-qPCR.
Additional file 6
Differential cell counts in peripheral blood of domestic pigs. The mean number (in K/µL) of leukocytes (WBC), lymphocytes (LYM), monocytes (MON) and neutrophils (NEU) were determined using an automated XT-2000iV hematology analyzer. Error bars represent ±SD. Reference value ranges for the tested blood parameters in domestic pigs: grey-dotted line. DPI = day post inoculation of group 1A and 1B.
Additional file 7
T-cell related responses upon infection in blood lymphocytes of domestic pigs. Absolute number and percentage of T helper cells (CD4+CD8-) and γδ T cells (γδTCR+) in peripheral blood lymphocytes is given. DPI = day post inoculation of group 1A and 1B.
Additional file 8
B-cell related responses upon infection in blood lymphocytes of domestic pigs. Blood lymphocytes were immune-stained to determine the frequency of different B-cell subpopulations by FACS analysis: naïve B-cells (CD2+CD21+), activated B-cells upon antigen contact (CD2-CD21+) and antibody-forming and/or memory B-cells (CD2+CD21-). DPI = day post inoculation of group 1A and 1B.
Additional file 9
Measurements of the rectal temperature and body weight. WB = naturally HEV infected wild boar; DP = domestic pigs in contact with naturally HEV infected wild boar.
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