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HEPATITIS E VIRUS IN HEMATOPOIETIC STEM CELL TRANSPLANT RECIPIENTS

by

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Thesis presented to *Escola Superior de Biotecnologia* of the *Universidade Católica Portuguesa* to fulfill the requirements of Master of Science degree in Applied Microbiology

by

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RESUMO

Objetivo: O vírus da Hepatite E (HEV) causa hepatite em indivíduos saudáveis, no entanto, em países desenvolvidos, a infeção por HEV pode evoluir para crónica em pacientes imunocomprometidos. Pouco se sabe sobre a infeção por HEV em recetores de transplante de células-tronco hematopoiéticas (HSCT), e o seu estado prolongado de imunossupressão torna-os um grupo de risco importante. Resumimos todas publicações sobre infeções por HEV em recetores de HSCT e desenvolvemos um estudo retrospetivo epidemiológico para caracterizar a prevalência de HEV num coorte de HSCT alogénicos.
Métodos: O estudo I foi realizado para resumir todos os dados publicados sobre infeções por HEV em recetores de HSCT, realizando uma revisão sistemática da literatura. A pesquisa bibliográfica foi conduzida na PubMed e Scopus e as guidelines PRISMA foram seguidas. O software MetaXL foi utilizado na análise estatística para estimar a prevalência geral de infeção por HEV de acordo com as diferentes abordagens de diagnóstico (deteção de RNA HEV e anti-HEV IgM/IgG). O estudo II foi desenvolvido como um estudo retrospetivo num coorte de 196 pacientes submetidos a alo-HSCT entre 2016-2018, no qual rastreamos o RNA HEV por RT-PCR em tempo real e para anti-HEV IgM e IgG usando um imunoensaio enzimático.
Resultados: No estudo I, foram encontrados 7 manuscritos que relatam uma prevalência geral de anti-HEV IgM/IgG de 12,0% (IC95%: 0,16-28,5) e prevalência de RNA HEV de 1,50% (IC95%: 0,70-2,60). A prevalência isolada de anti-HEV IgM foi de 2,00% (IC95%: 0,30-4,50) e a anti-HEV IgG foi de 11,4% (IC95%: 1,80-26,3). O estudo II revelou uma prevalência de anti-HEV IgG de 19,9%. Além disso, encontramos uma prevalência de infeção recente/ativa por HEV de 4,1%, com 6 casos positivos para RNA HEV e 2 casos positivos para anti-HEV IgM e IgG.
Conclusão: Nos últimos anos, alguma atenção foi dada a este grupo de imunocomprometidos, mas ainda há um número reduzido de estudos. Este estudo mostra que os recetores de alo-HSCT estão em risco de infeção por HEV, portanto devem ser rastreados antes do transplante e durante episódios de elevação das enzimas hepáticas após transplante com o teste de RNA HEV. No entanto, são necessários mais estudos para aumentar a nossa compreensão da epidemiologia do HEV em receptores de HSCT, visto que pode ser um fator importante no resultado dos pacientes.
Palavras-chave: Vírus da hepatite E; Transplante de células-tronco hematopoiéticas; prevalência

ABSTRACT

Aim: Hepatitis E virus (HEV) causes hepatitis in healthy individuals, however, in developed countries, HEV infection can evolve to chronic hepatitis in immunosuppressed patients. Little is known of HEV infection in hematopoietic stem cell transplant (HSCT) recipients, and their prolonged immunosuppression state makes them an important risk group. We summarize all published data regarding HEV infections in HSCT recipients and developed an epidemiology study to characterize HEV prevalence in a retrospective cohort of allo-HSCT.

Methods: Study I was performed to summarize all published data regarding HEV infections in HSCT recipients by performing a systematic review of the literature. Literature search was conducted in PubMed and Scopus and Preferred Reporting Items for Systematic Reviews and Meta-Analyses guidelines were followed. The MetaXL software was used for statistical analysis to estimate the overall prevalence of HEV infection according to the different diagnostic approaches (HEV RNA and anti-HEV IgM/IgG detection). Study II was developed as a retrospective study in a cohort of 196 patients submitted to allo-HSCT between 2016-2018, on which we screened for HEV RNA by real-time RT-PCR and for anti-HEV IgM and IgG using an enzyme immunoassay.

Results: In study I, we found 7 manuscripts reporting an overall anti-HEV IgM/IgG prevalence of 12.0% (95% CI: 0.16-28.5) and HEV RNA prevalence of 1.50% (95% CI: 0.70-2.60). Isolated anti-HEV IgM prevalence was 2.00% (95% CI: 0.30-4.50), and anti-HEV IgG was 11.4% (95% CI: 1.80-26.3). Study II revealed an anti-HEV IgG prevalence of 19,9%. Furthermore, we found a prevalence of recent/active HEV infection of 4.1%, with 6 positive cases for HEV RNA, and 2 positive cases for both HEV IgM and IgG.

Conclusion: Over the last years, some attention has been given to this group of immunocompromised patients, but there is still a small number of studies. This study shows that recipients of allo-HSCT are at risk for HEV infection. Therefore, allo-HSCT recipients should be screened prior transplantation, and during episodes of liver enzyme abnormalities post-transplantation, with HEV RNA testing as the preferred diagnostic method in these immunocompromised patients. Nevertheless, more studies are needed to increase our understanding of the epidemiology of HEV in HSCT recipients, as it may be an important factor in the outcome of patients.

Keywords: Hepatitis E Virus; Hematopoietic Stem Cell Transplantation; prevalence

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ABBREVIATIONS

Allo-HSCT: Allogeneic-Hematopoietic Stem Cell Transplantation

ALT: Alanine Aminotransferase

AP: Alkaline Phosphatase

ALT: Alanine Transaminase

AST: Aspartate Aminotransferase

Auto-HSCT: Autologous-Hematopoietic Stem Cell Transplantation

ATG: Antithymocyte Globulin

BM: Bone Marrow

cDNA: Complementary Deoxyribonucleic Acid

CI: Confidence Interval

CMV: Cytomegalovirus

C.O.: Cutt-off

CsA: Cyclosporine

DNA: Deoxyribonucleic Acid

EBV: Epstein-Barr Virus

ECDC: European Centre for Disease Prevention and Control

EIA: Enzyme Immunoassay

ELISA: Enzyme-Linked Immunosorbent Assay

GVHD: Graft *versus* host Disease

HBV: Hepatitis B Virus

HCV: Hepatitis C Virus

Hel: Helicase

HEV: Hepatitis E Virus

HHV-6: Human Herpevirus 6

HLA: Human Leucocyte Antigen

HRP: Horseradish Peroxidase

HSC: Hematopoietic Stem Cell

HSCT: Hematopoietic Stem Cell Transplantation

Hsp90: Heat Shock Protein 90

HSV: Herpes Simplex Virus

HVR: Hypervariable Region

IgG: Immunoglobulin G

IgM: Immunoglobulin M

MA: Myeloablative conditioning

Met: Methyltransferase

MMF: Mycophenolate Mofetil

MTX: Methotrexate

OR: Odds Ratio

ORF: Open Reading Frame

OS: Overall Survival

***p*:** p-value

PCP: Papain-like Cysteine Protease

PCR: Polymerase Chain Reaction

PFS: Progression Free Survival

PRISMA: Preferred Reporting Items for Systematic Reviews and Meta-Analyses

PTLD: Post-transplant Lymphoproliferative Disease

RdRp: RNA-dependent RNA polymerase

RIC: Reduced Intensity Conditioning

RNA: Ribonucleic Acid

RT: Reverse Transcriptase

RT-PCR: Reverse Transcription-Polymerase Chain Reaction

RT-qPCR: Reverse Transcription-quantitative Polymerase Chain Reaction

SOT: Solid Organ Transplantation

TBI: Total Body Irradiation

TMB: Tetramethylbenzidine

UCB: Umbilical Cord Blood

VLP: Virus-like-particles

VZV: Varicella Zoster Virus

WHO: World Health Organization

γGT: gamma-glutamyl-transferase

I. INTRODUCTION

1. Hematopoietic Stem Cell Transplantation

Hematopoietic stem cells (HSC) are multipotent cells with indefinite cell division potential that reside in bone marrow, being able to differentiate into various lineages of mature hematologic cells (Felfly and Haddad 2014; Park, Yoo, and Kim 2015). Hematopoietic Stem Cell Transplantation (HSCT) is an established curative treatment for patients with hematological, oncological, hereditary, and immunological diseases (Appelbaum 2007; Copelan 2006). Since the first successful bone marrow transplant in 1956, performed by Dr E. Donnall Thomas in Cooperstown, New York (Thomas et al. 1957), thousands of patients each year with conditions affecting the blood or bone marrow have benefited from this treatment.

1.1. General overview

HSCT involves the administration of healthy multipotent hematopoietic stem cells of any donor type and source, to a recipient with dysfunctional or depleted hematopoietic system with the intention of replacing it in total or in part (Ljungman et al. 2010; Gratwohl et al. 2010; Stephen J. Forman and Ryotaro Nakamura 2015). Generically, HSCT is classified depending on the source of the stem cells: autologous and allogenic.

Autologous HSCT (auto-HSCT) is usually used to restore the body's ability to make normal blood cells after high dose chemotherapy or radiation and is characterized by using the patient's own stem cells, previously collected. The advantage of auto-HSCT is that there is no risk of graft rejection and development of graft-*versus*-host disease (GVHD) since the infusion is made with the patient's own cells (Khaddour and Mewawalla 2019; Ali, Adil, and Shaikh 2015). Allogeneic HSCT (allo-HSCT) involves the infusion of stem cells collected from a healthy donor into a recipient after high dose chemotherapy or radiation. Allo-HSCT is used to replace a defective host bone marrow or immune system with a healthy donor (Korrapati and Nanganuru 2014). Considering the risk of graft rejection and GVHD, the donors of cells have to come from a Human Leukocyte Antigen (HLA) matched family member, unrelated matched donor or mismatched family donor (haploidentical) (Khaddour and Mewawalla 2019). A major obstacle to a successful allo-HSCT is the limited number of HLA-matched related donors

within families, and the chance of finding a matched unrelated donor is strongly dependent on the patient's ethnic background (Lucarelli et al. 2012).

To achieve a successful HSCT, ablation of the recipient's bone marrow is necessary, which requires conditioning regimen with high-dose chemotherapy and/or radiotherapy (Bacigalupo et al. 2009). The host stem cells must be eradicated to prevent rejection of the donor's cells and to create an environment that supports proliferation and differentiation for the donor's stem cells. Moreover, the conditioning regimen eliminates the underlying disease (Lucarelli et al. 2012; Juric et al. 2016; Bacigalupo et al. 2009). Conditioning regimen can be defined in two categories: myeloablative conditioning (MA) and non-myeloablative/reduced-intensity conditioning (RIC) (Bacigalupo et al. 2009). Myeloablative conditioning is a high-dose intensity therapy that consists of total body irradiation (TBI) and/or use of alkylating agents (Bacigalupo et al. 2009), and its major disadvantage is the myelotoxicity and considerable morbidity and mortality (Juric et al. 2016). RIC uses lower, less toxic doses of chemotherapy and radiation than MA and its use has expanded HSCT to high risk patients, such as older patients or patients with several comorbidities (Gratwohl et al. 2010).

Bone marrow (BM), umbilical cord blood (UCB) or peripheral blood stem cells (PBSC) are frequently used as the source for stem cells. For allo-HSCT, all three stem cell sources are used and have their specific advantages and disadvantages. Bone marrow is a great source for stem cells and is collected from the donor by aspiration of cells from the posterior iliac crests (Copelan 2006). Despite the fact that hematopoietic stem cells are at low concentrations in the bloodstream, the administration of growth factors rapidly increases HSC concentration allowing its collection by apheresis. Therefore, PBSC has become the preferred choice in auto-HSCT because of a more rapid hematopoietic reconstitution (Calandra et al. 2008). PBSCs are associated with more rapid engraftment but are also associated with an increased risk of chronic GVHD compared with BM, which makes it a less attractive option for children, or for some patients with early stage disease, making BM the preferred choice in these situations (Copelan 2006; Ljungman et al. 2010). UCB may be used in the context of HLA-identical allo-HSCT, nevertheless as this is quite a rare situation, unrelated UCB cells are more commonly used when patients lack an HLA-identical sibling or a well-matched unrelated donor. An additional advantage is that UCB cells can be obtained rapidly and may, therefore, be the best option when a patient needs an urgent HSCT (Copelan 2006; Ljungman et al. 2010).

The most important complication of allo-HSCT is GVHD. GVHD is an immune response accentuated, and possibly stimulated by an injury resulting from the preparative regimen used before transplantation (Copelan 2006). It can affect one or more organs to varying degrees, with the most frequently affected being the skin, gastrointestinal tract and liver. The prophylactic administration of immunosuppressive drugs is an important part of the pre-transplant and post-transplant treatment (Lucarelli et al. 2012). GVHD can be categorized into acute and chronic. Acute GVHD usually develops within three months. Prophylaxis is usually achieved with calcineurin inhibitors, methotrexate, and anti-thymocyte globulins. Severity of acute GVHD is estimated from grade I to VI, and treatment with either high dose prednisone or methylprednisolone is indicated in higher grades. Chronic GVHD occurs after three months. Treatment is similar to that of acute GVHD, but the duration is usually over 2 years. (Khaddour and Mewawalla 2019)

1.2. Risk factors for morbidity and mortality

HSCT results in more cures and remissions than alternative treatments but also causes greater morbidity and mortality. Although the mortality rate for autologous transplantations and allogeneic transplantations are very low, some percentage of patients with advanced cancer die from complications related to transplantation (Copelan 2006). The main risk factors for the outcome are the stage of the disease, the age of the patient, the time interval from diagnosis to transplant and, for allo-HSCT, the donor/recipient histocompatibility and the donor/recipient gender combination. Survival rates decrease with advanced disease stage, increasing age, increasing time from diagnosis to transplant, increase in HLA disparities, and for male recipients having a female donor. All components should be integrated into risk assessment and decision making for a transplant (Ljungman et al. 2010). Indeed, the principal risk factor is HLA mismatch, but it may occur despite an HLA-matched donor and the use of preventive measures. The incidence of GVHD can be reduced by in vitro T-cell depletion of the graft before transplantation, but this does not improve disease free survival, because the rates of graft rejection and relapse increase. (Copelan 2006). Graft failure is associated with increased risk of infection and post-transplant mortality and is the process of which there is a loss of bone marrow function after reconstitution following infusion of hematopoietic stem cells or if there is no gain of function after infusion. The incidence of failure is highest when there is a high HLA disparity that usually occurs in the case of cord blood and

haploidentical donors and is lowest with autologous and matched donor siblings. Factors responsible for graft rejection include functional residual host immune response to the donor cells, a low number of infused cells, in vitro damage during collection and cryopreservation, inadequate preparative regimen and infections (Khaddour and Mewawalla 2019).

1.2.1. Viral infections

HSCT recipients, especially those who have received allo-HSCT are at increased risk for many infectious complications owing to impaired cell-mediated immunity and a prolonged immunocompromised state. These complications are influenced by factors such as the amount of time after HSCT, the prophylactic strategy used, comorbidities, pathogen exposure, and the degree of immunosuppression. Indeed, the prevention of bacterial, fungal, and viral infections is a priority for HSCT patients, since they are associated with high morbidity and mortality (Gyurkocza and Sandmaier 2014).

There are three phases of opportunistic infections among allo-HSCT recipients according to time after transplantation and the presence or absence of GVHD (Copelan 2006) – Figure 1. Phase I, or the pre-engraftment phase (days 0-45 post-HSCT), is characterized by prolonged neutropenia and breaks in the mucocutaneous barriers, which increase the patient's risk of bacterial and fungal infections. Additionally, herpes simplex virus reactivation may occur. Phase II, or the post-engraftment phase (days 30-100 post-HSCT), is characterized by impaired cell-mediated immunity and is directly related to the severity of GVHD and immunosuppressive therapy. Cytomegalovirus (CMV), *Pneumocystis jiroveci*, and *Aspergillus* species are common pathogens during this phase. During phase III, or the late phase (more than 100 days post-HSCT), the risk of infection corresponds to the severity of the patient's GVHD during the first two phases. Common pathogens include CMV, varicella-zoster virus (VZV), and infections from encapsulated bacteria, such as *Streptococcus pneumoniae* (Tomblyn et al. 2009).

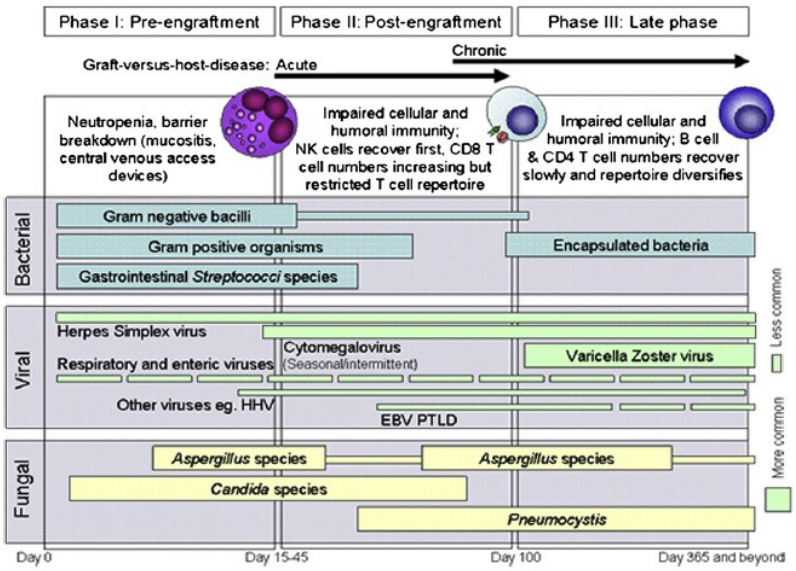

Figure 1. Phases of infections following allogeneic hematopoietic stem cell transplantation (Tomblyn et al. 2009)

Overall, the viruses of major importance in HSCT recipients include herpes simplex virus (HSV), VZV, CMV, Epstein-Barr virus (EBV), respiratory viruses (like influenza, parainfluenza, respiratory syncytial virus, adenovirus), human herpes virus 6 (HHV-6), hepatitis B (HBV), and hepatitis C (HCV) (Tomblyn et al. 2009).

2. Hepatitis E Virus

2.1. History

Hepatitis E virus (HEV) was discovered in 1978 in Kashmir, India in the first described epidemy of this virus (M. S. Khuroo 1980). The outbreak was initially classified as non-A, non-B Hepatitis and resulted in 52,000 estimated patients with icteric hepatitis and 1700 deaths. The disease affected young adults, ranging from 15 to 45 years, and was self-limiting without progress to chronicity nor cirrhosis, nevertheless, in pregnant women a high fetal and perinatal mortality was associated (Mohammad S. Khuroo, Khuroo, and Khuroo 2016). Years later, a similar outbreak of non-A, non-B Hepatitis was reported in a Soviet military camp in Afghanistan (Balayan et al. 1983). A few outbreaks have occurred since then, with the largest being described in China between 1986 and 1988 and affecting 120,000 patients (Aye et al. 1992).

In 1983, a human volunteer who had previously contacted with hepatitis A virus, orally self-administrated pooled stool extracts from patients with the disease, and after some days developed severe acute hepatitis with jaundice and elevated liver tests. The analysis of stool samples by immune electron microscopy analysis showed virus-like-particles (VLP) which confirmed the viral etiology of this disease. Then, scientists experimentally infected some primates with the agent and started to study the physicochemical properties of the agent (Bradley, Purdy, and Reyes 1991). In 1990, partial cloning of the virus was done (Reyes et al. 1990), which was followed by sequencing the full-length HEV genome (Tam et al. 1991; C. C. Huang et al. 1992).

2.2. Structure and molecular properties

HEV belongs to the *Hepeviridae* family, genus *Orthohepevirus* (Smith et al. 2014; 2016) – Figure 4. HEV is a small (27–34 nm) RNA virus with an icosahedral capsid (Tam et al. 1991). The virus was described as a non-enveloped virus for many years, however, HEV can be "masked" by the membrane of the host cells, and be resistant to antibodies when this form is in blood (Takahashi et al. 2010; Chapuy-Regaud et al. 2017). When HEV particles were released by the cellular exosomal pathway, they appeared to be similar to a "quasi-enveloped" virus. The resistance of the "quasi-enveloped" form is caused by the absence of viral antigens on the surface (Feng et al. 2013).

HEV genome is a single-stranded, positive-sense RNA with approximately ~7.2 kb in length, that consists of a short 5´non-coding region that is capped with a 7 methyl-guanine, three open reading frames (ORFs) with distinct functions (ORF1, ORF2, and ORF3), and a short 3´non-coding region that ends in a poly(A) tail (Nassim Kamar et al. 2017), as shown in Figure 2.

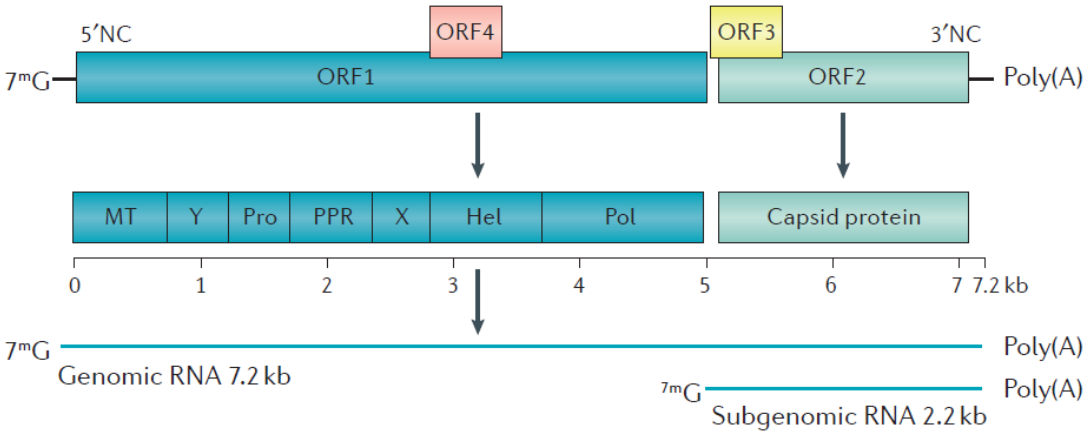

Figure 2. HEV genome and viral proteins (Nassim Kamar et al. 2017)

ORF1 encodes a set of proteins of ~1,700 amino acids, which contains eight functional domains: a methyltransferase (Met); a Y domain (Y); a papain-like cysteine protease (PCP); a hypervariable region (HVR); a X domain (X); a helicase (Hel); and a RNA-dependent RNA polymerase (RdRp) (Koonin et al. 1992). ORF1 is involved in viral replication, transcription, and polyprotein cleavage (Reyes et al. 1990; Koonin et al. 1992; Kaur et al. 1992; R. P. Holla et al. 2013). ORF4 has been recently identified, situated within the ORF1 region of the HEV genome, encoding a 20 kDa protein which interacts with host and viral proteins to control the activity of the viral RdRp, however, ORF4 is unique to genotype HEV-1 strains (Nair et al. 2016).

ORF2 includes the 660 amino-acid virus capsid protein (Jameel et al. 1996) which has three glycosylation sites (Asn132, Asn310, and Asn562) and an amino-terminal signal peptide (Zafrullah et al. 1999). The capsid protein contains 4 domains: N terminals; shell domain; middle domain; and protruding domain, which contains a putative receptor binding domain and is the major target for neutralizing antibodies (Guu et al. 2009; Yamashita et al. 2009). The capsid consists of 180 copies arranged as an icosahedron (Xing et al. 2010), being involved in virus assembly, encapsidation, binding, and the host immune response to the virus (Surjit, Jameel, and Lal 2004). Immunological and

structural studies of this region have contributed to the development of an HEV vaccine (Xing et al. 2011).

ORF3 is located between ORF1 and ORF2, partially overlapping with ORF2 (Debing, Moradpour, et al. 2016). It encodes for a small 113 amino acid phosphoprotein with approximately 13 kDa (Graff et al. 2006) and seems to be involved in viral assembly and egress, interacting with the capsid protein (Tyagi et al. 2002).

2.3. Life Cycle

The mechanism of viral replication of HEV remains hypothetical. An example of the putative cycle is presented in Figure 3. HEV is a hepatotropic virus, multiplying mainly in hepatocytes, however immunohistochemistry experiments and detection of negative-sense HEV RNA indicate that replication can also occur in other tissues such as the gastrointestinal tract (Izopet et al. 2012; Williams et al. 2001), kidney (Geng et al. 2016), central nervous system (Drave et al. 2016) and placenta (Bose et al. 2014). The cellular receptor for HEV is not yet known, but the presence of heparan sulfate proteoglycans appears to be necessary for the binding of the virus to target cells (Kalia et al. 2009). Once it has bound, HEV enters the cell by endocytosis, which appears to be dynamin-2, clathrin, and membrane cholesterol-dependent (Kapur et al. 2012; P. Holla et al. 2015).

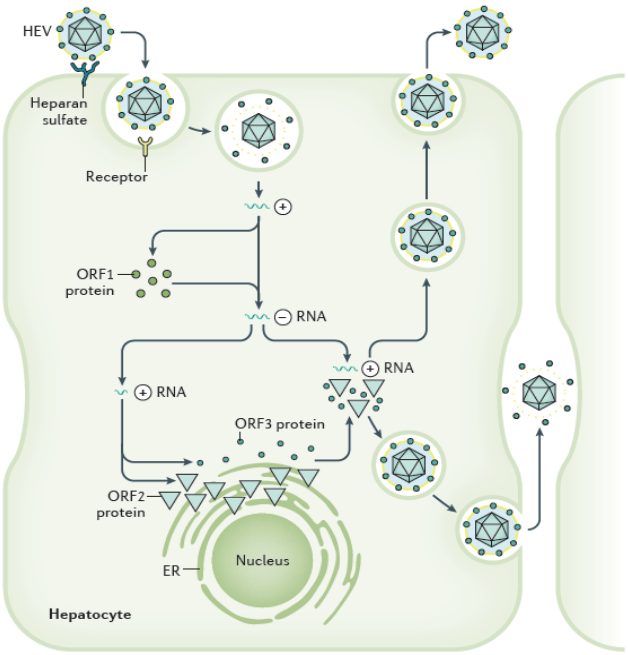

Figure 3. HEV life cycle (Nassim Kamar et al. 2017)

The pathway of the virus within the cell remains unknown, but heat shock protein 90 (Hsp90) and tubulin could play a role in the intracellular transport of HEV virions (Zheng et al. 2010). Subsequently, the RNA is released in the cytoplasm of the cell, but the mechanisms and exact localization of this process have not been explored. Once the viral RNA is free in the cytosol, translation of the ORF1 polyprotein will occur because of its 50 cap. Following the translation of methyltransferase, protease, helicase, and RNA polymerase activities, the genomic RNA is copied into a strand of negative RNA, which then allows the synthesis of genomic and subgenomic RNAs (Purdy et al. 1993). ORF2 and ORF3 are translated subsequently to produce the structural proteins (capsid protein and phosphoprotein) that will allow the encapsidation of the newly synthesized viral RNA strands. This makes it possible to obtain new viral particles (Graff et al. 2006; Yamada et al. 2009); a glucose-regulated protein belonging to the Hsp70 family (Grp78) may play a role in the folding and assembly of the capsid proteins (Yu et al. 2011). The proteins encoded by ORF2 and ORF3 interact, which suggests a role for ORF3 in the assembly of viral particles (Tyagi et al. 2002). HEV is not cytolytic (Tanaka et al. 2007). Furthermore, studies have shown the requirement of the exosomal pathway for virion release (Nagashima et al. 2014; 2017). Recent studies suggest that viruses secreted into the bloodstream are associated with the ORF3 protein and lipids, but viruses secreted into the bile are non-enveloped (Nagashima et al. 2014; 2011).

2.4. HEV genotypes

The genus *Orthohepevirus* A includes eight genotypes (HEV-1 to HEV-8): HEV-1 and HEV-2 have been detected only in humans; HEV-3 and HEV-4 have been detected in humans and several animal species (domestic pig, wild boar, Cervidae) (Teshale e Hu 2011; Mohammad S. Khuroo, Khuroo, e Khuroo 2016); HEV-5 and HEV-6 have been detected only in wild boars (Takahashi et al. 2011; 2014); HEV-7 has been only described in one report in humans; while the new genotype HEV-8 has been associated with Bactrian camels and also humans (Woo et al. 2016) – Figure 4.

There is no evidence that animal reservoirs for HEV1 and HEV2 exist, although non-human primates can be infected with HEV-1 (Purcell et al. 2013), natural infection of this reservoir and consecutive zoonotic transmission has not been reported.

Experimental infection of pigs with HEV-1 or HEV-2 was unsuccessful suggesting that these genotypes cannot cross the species barrier (X. J. Meng, Halbur, Haynes, et al. 1998).

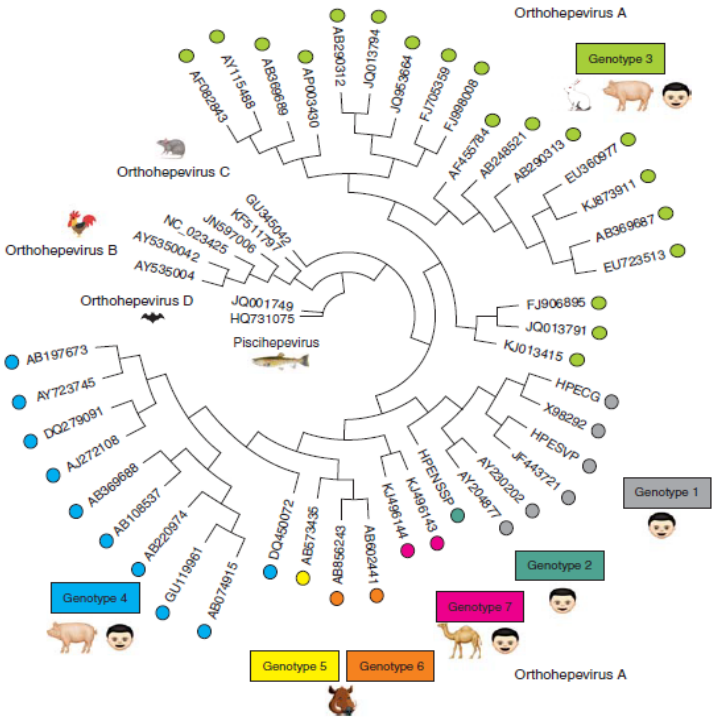

Figure 4. Phylogenetic tree of hepatitis E virus strains. Phylogenetic tree is based on full-length sequences of HEV strains (Kenney and Meng 2019).

2.5. Epidemiology

According to World Health Organization (WHO), HEV infection is one of the most common cause of acute viral hepatitis and has a large distribution worldwide, with the WHO estimating 20 million infections, >3 million acute cases, and >57,000 HEV-related deaths, annually (World Health Organization 2018; 2015).

HEV can present two distinct epidemiological patterns observed in different regions of the globe (Nassim Kamar et al. 2017; Donnelly et al. 2017; Horvatits et al. 2019) - Table 1, Figure 5. These patterns seem to be correlated with the distribution of HEV genotypes, transmission routes, source of virus infection, disease prevalence, and, in some cases, clinical characteristics of the disease. The epidemiology and clinical features of HEV infection are primarily determined by the predominant genotype in the region and their respective hosts (Teshale, Hu, and Holmberg 2010). Genotypes HEV-1

and HEV-2 are endemic in developing countries, where they cause water-borne outbreaks. These are obligate human pathogens, transmitted via fecal-oral route and clinical presentation with HEV-1 and HEV-2 infection is indistinguishable from any other cause of acute viral hepatitis (Nassim Kamar et al. 2017). The most common mode of HEV transmission in developed countries is believed to be food-borne zoonosis by genotypes HEV-3 and HEV-4 (Scobie and Dalton 2013). Host species for HEV-3 and HEV-4 include pigs, deer, rabbits, mongoose, cattle, sheep and horses (X. J. Meng, Halbur, Shapiro, et al. 1998; Tei et al. 2003a; Cossaboom et al. 2011; Nidaira et al. 2012; Wang et al. 2002; W. Zhang et al. 2008).

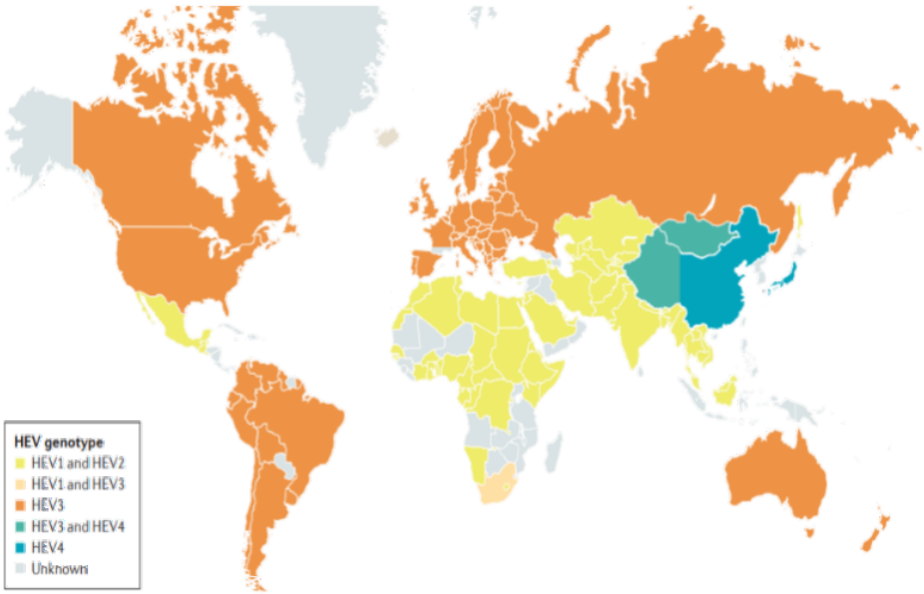

Figure 5. Global distribution of HEV genotypes (Nassim Kamar et al. 2017)

Table 1. HEV characteristics according to genotypes. Adapted from (Nassim Kamar et al. 2017; Donnelly et al. 2017; Horvatits et al. 2019)

Parameter	HEV-1	HEV-2	HEV-3	HEV-4
Geographical distribution	Asia, Africa, Latin America	Mexico, West Africa	Worldwide	China, East Asia, Central Europe
Reservoir	Humans		Animals (mainly swines)	
Pattern of spread	Epidemic, Sporadic		Autochthonous, Sporadic	
Transmission	• Contamination of drinking water supplies with human faeces • Vertical • Iatrogenic		• Consumption of contaminated animal products or contact with infected animals • Iatrogenic	
Hepatic manifestations	• Self-limiting acute hepatitis • Acute liver failure • Risk factors for severity are pregnancy and pre-existing chronic liver disease		• Self-limiting acute hepatitis • Acute liver failure • Chronic hepatitis • Risk factors for severity is pre-existing chronic liver disease	
Extrahepatic manifestations	• Neurological manifestations • Renal manifestations • Pancreatitis		• Neurological manifestations • Renal manifestations • Hematological manifestations	

Hepatitis E is highly endemic in several parts of Asia (south, central and southeast Asia), Africa, the Middle East and Mexico (Rakesh Aggarwal 2011a), being HEV-1 and HEV-2 genotypes the most prevalent in these areas with limited access to water and inadequate sanitary conditions (Nassim Kamar, Dalton, et al. 2014; Okamoto 2007; Rakesh Aggarwal and Gandhi 2010). HEV-1 has a more widespread distribution than HEV-2, which has been reported only in Africa and in an outbreak in Mexico (Nassim Kamar et al. 2017). HEV-1 and HEV-2 cause an estimated 20.1 million new infections annually in Asia and Africa, with 3.4 million cases of acute hepatitis E, 70,000 deaths from acute liver failure and 3,000 stillbirths (Rein et al. 2012). Overall morbidity rates are higher among teenagers and young adults (Rakesh Aggarwal 2011b), pregnant women and patients with pre-existing chronic liver disease, leading to fulminant hepatitis (Rakesh Aggarwal 2011a; Acharya et al. 2007).

The prevalence of anti-HEV Immunoglobulin G (IgG) in Africa ranges from 4.6 to 10.7% in the general population (Goumba, Yandoko-Nakouné, and Komas 2010; World Health Organization 2018), while Asia shows a higher prevalence with anti-HEV

IgG reaching 34.8% to 94% (World Health Organization 2018; Ren et al. 2014; You et al. 2013; Izopet et al. 2015; Kang et al. 2017).

Are considered low endemic areas for hepatitis E regions with well-controlled water supplies and adequate sanitary conditions, such as Europe, high-income areas in Asia (such as Japan, Taiwan, South Korea, and Hong Kong), and the Americas. Here, the disease is characterized by sporadic cases (Domanović et al. 2017; World Health Organization 2018; Fierro et al. 2016; Panduro-Cerda et al. 2011), associated with zoonotic transmission by HEV-3 and HEV-4 from domestic animals, most often from pigs (Harry R Dalton, Kamar, and Izopet 2014). Additionally, HEV can be iatrogenically transmitted between humans through infected blood or blood products, especially HEV-3 in Europe (Hewitt et al. 2014), although cases have also been reported in Japan (for HEV-3 and HEV-4) and China (HEV-1 and HEV-4) (Satake et al. 2017; L. Zhang et al. 2017).

A survey performed in 30 countries in the European Union/European Economic Area showed that the number of reported cases of HEV infection has increased from 514 per year in 2005 to 5,617 in 2015, with most infections being locally acquired (Aspinall et al. 2017). A recent meta-analysis identified 73 studies of HEV seroprevalence in Europe with estimates ranging from 0.6% to 52.5%, increasing with age but unrelated to gender and showed a high variability in seroprevalence rates between regions. (Hartl et al. 2016). In Portugal, reported anti-HEV IgG prevalence in the general population was 16.3% (Nascimento et al. 2018); while in Finland, Norway and Germany reported seroprevalences were 27.6%, 11.4% and 16.8%, respectively (Kantala et al. 2009; Olsøy et al. 2019; Faber et al. 2012). Furthermore, estimates show 68 000 and 100 000 HEV infections occur annually in France and the United Kingdom, respectively (Van Cauteren et al. 2017; Hewitt et al. 2014). Many countries have undertaken epidemiological studies of HEV seroprevalence in their respective healthy blood donors, such as Scotland, Netherlands, Spain, France and Southwest England, reporting an anti-HEV IgG prevalence ranging from 6.1% to 26.7% (Thom et al. 2018; Slot et al. 2013; Sauleda et al. 2015; J. M. Mansuy et al. 2016; Harry R. Dalton, Stableforth, et al. 2008). Significant differences in the seroprevalence of populations inside the same countries exist, such as been reported in France, Italy, and Poland, areas considered hyperendemic for HEV (Lapa, Capobianchi, and Garbuglia 2015), that may be explained by food habits and differences in culinary practices between regions (Capai, Falchi, and Charrel 2019; Hartl et al. 2016). This high variability of seroprevalence rates in Europe are attributed to the

different geographic area and populations studied, as well as the commercial immunoassays used, since it has been demonstrated that the assays used in anti-HEV Immunoglobulin M (IgM) and IgG detection have a wide variation in sensitivity and specificity (Bendall et al. 2010; Pas et al. 2013; Avellon et al. 2015).

In the Americas, the anti-HEV IgG seroprevalence ranges from 3 to 31%, which can be explained by the sporadic HEV cases reported in the area (World Health Organization 2018; Lopes dos Santos et al. 2010; Mirazo et al. 2011). In the United States, initial estimates of anti-HEV IgG seroprevalence of 21% were recently updated to 6% (Ditah et al. 2014). Analysis of HEV RNA in plasma samples collected from 96 centers across the United States showed that 0.002% were positive (Roth et al. 2017). The less-frequent consumption of game or organ meat in the United States in comparison with Europe, together with the lack of knowledge about this virus among clinicians, might explain the low prevalence of HEV infection in the United States (Nelson, Kmush, and Labrique 2011; Ditah et al. 2014).

2.6. Transmission

Hepatitis E is mainly transmitted by a fecal-oral route through contaminated water ingestion (Nassim Kamar, Bendall, et al. 2012; X.-J. Meng 2013) and through the consumption of undercooked pork and boar meat and contact with pigs, as documented in several case reports (World Health Organization 2010; Nassim Kamar, Bendall, et al. 2012; X.-J. Meng 2013; Pavio, Meng, and Renou 2010; Sonoda et al. 2004; Tei et al. 2003b). Transmission via contaminated shellfish (Claire Crossan et al. 2012) and soft fruits (Brassard et al. 2012) is also recognized as a potential source of food-borne transmission. Transmission via blood transfusion is increasingly recognized as a risk factor (Huzly et al. 2014; Hauser et al. 2014; Hewitt et al. 2014) - Figure 6.

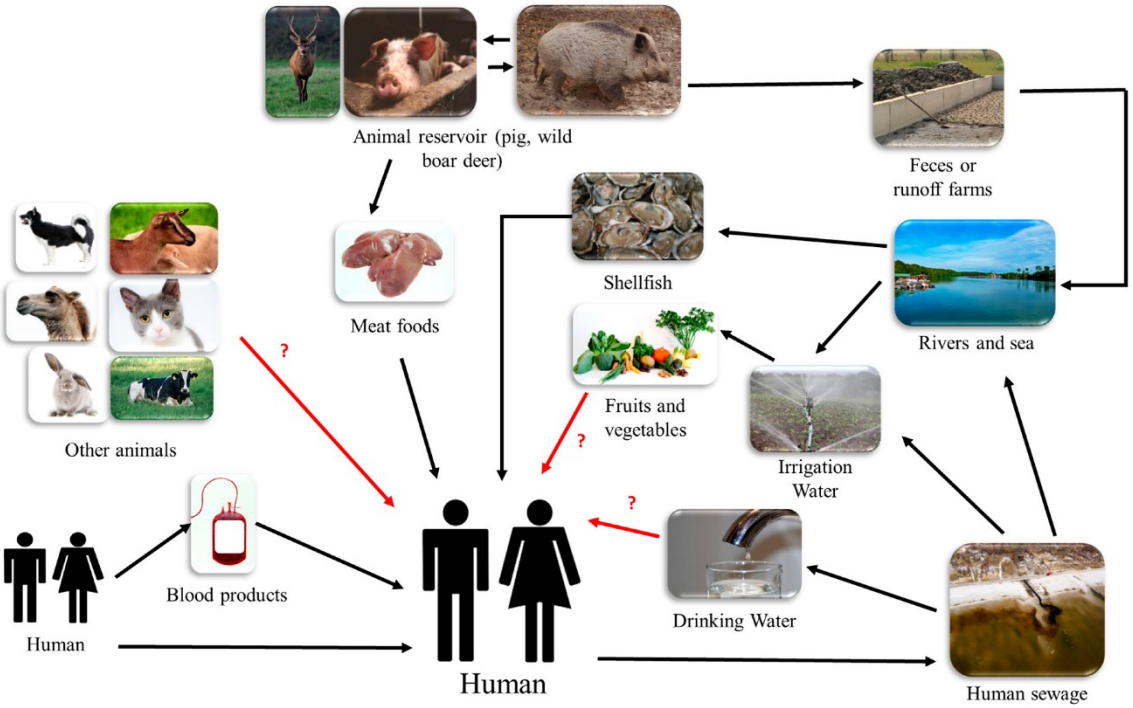

Figure 6. Representation of the different routes of transmission of HEV. Black arrows for confirmed transmission routes; red arrows with "?" for suspected and not confirmed transmission routes (Capai, Charrel, and Falchi 2018).

2.6.1. Waterborne transmission

Humans are the main reservoir of HEV-1 and HEV-2, and the transmission occurs via fecal-oral route mainly in developing countries where wastewater treatment is inadequate (Nassim Kamar et al. 2017; World Health Organization 2010), however transmission is also possible in developed countries when untreated water is ingested, since HEV is resistant in the environment because of its non-enveloped form (Nassim Kamar et al. 2017). The incubation period of HEV infection in outbreaks in which the period of water contamination was known has varied from 2 to 10 weeks, and are usually short lived and unimodal (Nassim Kamar et al. 2017). However, some outbreaks have been multimodal and lasted for several months, caused by prolonged contamination of drinking water sources (Naik et al. 1992).

Vegetables and fruits irrigated with contaminated water are a possible route of transmission (Capai, Falchi, and Charrel 2019). HEV-3 RNA was detected in red fruits, strawberries, green leaves and spices (Doceul et al. 2016), likely via exposure to contaminated water. For example: in Canada swine HEV was detected in strawberries in 1.6% of the samples tested (Brassard et al. 2012); a European study of the berry fruit

supply chain identified HEV in 2.6% of raspberries at point of sale (Maunula et al. 2013); and a recent European study identified HEV in 5% of irrigation water samples from leafy green vegetable production chains (Kokkinos et al. 2017).

2.6.2. Zoonotic transmission

HEV-3 and HEV-4 are abundant in domestic swine from all over the world (Doceul et al. 2016). Direct and indirect contact with infected animals or consumption of contaminated food products are the main transmission routes of HEV-3 and HEV-4 to humans (Doceul et al. 2016). HEV-3 and HEV-4 RNA or specific anti-HEV antibodies have also been detected in a wide range of different animal species such as other Suidae (for example, wild boar (J. Schlosser et al. 2014)), wild fauna (such as sika deer, red deer, roe deer, mongooses (Doceul et al. 2016) or rabbits (Izopet et al. 2012)) and domestic animals (such as goats, sheep, buffaloes, yellow cattle, workhorses, cats and dogs (Doceul et al. 2016; Yan et al. 2016)), implying that these animals are potential sources of HEV exposure. For example, in Germany, 33% of wild boar and 50% of domestic pigs are seropositive for anti-HEV IgG (Denzin and Borgwardt 2013; Dremsek et al. 2013). HEV infection has been identified in more than 80% of some pig herds in the USA, Canada and in England (Grierson et al. 2015; Yoo et al. 2001), although Scottish herds have a lower seroprevalence of around 62% (C. Crossan et al. 2015). In a more recent report on English and Northern Irish pigs, 93% of slaughter age animals were seropositive (Grierson et al. 2015).

HEV-3 and HEV-4 infection in animals is asymptomatic, but the virus is excreted in large quantities in the feces of infected animals, which contributes to the maintenance of the virus in herds. HEV excretion is the highest in growing pigs of 3–5 months of age (Casas et al. 2011). Infected pork liver, pork products containing liver and other pork meat consumed raw or undercooked have been identified as sources of HEV infections (Colson et al. 2010; Renou, Afonso, e Pavio 2014; World Health Organization 2010; Nassim Kamar, Bendall, et al. 2012; X.-J. Meng 2013; Pavio, Meng, e Renou 2010; Sonoda et al. 2004; Tei et al. 2003b). In France, it was reported a correlation between HEV infection and consumption of pork liver sausage ficatellu, a liver product that contains at least 30% pork liver and does not undergo a heating step during production (Renou, Afonso, and Pavio 2014; Berto et al. 2013; Colson et al. 2010). One survey that analyzed food products in France showed that 30% of ficatelli samples were positive for

the presence of HEV RNA (Pavio, Merbah, and Thébault 2014). In addition, HEV-3 and HEV-4 were also detected in the milk of infected cows (F. Huang et al. 2016). Personnel with professional occupation with pigs, such as farmers, veterinarians and individuals working in the slaughterhouse, are more frequently HEV seropositive than the general population (Lange et al. 2017; Galiana et al. 2008; Pérez-Gracia et al. 2007; Renou et al. 2007; Colson et al. 2007), as are forestry workers (Chaussade et al. 2013).

Regarding the other genotypes, only one case of transmission of HEV-7 to an immunocompromised individual through consumption of camel meat and milk has been described; this individual developed severe hepatitis E and required liver transplantation (Lee et al. 2016). HEV-5 and HEV-6 have been detected in wild boar in Japan, but no infection of humans has been described (Li et al. 2015).

HEV bioaccumulation was demonstrated in oysters under experimental conditions (Grodzki et al. 2014), and in the West of Scotland, 92% of tested wild caught mussels were HEV RNA positive and consumption of undercooked/raw shellfish is another viable route of transmission, thereby multiplying potential sources of human exposure (Claire Crossan et al. 2012).

2.6.3. Iatrogenic transmission

Although less common than waterborne or zoonotic transmission, HEV can be transmitted iatrogenically between humans through infected blood and blood products. Transmission of HEV-3 and HEV-4 by transfusion of blood products (including red cells, platelets, and even pathogen-inactivated fresh frozen plasma) has been reported in many Western (and some Asian) countries (Hewitt et al. 2014; Matsubayashi et al. 2008; Hauser et al. 2014). Transfusion-associated HEV infection has been documented for HEV-3 in many European countries (Hewitt et al. 2014), for HEV-1 and HEV-4 in China (L. Zhang et al. 2017) and for HEV-3 and HEV-4 in Japan (Satake et al. 2017). Just a minority of recipients of infected blood or blood products develops symptomatic hepatitis E, and the risk of developing chronic HEV infection is considerable only when infected blood or blood components are given to immunosuppressed individuals (Hewitt et al. 2014).

The incubation period for HEV-3 infection in immunosuppressed patients with blood-borne HEV infection has been demonstrated to be 50-60 days, compared with less than 30 days for immunocompetent patients with HEV-1 infection (Pischke et al. 2016).

The presence of anti-HEV IgG in recipients does not necessarily protect the recipient from transfusion-transmitted infection, as low levels of antibody do not prevent reinfection (S. A. Baylis et al. 2015). Transmission of HEV infection can also occur via solid organ transplantation (SOT) of liver and non-hepatic grafts. Schlosser described a case of a 73 year old man in whom HEV transmission occurred after transplantation of an HEV-infected liver from a donor with occult HEV infection (B. Schlosser et al. 2012).

2.6.4. Other routes of Transmission

Person-to-person transmission of HEV-1 or HEV-2 is infrequent in both sporadic and epidemic settings (Rakesh Aggarwal and Naik 1994; Somani et al. 2003). However, vertical transmission of HEV-1 and HEV-2 from a woman to her child during pregnancy is well documented (M. S. Khuroo, Kamili, e Jameel 1995; M. S. Khuroo, Kamili, e Khuroo 2009). HEV-1 infection frequently determines symptomatic disease in pregnant women that can be severe, especially in the third trimester determining acute liver failure with a mortality rate of 15–25% (Naidu and Viswanathan 1957; Sharma et al. 2017). Infection with HEV-1 during pregnancy is also related to an increased risk of adverse outcomes of pregnancy as spontaneous abortion, fetal death in utero, and premature delivery in patients with icteric hepatitis or with acute liver failure caused by HEV (Jilani et al. 2007; Salam et al. 2013). In addition, vertical transmission can result in complications to the fetuses and neonates, such as anicteric or icteric hepatitis, hypoglycemia and neonatal death (Sharma et al. 2017; Jilani et al. 2007; Salam et al. 2013; Kumar et al. 2014; Bose et al. 2011).

In industrialized countries, the rare cases of infection reported in pregnant women with HEV-3 did not lead to infection in the newborn (Lachish et al. 2015; Anty et al. 2012; Shaikh and Nelson-Piercy 2006; Tabatabai et al. 2014). The contribution of these routes to the overall burden of hepatitis E has not been determined but is likely small.

2.7. Diagnosis

The diagnosis of Hepatitis E by the study of clinical signs is quite complicated, considering the short period of HEV viremia, but also because the majority of cases are asymptomatic, and the clinical forms are indistinguishable from other causes of acute

viral hepatitis. The detection of infection markers is therefore required to diagnose Hepatitis E or previous exposure to HEV.

HEV infection can be diagnosed either indirectly by the demonstration of anti-HEV IgM and IgG antibodies or directly by detecting HEV RNA using a quantitative reverse transcription polymerase chain reaction (RT-qPCR) in serum, plasma or stool samples, however alanine aminotransferase activity is also a marker of infection (Nassim Kamar et al. 2017; R. Aggarwal et al. 2000; Rakesh Aggarwal 2013).

HEV pathogenesis consists of three distinct stages: the incubation period, acute hepatitis E and a convalescent phase characterized by a gradual recovery – Figure 7.

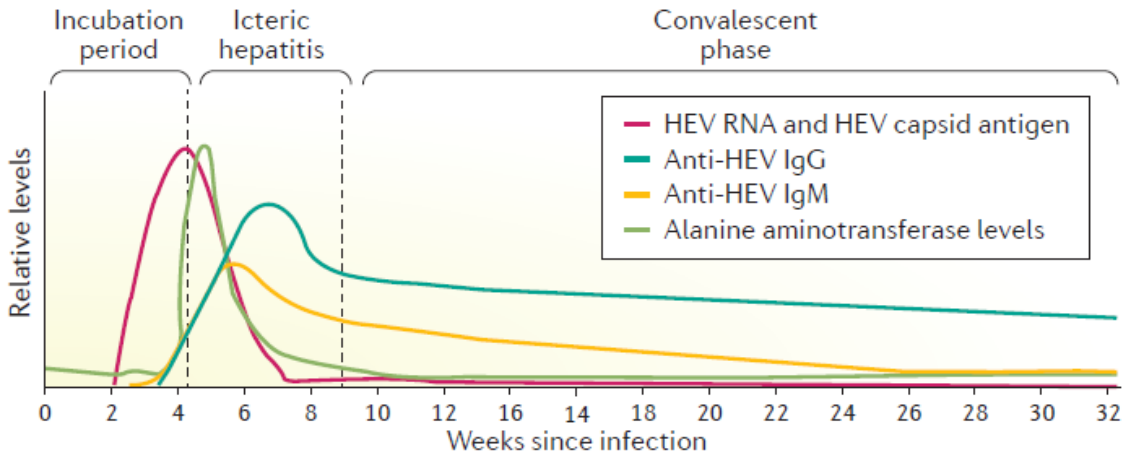

Figure 7. Hepatitis E biomarkers (Nassim Kamar et al. 2017)

The incubation period is characterized by nonspecific symptoms, such as malaise, fever, body aches, nausea, and vomiting. The icteric phase is marked by the production of dark-colored urine and jaundice, which most often resolve spontaneously after a few days to weeks, known as the convalescent phase (Harry R. Dalton, Bendall, et al. 2008).

HEV RNA becomes detectable in the blood and stool during the incubation period and persists for 4 weeks in blood and 6 weeks in feces. Capsid antigen persists in the blood for approximately the same duration (Wen et al. 2015). An initial incubation period of 2–6 weeks usually precedes the short-lived IgM response, which is detected on average at the 8 week around the time the levels of liver enzymes, such as serum alanine aminotransferase (a marker of liver inflammation or injury) increase and persist for 6–9 months (S. Huang et al. 2010). The detection of these IgM antibodies is a marker of

recent infection. The anti-HEV IgG response reaches its peak four weeks later than IgM, and persists for several years after the infection, although the exact duration of this response remains uncertain. (M. S. Khuroo et al. 1993; Nassim Kamar et al. 2017). IgG is a marker of a past infection.

2.7.1. Anti-HEV antibodies detection

Two serological markers can be used to investigate the presence of past or recent HEV infection. The first is anti-HEV IgM, which indicates the acute phase, and the second is anti-HEV IgG, which indicates current infection when observed together with anti-HEV IgM detection or past contact when it is detected alone (Domanović et al. 2017; J. Zhang et al. 2014). To detect anti-HEV antibodies, several serological methods are available including enzyme immunoassay (EIA) and immunochromatography. Enzyme immunoassays are used with recombinant ORF2 proteins and/or ORF3 proteins from an HEV-1 strain as antigens. Antibodies in patients infected with other genotypes generally cross-react adequately owing to shared ORF2 and ORF3 epitopes (Nassim Kamar et al. 2017). Determining the anti-HEV IgG concentration could be useful for estimating the risk of reinfection after natural infection or after vaccination in clinical trials. One study demonstrated that immunocompromised patients with low anti-HEV IgG concentration (<7 WHO units per ml) could become reinfected, with a risk of developing chronic hepatitis (Abravanel et al. 2014).

The diagnostic performance of IgM and IgG assays varies considerably and must be evaluated carefully as studies have shown a high heterogeneity between commercial tests (Bendall et al. 2010; Vollmer et al. 2016; Avellon et al. 2015; Wenzel et al. 2013; Norder et al. 2016). There is currently no consensus across laboratories for HEV testing, and this variability may at least in part account for the differences in reported rates of anti-HEV antibody in various populations. In the recent meta-analysis of HEV seroprevalence in Europe (Hartl et al. 2016), seroprevalence again varied depending upon the assay used, with the Wantai assay reporting significantly higher seroprevalence rates across all cohorts tested. As a result, it can be difficult and unreliable to compare data from different populations obtained by different laboratory methods.

2.7.2. Viral RNA detection and quantification

HEV RNA detection and quantification in blood, feces or other bodily fluids is the gold standard for detecting an active HEV infection (acute or chronic). The most commonly used technique is RT-qPCR targeting conserved regions across HEV genotypes, usually ORF2 and ORF3 (Abravanel et al. 2013; Abravanel, Sandres-Saune, et al. 2012).

Assays based on RNA amplification require the WHO international reference panel for validation and comparison of their performance (Sally A. Baylis et al. 2013). WHO established a genotype 3a HEV strain as the International Standard strain for HEV RNA, with an assigned unit of 250 000 IU/mL. The limit of detection of current assays is 7–80 IU/mL (Abravanel et al. 2013; Sauleda et al. 2015), which is relevant to take into account not only for diagnosis but also for defining an optimal strategy (testing of individual samples versus minipool testing) for blood screening. RNA testing is usually useful when serology is difficult to interpret due to cross-reactivity with polyclonal antibody immune response and in immunocompromised patients, in which the antibody response may be undetectable. Therefore, HEV RNA PCR is the favored diagnostic test, particularly in the immunocompromised.

This method is also vital for genotyping for epidemiological purposes (Zhao and Wang 2016). HEV genotypes and subgenotypes can be characterized by sequencing different regions of the HEV genome, such as the ORF2 or ORF1 region encoding RNA polymerase. Sequencing information can be used to perform molecular epidemiology to identify the source of infection or to detect mutations in the gene encoding RNA polymerase in patients in whom ribavirin therapy fails (Debing, Ramière, et al. 2016; Lhomme et al. 2015).

2.7.3. Capsid antigen detection

Acute HEV infections can be diagnosed by detecting the HEV capsid antigen via a commercial sandwich EIA. The specificity of this assay is 100%, and the diagnostic sensitivity for an acute HEV infection is 91%, with no significant difference between immunocompetent (88%) and immunocompromised (94%) patients (Trémeaux et al. 2016). As testing for HEV capsid antigen is technically simpler, less expensive and faster than quantification of HEV RNA levels, this test could become an alternative for diagnosing HEV infections in laboratories with no molecular diagnostic facilities.

2.8. Clinical manifestations

In humans, the acute form of the disease can be caused by strains belonging to four genotypes: HEV-1, HEV-2, HEV-3, and HEV-4 (Rakesh Aggarwal 2011b). The course and clinical presentation of HEV infection are highly variable and the mechanisms leading to the different clinical outcomes are only partially understood. HEV multiplies in the liver and reaches the digestive tract via the bile, although it is non-cytopathic (Bouwknegt et al. 2009) - Figure 3. Infectious HEV virions are secreted by patients with acute hepatitis E into stool and urine (Geng et al. 2016), and the outcome of acute HEV infection is determined by the strength of the host immune response (Suneetha et al. 2012; Abravanel et al. 2016). Once HEV infection is cleared, patients develop immunity against HEV. Reinfection is possible, although the likelihood of developing symptomatic hepatitis is lower during subsequent infections (Pischke et al. 2016; J. Zhang et al. 2014).

When HEV infection is suspected on the basis of clinical symptoms or an isolated increase in alanine aminotransferase, anti-HEV IgM is measured first, given the good performance and wide availability of this assay. Although, in patients who are immunocompromised anti-HEV IgM and IgG may be negative and therefore RNA testing by PCR must be employed (Donnelly et al. 2017). A study performed by Pas et al. demonstrated that anti-HEV IgM could only be detected in 7/16 immunocompromised patients compared with 18/18 immunocompetent patients in the acute phase of infection, suggesting a delayed immune response and abnormal IgM antibody kinetics in the immunocompromised group (Pas et al. 2013). Accordingly, HEV RNA should be tested in three clinical settings: when the anti-HEV IgM is negative and alanine aminotransferase activity is elevated, when the HEV RNA in blood and stool persists for 3–6 months, to identify a chronic infection, and when a recent reduction in immunosuppression has been made or antiviral therapy has been started, to monitor chronic infection (Nassim Kamar et al. 2017).

2.8.1. Immunocompetent individuals

Most cases of acute hepatitis E in immunocompetent individuals have a favorable outcome, with biological parameters normalizing spontaneously within three months without the need for antiviral therapies (Nicand, Bigaillon, and Tessé 2009). Symptoms are similar to those of other types of acute viral hepatitis and include fever, anorexia,

nausea, fatigue, myalgia and jaundice (Rein et al. 2012), with cholestatic forms occurring in 20% of cases (Irshad 1999). Routine laboratory testing usually detects an increase in alanine and aspartate aminotransferase (ALT, AST) levels, accompanied by an increase of alkaline phosphatase (AP), gamma-glutamyl-transferase (γGT), and bilirubin levels. The ALT level increases usually between 1000–3000 IU/L, but extreme values can be seen, and ALT elevation is commonly higher than AST elevation (Wedemeyer, Pischke, and Manns 2012).

The hepatic manifestations following HEV infection vary from an entirely asymptomatic disease course or mild systemic illness to icteric acute hepatitis and fulminant or acute liver failure. Cases leading to death correspond to the acute/fulminant forms (H. R. Dalton et al. 2007). Fulminant hepatitis is frequent among people with underlying liver diseases even in industrialized countries after HEV infection (Xin and Xiao 2016).

Pregnant women, particularly in the second and third trimesters, are at high risk of developing symptomatic disease following HEV-1 and HEV-2 infections, and a large proportion of these women progresses to acute liver failure. The newborn babies of these mothers are at risk of acquiring HEV infection and developing complications (Jilani et al. 2007).

There are no recommendations for treating HEV infected patients with antiviral regimens, however a combination of ribavirin and pegylated alpha interferon shows efficacy against HEV (Tanaka et al. 2007). Whether early treatment with ribavirin hastens viral clearance or minimizes the risk of liver failure remains to be determined. A small number of individuals with acute hepatitis E have been treated with ribavirin (Péron et al. 2016; Gerolami et al. 2011), most of whom were infected with HEV-3 (Pischke et al. 2013; Goyal et al. 2012). The studies show a rapid normalization of liver enzymes and clearance of HEV RNA, nevertheless, these data are based on a highly variable dose and duration of ribavirin therapy. Thus, spontaneous improvement cannot be ruled out.

2.8.2. Immunocompromised individuals

Immunocompromised patients are amongst those at higher risk of HEV infection and disease development. The majority of immunosuppressed patients are asymptomatic and present with persistent, mildly abnormal liver function tests (Nassim Kamar, Garrouste, et al. 2011), nevertheless chronic infection can occur in immunosuppressed

patients, such as individuals with HIV, hematological malignancies, or transplant-related patients (Abravanel, Mansuy, et al. 2012; Nassim Kamar, Dalton, et al. 2014; Nassim Kamar et al. 2017; Ankcorn et al. 2018).

Chronicity is defined as a persistent viremia at least three to six months after the diagnosis associated with a rapid evolution towards liver fibrosis, cirrhosis and graft rejection (Nassim Kamar, Garrouste, et al. 2011; N. Kamar et al. 2013). Within a few years after the infection, approximately 10% of the infected patients developed cirrhosis (Nassim Kamar, Garrouste, et al. 2011; N. Kamar et al. 2008). No HEV reactivation was observed after HEV clearance (N. Kamar, Izopet, and Rostaing 2012), nonetheless reinfections have been described and can lead to chronic hepatitis (Abravanel et al. 2014).

To date, chronic HEV infection has not been documented with HEV-1 or HEV-2 infection, with the vast majority observed with HEV-3 (Hoofnagle, Nelson, and Purcell 2012; de Niet et al. 2012; Gérolami, Moal, and Colson 2008; Te et al. 2013; Renou et al. 2010; Tavitian et al. 2010; Gauss et al. 2012), however, chronic infection with HEV-4 can occur (Wu et al. 2017; Geng et al. 2014).

AST and ALT are less elevated in patients who progress to chronic HEV infection; the mean ALT is 300 IU/L in chronic disease, and 1000 IU/L in acute disease (Murali, Kotwal, and Chawla 2015). No correlation was found between the viral load and the risk of progression to fibrosis (Murali, Kotwal, and Chawla 2015).

The first treatment approach when patients are under immunosuppressive therapy, like transplant recipients, is the reduction of the doses, if feasible (Nassim Kamar, Rostaing, Abravanel, Garrouste, Esposito, et al. 2010). For patients who cannot reduce immunosuppression or who fail to clear the virus despite a reduction in immunosuppression, ribavirin monotherapy is the treatment of choice for the majority of patients (Nassim Kamar, Rostaing, Abravanel, Garrouste, Lhomme, et al. 2010; Nassim Kamar, Izopet, et al. 2014; Debing et al. 2014; Mallet et al. 2010; Pischke et al. 2013; Tavitian et al. 2015; Galante et al. 2015). A negative HEV RNA concentration in the feces is necessary to confirm successful viral clearance (Abravanel et al. 2015). There is no definitive guide to the treatment duration, although the majority of reports have shown that a three-month course of ribavirin seemed to be a good duration of therapy for most patients with chronic infection (Nassim Kamar, Izopet, et al. 2014). Pegylated alfa interferon is not recommended in patients who have received SOT due to an appreciable risk of acute graft rejection (Rostaing et al. 1995). Plus, liver transplantation is considered when there is fulminant hepatitis and the patient's condition is life-threatening.

2.8.3. Extrahepatic manifestations

HEV infection may also present with extrahepatic manifestations. In vitro data have identified that HEV can replicate in non-liver cells including human intestine (Emerson et al. 2004) and has been described in association with a range of neurological manifestations (Harry R. Dalton et al. 2016; Nassim Kamar, Bendall, et al. 2011; Woolson et al. 2014). The reported cases are mainly associated with HEV-3 infection in Europe or in the context of HEV-1 infection in Asia, with more than 90% of cases documented in immunocompetent individuals, but also occur in the context of chronic infection with HEV-3 in immunocompromised patients. The best documented neurological manifestations associated with HEV infection are Guillain–Barré syndrome, neuralgic amyotrophy, encephalitis and myelitis (Harry R. Dalton et al. 2016). The association between HEV infection and Guillain–Barré syndrome is supported by three case-control studies from Bangladesh (Geurtsvankessel et al. 2013), the Netherlands (van den Berg et al. 2014), and Japan (Fukae et al. 2016) that show evidence of very early HEV infection in 5–11% of patients with Guillain–Barré syndrome, significantly higher than in healthy controls. Most of the cohort studies and case studies of HEV infection in patients with neuralgic amyotrophy are from Europe with HEV-3 infection (Harry R. Dalton et al. 2016).

Other recognized extrahepatic manifestations of HEV infection include renal impairment. Impaired renal function has been observed in both acute and chronic HEV infection (Nassim Kamar et al. 2005; Nassim Kamar, Weclawiak, et al. 2012). Renal biopsy samples from patients infected with HEV-1 and HEV-3 show evidence of glomerular disease patterns (Nassim Kamar, Weclawiak, et al. 2012; Ali, G. et al. 2001), including membranoproliferative glomerulonephritis with or without cryoglobulinaemia and membranous glomerulonephritis (Nassim Kamar, Weclawiak, et al. 2012; Taton et al. 2013; Guinault et al. 2016). The clearance of HEV was associated with an improvement in renal function and a decline in the proteinuria level in most patients (Nassim Kamar, Weclawiak, et al. 2012; Guinault et al. 2016; Bello et al. 2015).

2.9. Prevention

Prevention strategies for waterborne genotypes HEV-1 and HEV-2 have focused on reducing the risk of exposure through improvements in potable drinking water and sanitation in endemic areas (Nelson, Kmush, and Labrique 2011; Haque et al. 2015). During an HEV outbreak, improvement of water quality, often through boiling or chlorination, results in rapid declines in disease incidence. Conversely, failure to chlorinate the water supply has led to some of the largest documented HEV epidemics (Naik et al. 1992). Recently, WHO has published specific guidelines to prevent waterborne HEV infection (World Health Organization 2014).

HEV-3 and HEV-4 transmission has been largely foodborne and linked to the consumption of infected organ meats, game, and shellfish. Infections can be prevented by careful preparation of these foods. HEV present in contaminated meat is resilient to low-temperature cooking (~56°C) typical of medium-to-rare cooking conditions in restaurants (Feagins et al. 2008). Complete particles of HEV are vulnerable to boiling or frying and they become inactivated after 5 minutes at temperatures above 90°C. To completely inactivate HEV in the food, an internal temperature of 71°C for 20 min is necessary (Barnaud et al. 2012). In vitro data showed that heating for >2 minutes at 70 °C eliminates HEV infectivity, whereas infective HEV could be detected despite storage at room temperature after 28 days (Johne et al. 2016). In the environment, the virus might be more heat-labile at slightly lower temperatures (Emerson, Arankalle, and Purcell 2005). For pregnant women at high risk of fulminant hepatitis, the consumption of “at-risk” foods is strongly discouraged.

With regard to the prevention of transmission via contaminated blood products, there is no evidence at present to support the need for HEV negative blood components for pregnant women. At present in England, and more recently in Scotland, NHS Blood and Transplant recommend that HEV negative blood should be used in patients who have undergone allogeneic stem cell transplant or solid organ transplant (NHS Blood and Transplant 2016).

Despite the obvious risk of HEV transmission, prevention strategies are currently insufficient and infrequently implemented, thus a vaccine against HEV is highly desirable, particularly for residents living in highly endemic areas and for those at high

risk of developing complications. Since all HEV genotypes belong to the same serotype, it is thought that one HEV vaccine should provide protection against all HEV genotypes.

Currently, HEV 239 vaccine (Hecolin, Xiamen Innovax Biotech, China) is the only vaccine that is commercially available against HEV, approved by the Chinese government (Jun Zhang et al. 2015), however, it has not yet been approved in other countries. The HEV 239 vaccine showed an efficacy of 100% after 12 months and 86.8% after a follow-up of 4.5 years (Jun Zhang et al. 2015), inducing effective neutralizing antibodies against HEV (Jun Zhang et al. 2009; Cheng et al. 2012).

II. AIMS

Allo-HSCT recipients are at higher risk of HEV infection, with progression to chronicity, and the screening of HEV is greatly recommended. Over the last years, some attention has been given to this group of immunocompromised patients, but there is still a small number of studies in allo-HSCT. Thus, more studies are needed to increase our understanding of the epidemiology of HEV in HSCT recipients.

Therefore, the purpose of this study was:

- Summarize all published data regarding HEV infections in HSCT recipients by performing a systematic review of the literature;
- Determine the prevalence of HEV infection among allogeneic HSCT recipients;
- Characterize HEV infection in allogeneic HSCT recipients.

III. STUDY I

STUDY I

In this study, we aimed to summarize all published data regarding HEV infections in HSCT recipients by performing a systematic review of the literature. This study (appendix II) is already published as:

> Sara Cruz, Carla Campos, Mafalda Timóteo, Ana Tavares, Maria São José Nascimento, Rui Medeiros, Hugo Sousa. HEPATITIS E VIRUS IN HEMATOPOIETIC STEM CELL TRANSPLANT RECIPIENTS: A SYSTEMATIC REVIEW. J Clin Virol. 2019 Oct;119:31-36. doi: 10.1016/j.jcv.2019.08.002. Epub 2019 Aug 2.

1. MATERIALS AND METHODS

1.1. STUDY DESIGN

Preferred Reporting Items for Systematic Reviews and MetaAnalysis (PRISMA) guidelines were followed in the preparation of this systematic review. Different queries, including MeSH terms, were tested and the literature search was performed with the query that obtained more representative manuscripts: 'hepatitis E virus AND stem cell transplantation'. PubMed and Scopus databases were searched, independently by two of the authors, for published manuscripts on 31st January 2019 without restrictions on time period, sample size or population.

The eligibility criteria applied to studies were: 1) HEV infection (present or past) identified by the presence of HEV RNA and/or HEV-specific antibodies (IgM/IgG) in tested samples; 2) HSCT recipients; 3) provide prevalence data. The exclusion criteria applied were: 1) duplicate data; 2) other types of manuscripts (reviews, case reports, comments or letters to the editor); 3) no access to abstracts and/or full texts; and 4) other languages rather than English, Spanish or Portuguese. Manuscript titles and abstracts were screened according to the eligibility criteria and selected manuscripts were fully reviewed for data extraction (author, publication date, country, population, age range, type of HSCT, HEV detection methods and the number of positive and negative cases).

1.2. Statistical analysis

All data was inserted in a database that was used for prevalence analysis and comparison between studies. Prevalence analysis was performed using the MetaXL program version 5.3 (EpiGear International, Sunrise Beach, Queensland, Australia). The overall prevalence of HEV infection was estimated using the different approaches of diagnosis (HEV RNA or anti-HEV IgM/IgG detection) in HSCT recipients pooling the study data using the random effects model. The random effects model was used since a considerable heterogeneity among studies was expected, due largely to the different settings (populations, types of patients, age, gender, diagnostic methods) in which studies were conducted. The double arcsine transformation method was used for variance stabilization (Barendregt et al. 2013) considering a 95% confidence interval and a 5% statistical significance level (p<0.050).

2. RESULTS

2.1. Study selection and description

The literature search retrieved a total of 73 manuscripts from both databases, and after duplicates removal, a total of 54 records were screened - Figure 8. After applying inclusion/exclusion criteria, 41 records were excluded: language (n=1), reviews (n=18), other types of articles such as Case Reports and Letter to the Editor (n=9), and studies not related to HSCT patients or HEV detection (n=13). A total of 13 full-text articles were assessed for full-reading of which 6 were excluded: 1 review, 4 case reports and 1 was not performed in HSCT patients. The bibliography of the selected manuscripts was reviewed to identify any new publications and no other article was added to the analysis.

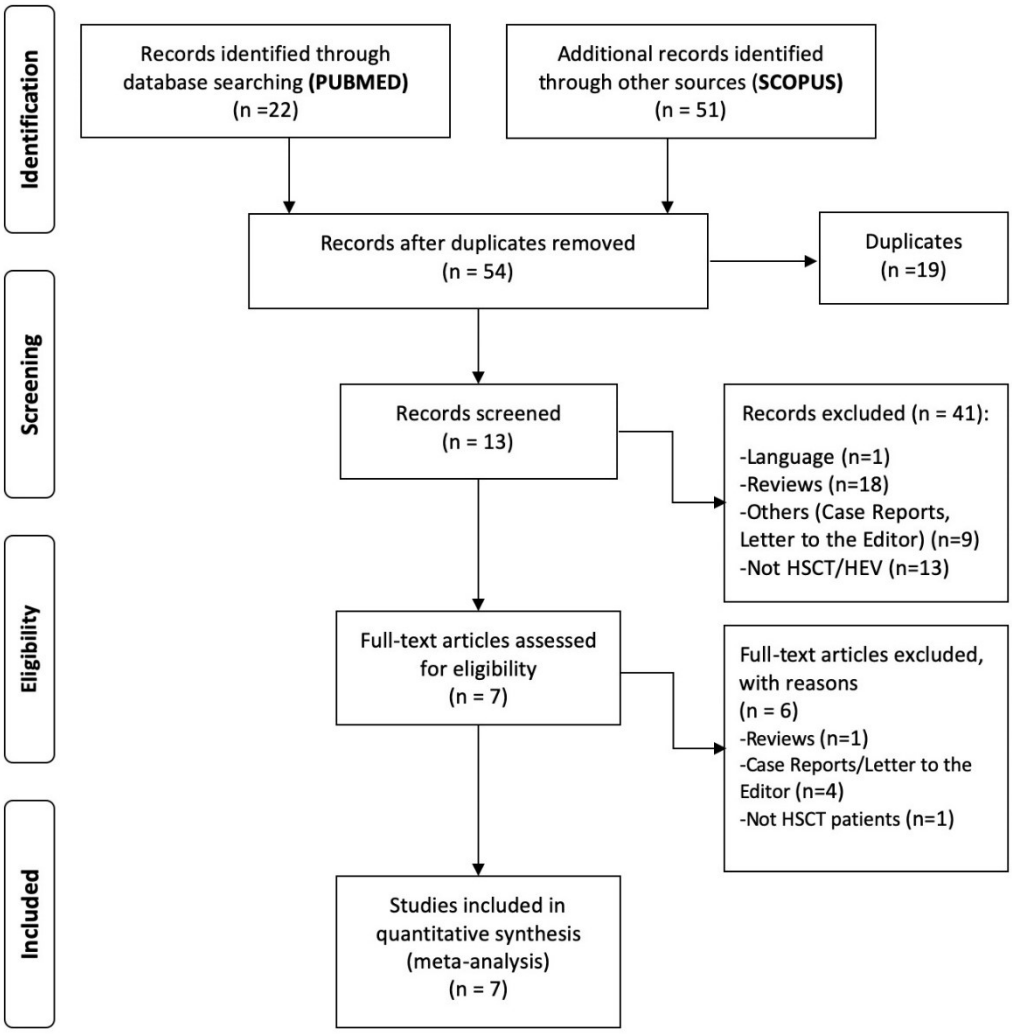

Figure 8. PRISMA flow diagram

After the full revision process, we included 7 manuscripts for data analysis (Abravanel, Mansuy, et al. 2012; Ankcorn et al. 2018; Tang et al. 2019; Willemse et al. 2017; Versluis et al. 2013; Koenecke et al. 2012; Reekie et al. 2018) -Table 2. Overall, these 7 studies included a total of 1178 HSCT patients from different countries: six studies were performed in Europe (United Kingdom, Netherlands, France, and Germany) (Abravanel, Mansuy, et al. 2012; Ankcorn et al. 2018; Willemse et al. 2017; Versluis et al. 2013; Koenecke et al. 2012; Reekie et al. 2018) and one study in China (Tang et al. 2019). These studies evaluated the prevalence of HEV infection based on the detection of HEV RNA (n=7) and/or the presence of anti-HEV IgM (n=3)/IgG (n=4). Phylogenetic analysis was performed in only three of these seven studies, revealing only HEV-3 genotype (Ankcorn et al. 2018; Versluis et al. 2013; Reekie et al. 2018).

Table 2. Details of the studies reporting HEV infection in HSCT recipients used in data analysis

First author (country, year)	Patients, n	Type of HSCT	HEV Diagnostic Methods	Assay	Positive, n	Prevalence
Tang FF et al. (China, 2019)	177	Allo-HSCT	Anti-HEV IgM Anti-HEV IgG	EIA (MP Diagnostics ®)	5	2.82%
			HEV RNA	Commercial RT-qPCR	2	1.13%
Reekie I, et al. (England, 2018)	259	Allo/Auto-HSCT	HEV RNA	In-house RT-qPCR	1	0.39%
Ankcorn MJ, et al. (United Kingdom, 2018)	144	Allo-HSCT	HEV RNA	In-house RT-qPCR	3	2.08%
Willemse SB et al. (Netherlands, 2017)	130	Allo-HSCT	HEV RNA	Commercial RT-qPCR	5	3.85%
Verluis J, et al. (Netherlands, 2013)	328	Allo-HSCT	Anti-HEV IgM Anti-HEV IgG	EIA (Wantai ®)	2 41	0.61% 12.5%
			HEV RNA	In-house RT-qPCR	8	2.44%
Abravanel F, et al. (France, 2012)	88	Allo/Auto-HSCT	Anti-HEV IgM Anti-HEV IgG	EIA (Adaltis ®)	3 11	3.41% 12.5%
			Anti-HEV IgG	EIA (Wantai ®)	32	36.4%
			HEV RNA	In-house RT-qPCR	0	0.00%
Koenecke C, et al. (Germany, 2012)	52	Allo-HSCT	Anti-HEV IgG	EIA (Abbott ®)	3	5.77%
			HEV RNA	In-house Nested RT-PCR	0	0.00%

Here, we briefly resume the data from all included studies. In China, a study evaluated HEV infection in 177 haploidentical-HSCT recipients that presented unexplained elevated transaminases after transplant, identifying 7 patients with an acute/current HEV infection (2 positives for HEV RNA and 5 positives for anti-HEV IgM/IgG) (Tang et al. 2019). In the United Kingdom, two studies identified a total of 4 recipients with an acute HEV infection based in the presence of HEV RNA: in one study, 259 HSCT recipients (111 allo-HSCT, 145 auto-HSCT, and 3 CD34 top-up procedures) were evaluated and only 1 HEV RNA positive patient was identified (Reekie et al. 2018);

while the other study analyzed 144 allo-HSCT recipients and 3 patients were positive for HEV RNA (Ankcorn et al. 2018). In the Netherlands, two studies analysed HEV infection in HSCT: one study was performed in 130 allo-HSCT recipients with elevated alanine aminotransferase (ALT), identifying 5 HEV RNA positive patients (Willemse et al. 2017); the other study analyzed 328 allo-HSCT recipients transplanted over a 5-year period, identifying a total of 10 patients with a current infection (8 were positive for HEV RNA and 2 were positive for anti-HEV IgM) and 41 patients were seropositive for anti-HEV IgG (Versluis et al. 2013). In France, a study with 88 HSCT recipients (72 allo-HSCT and 16 auto-HSCT) revealed that none patient tested positive for HEV RNA, while 3 were positive for anti-HEV IgM (Abravanel, Mansuy, et al. 2012). Interestingly, this study found a seroprevalence of anti-HEV IgG of 12,5% (11/88) and 36,4% (32/88) in the same group of HSCT recipients when using two different enzyme immunoassays methods. In the study from Germany with 52 allo-HSCT recipients with elevated ALT, but without any specific cause of hepatitis, none of them tested positive for HEV RNA, while 3 were positive for anti-HEV IgG (Koenecke et al. 2012).

2.2. HEV infection prevalence analysis

We have performed an analysis of the prevalence of HEV infection in HSCT recipients according to the different approaches of diagnosis. The diagnosis of HEV positive cases is based on the identification of a patient with an HEV RNA positive sample (serum, plasma, blood or feces) or both anti-HEV IgM and IgG positive sample, nevertheless, the seroprevalence of the infection is referred to the detection of anti-HEV IgG only.

Considering the analysis of HEV RNA detection, a total of 1178 HSCT patients were studied and only 19 were positive, which gives an overall prevalence of 1.50% (95% CI: 0.70-2.60) with no significant difference between studies (p=0.090; Figure 9).

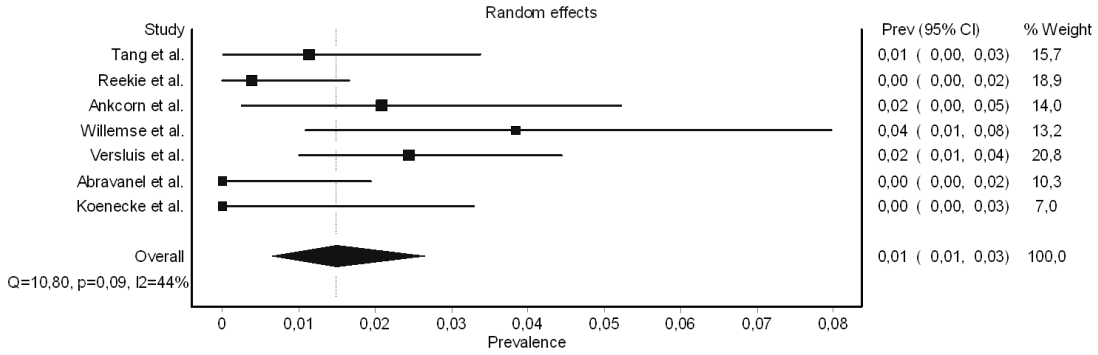

Figure 9. Forest plot of the overall prevalence based on the detection of HEV RNA

The analysis of anti-HEV IgM and IgG was performed separately: the detection of anti-HEV IgM as marker of acute infection was tested in a total of 593 HSCT patients with only 10 positive cases, giving an overall anti-HEV IgM prevalence of 2.00% (95% CI: 0.30-4.50; p=0.060; Figure 10); while the detection of anti-HEV IgG, a marker of past infection, was described in a total of 645 samples, 82 HSCT recipients were found positive to anti-HEV IgG giving an overall IgG seroprevalence of 11.4% (95% CI: 1.80-26.3), with statistically significant differences between the studies (p<0.001; Figure 11).

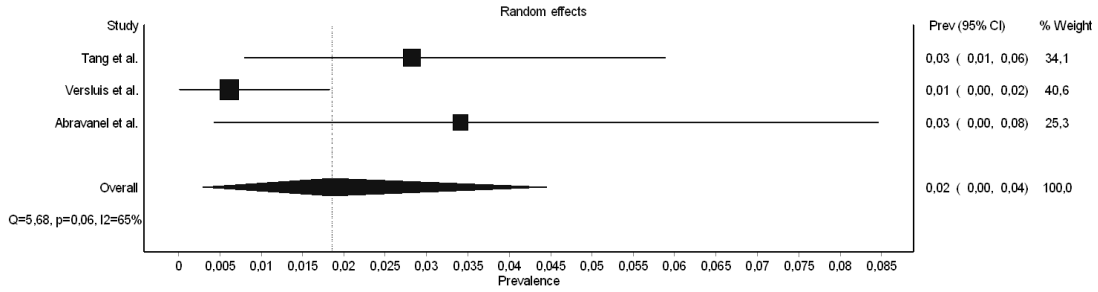

Figure 10. Forest plot of the overall prevalence based on the detection of anti-HEV IgM

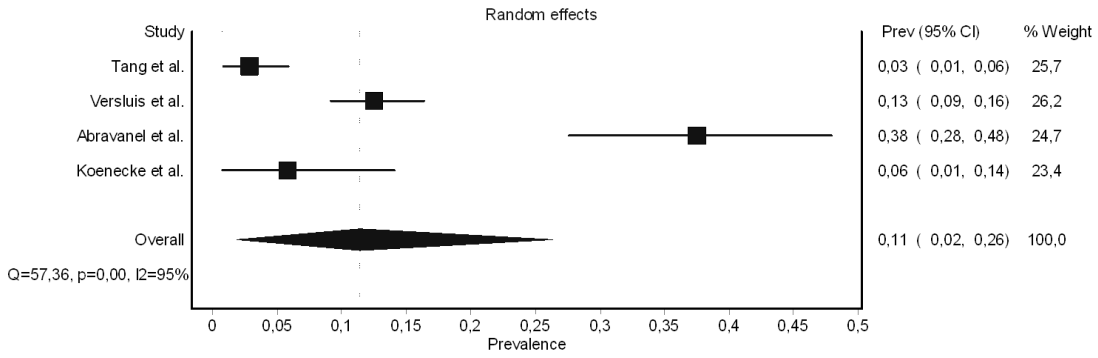

Figure 11 - Forest plot of the overall prevalence based on the detection of anti-HEV IgG

3. DISCUSSION

The evidence that most of the HEV-3 infections become chronic in immunocompromised hosts, especially those after transplantation, makes these patients an important group of study (Singh et al. 2018; W. Webb and Dalton 2019). Since the prevalence and incidence of HEV in HSCT recipients is largely unknown, we have performed a systematic review to understand the burden of HEV infection in this group of immunocompromised patients.

We found that there is a wide variation in HEV infection definition in literature, although in accordance with a recent surveillance report by the *European Centre for Disease Prevention and Control* (ECDC), concerning hepatitis E virus in Europe, a positive case is considered in a patient with an HEV RNA positive sample (serum, plasma, blood or feces) or both anti-HEV IgM and IgG positive sample (Aspinall et al. 2017). HEV RNA is detected usually between 2 and 8 weeks when viremia reaches its peak before strong declining, then, around 3 weeks after clinical symptoms, HEV RNA becomes undetectable, with the virus continuing to be shed in the stool for another 1 to 2 weeks. On the other hand, the anti-HEV IgM immune response remains detectable for 3–12 months and the IgG response reaches its peak four weeks later than IgM, remaining detectable for several years, although the exact duration of response remains uncertain (Nassim Kamar et al. 2017; W. Webb and Dalton 2019).

The fact that there is no standardized case definition, in addition to the heterogeneity in the analytical sensitivity of the commercial HEV assays, makes the comparison of studies more difficult. In this systematic review, all the seven studies performed the detection of HEV RNA, the most robust marker of acute/active infection (Abravanel, Mansuy, et al. 2012; Ankcorn et al. 2018; Tang et al. 2019; Willemse et al. 2017; Versluis et al. 2013; Koenecke et al. 2012; Reekie et al. 2018), while four performed also the detection of anti-HEV IgM/IgG (Abravanel, Mansuy, et al. 2012; Tang et al. 2019; Versluis et al. 2013; Koenecke et al. 2012). The studies that performed HEV RNA detection as the diagnostic method for HEV infection in HSCT recipients revealed similar results between them, with an overall prevalence of HEV infection of 1.5%. Furthermore, we found that the overall prevalence of anti-HEV IgM was 2.0%, a

comparable value to the reported by the detection of HEV RNA, which is expected since both are markers of acute infection.

The analysis of anti-HEV IgG showed an overall seroprevalence of 11.4% in HSCT patients, with significant differences between the results of the different studies, mainly due to the study from France which revealed a much higher prevalence when compared to the others studies (Abravanel, Mansuy, et al. 2012). These results are difficult to compare due to the differences in the sensitivities and specificities of anti-HEV IgG commercial immunoassays (Bendall et al. 2010; Pas et al. 2013; Avellon et al. 2015; Hartl et al. 2016). Moreover, seroprevalence is greatly influenced by food habits (World Health Organization 2010; Hartl et al. 2016) and to differences in culinary practices between countries/regions (Hartl et al. 2016). Reported anti-HEV IgG seroprevalence not only varies between countries but also within countries, with significant variance between regional areas (Hartl et al. 2016). Indeed, even seroprevalences in Europe are very difficult to compare in consequence of these serology limitations and very different rates have been reported across the continent, ranging from 1.3-52% (Lapa, Capobianchi, and Garbuglia 2015; Scotto et al. 2014; J.-M. Mansuy et al. 2011). In Finland, anti-HEV IgG seroprevalence was found to be 27.6% in general population(Kantala et al. 2009), while in Norway, Germany and Portugal reported seroprevalences were 11.4%, 16.8% and 16.3%, respectively (Olsøy et al. 2019; Faber et al. 2012; Nascimento et al. 2018). Furthermore, several countries such as Netherlands, Spain, France, and Southwest England have performed these studies in healthy blood donors reporting an anti-HEV IgG prevalence ranging from 16.0 to 26.7% (Slot et al. 2013; Sauleda et al. 2015; J. M. Mansuy et al. 2016; Harry R. Dalton, Stableforth, et al. 2008). These facts contribute to the assumption that the anti-HEV IgG prevalence does not reflect the spread of HEV in the HSCT population, which reinforces the importance of better characterization in this group of patients.

HSCT recipients are at higher risk of HEV infection, which could lead to chronic infection, and the diagnosis of HEV infection is highly recommended. Over the last years, some attention has been given to this group of immunocompromised patients, but there is still a small number of studies in HSCT as demonstrated in the present systematic review. Therefore, more studies are needed to increase our understanding of the epidemiology of HEV in HSCT recipients.

IV. STUDY II

STUDY II

This study was developed as an epidemiology study to characterize HEV prevalence in a retrospective cohort of allo-HSCT recipients.

1. MATERIALS AND METHODS

1.1. TYPE OF STUDY AND POPULATION

A retrospective study was performed in all patients with different malignancies who had undergone allo-HSCT at the *Bone Marrow Transplant Service* from the *Portuguese Oncology Institute of Porto (IPO Porto)* between January 2016 to December 2018 (n=196).

Patient clinical records were reviewed to retrieve demographic characteristics (gender, age), clinicopathological information (underlying disease, conditioning regimen, HLA match, and stem cell source) and were stored in a database with unique codification. All procedures were approved by the Institution Ethical Committee (ref CES IPO 129/019) and the study did not interfere with routine clinical procedures.

1.2. SAMPLES COLLECTION

Serum samples were collected retrospectively from the laboratory archives at the *Virology Service of IPO Porto* and used for both viral RNA detection and anti-HEV antibodies detection. All samples were stored at -20ºC/-80ºC prior use.

1.3. DETECTION OF ANTI-HEV ANTIBODIES

For the detection of both anti-HEV IgM and IgG in serum samples, the Wantai HEV-IgM and Wantai HEV-IgG enzyme-linked immunosorbent assay (ELISA) (Wantai, Biological Pharmacy Co., Beijing, China) were used. Wantai HEV-IgM and HEV-IgG ELISA is an assay for qualitative determination of IgM and IgG class antibodies to HEV in human serum or plasma samples.

The HEV-IgM kit is an immunocapture assay that uses solid phase antibodies against human immunoglobulin M proteins (anti-μ chain). The specific anti-HEV IgM is detected by the addition of recombinant HEV ORF2 antigens. Briefly, the patient's serum sample is added, and during the first incubation step, any IgM-class antibodies will be captured in the wells. After a washing step using a microplate washer (Bio-Rad plate washer PW40) (Bio-Rad Laboratories, Redmond, WA, USA), recombinant HEV ORF2 antigen conjugated to HRP is added. During the second incubation, the HRP-conjugated antigens will specifically react with HEV IgM antibodies. After another washing step, chromogen solutions are added into the wells, and in presence of (anti-μ) - (anti-HEV-

IgM) - (HEV Ag-HRP) immunocomplex, the colorless chromogens turn in a blue-colored product. The reaction is stopped with sulfuric acid, and the blue color turns yellow.

The HEV-IgG kit employs solid phase, indirect ELISA method for the detection of IgG-class antibodies to HEV. Briefly, polystyrene microwell strips are pre-coated with HEV recombinant, and anti-HEV specific antibodies will be bound to the solid phase pre-coated HEV antigens during the first incubation step. After a washing step in a microplate washer (Bio-Rad plate washer PW40) (Bio-Rad Laboratories, Redmond, WA, USA), rabbit anti-human IgG antibodies conjugated to horseradish peroxidase (HRP-Conjugate) are added. During the second incubation step, these HRP-conjugated antibodies will be bound to any antigen-antibody (IgG) complexes previously formed. After another washing step, chromogen solutions containing tetramethylbenzidine (TMB) and urea peroxide are added to the wells and in presence of the antigen-antibody-anti-IgG (HRP) immunocomplex, the colorless chromogens are hydrolyzed by the bound HRP conjugate to a blue colored product. The reaction is stopped with sulfuric acid, and the blue color turns yellow.

In both assays, positive and negative controls were included in every ELISA run. HEV-IgM/HEV-IgG positive samples develop a yellow color, while wells containing negative samples remain colorless. The amount of color intensity was measured at 450nm in a Bio-Rad plate reader PR4100 (Bio-Rad Laboratories, Redmond, WA, USA) (software Magellan v 7.0 for PR4100) and is proportional to the amount of antibody captured in the wells. The results are calculated by relating each specimen absorbance (A) value to the Cut-off (C.O.), expressed as ratio to the cut-off value recommended by the manufacturer (A/C.O.), with a positive sample being A/C.O.≥1.

1.4. DETECTION OF HEV RNA

Viral Nucleic Acid extraction was performed from serum samples using *STARMag 96 X 4 Universal cartridge kit* (Seegene, Korea) on the Hamilton MicroLab STARlet IVD, according to recommended manufacturers' instructions.

HEV was identified using Real-Time Reverse Transcription-Polymerase Chain Reaction (RT-qPCR) technique. HEV RNA detection was performed using two protocols: **1)** RT-qPCR assay that targets the ORF 2/3 overlapping region using primers and probes from *Jothikumar et al. 2006*; and **2)** RT-qPCR assay that target the ORF 2 region using the *HEV ELITe MGB® Kit* (ELITech Group®, Puteaux, France) in combination with *ELITe*

InGenius® equipment. All samples were tested with protocol 1, while protocol 2 was used as a confirmation protocol, only tested3. in anti-HEV IgM and IgG positive samples.

1.4.1. Protocol 1

A protocol for the detection of HEV RNA by one-step real-time RT-PCR assay that targets the ORF2/3 overlapping region was used as previously described (Jothikumar et al. 2006)**.** The reaction was performed with 1x *iTaq Universal Probes reaction mix* (Bio-Rad Laboratories, Redmond, WA, USA), 0.5µL of *iScript reverse transcriptase*, 500 nmol of each primer (forward JVHEVF 5' – GGTGGTTTCTGGGGTGAC – 3' and reverse JVHEVR 5' – AGGGGTTGGTTGGATGAA – 3'), 250 nmol of probe (JVHEVP FAM-TGATTCTCAGCCCTTCGC-MGB-NFQ) and 5 µL of extracted nucleic acid, made up to 20 µL with RNase-free water.

Thermal profile consisted of reverse transcription at 50°C for 10min, followed by reverse transcriptase (RT) inactivation and initial denaturation of cDNA at 95°C for 3min and then 45 cycles of amplification with denaturation at 95°C for 15s, and annealing/extension at 55°C for 15s. The fluorescence signal was acquired on the FAM channel at the end of each cycle of annealing/extension.

RT-qPCR reactions were performed on a CFX Connect Real-Time System (Bio-Rad Laboratories, Redmond, WA, USA). All the steps during the preparation of the reaction mix and addition of samples were performed on ice. To minimize potential contamination, mastermix preparation and addition of the samples were made in a separate room, and samples weren't taken into the RT-PCR set-up room. Regarding amplification quality, positive and negative controls were included in each run: as negative control we used RNAse free water; and as positive control we used an HEV DNA standard of an oligonucleotide of the ORF 3 region of the HEV genome with 76bp of length: 5' – GGC GGT GGT TTC TGG GGT GAC CGG GTT GAT TCT CAG CCC TTC GCA ATC CCC TAT ATT CAT CCA ACC CCC TTC G – 3' (Applied Biosystems, Foster City CA, USA).

1.4.2. Protocol 2

The *HEV ELITe MGB® Kit* (ELITech Group®, Puteaux, France) commercial assay consists of a one-step method, with Reverse Transcription and Real-Time amplification reaction that targets the ORF2 region of the HEV genome using the *ELITe InGenius®* equipment (ELITech Group®, Puteaux, France). The HEV specific probe with ELITe MGB® technology is labeled with FAM fluorophore and is activated when hybridized with the specific product of the HEV amplification reaction. The assay was performed according to manufacturer instructions**.** Briefly, one reaction contained 5µL of HEV PreMix, 15µL of PCR MasterMix, 0.3µL of RT EnzymeMix and 10µl of extracted nucleic acid. Thermal profile consisted of denaturation at 95°C for 5min and then 40 cycles of amplification with denaturation at 95°C for 15s, annealing at 56°C for 30s and elongation at 72°C for 20s. Positive and negative controls were used: as negative control we used molecular grade water, and as positive controls we used a positive sample for HEV RNA. All samples were added a CPE as Internal Control.

1.5. Variable Definition

Hematological diseases were classified into the following categories: 1) Acute leukemia (acute myeloid leukemia, acute lymphoid leukemia); 2) Chronic myeloproliferative disorders (chronic myeloid leukemia,); 3) Chronic lymphoproliferative disorders (T-cell lymphoma, multiple myeloma, chronic lymphoid leukemia, Hodgkin's lymphoma, non-Hodgkin's lymphoma, plasmacytoid dendritic cell neoplasm); 4) Myelodisplasic syndrome (myelodysplastic/myeloproliferative syndrome); 5) Aplastic anemia (Fanconi Anemia, spinal cord aplasia/hypoplasia); and 6) others (hemoglobinopathies, paroxysmal nocturnal hemoglobinuria, primary immunodeficiency, familial hemophagocytic lymphohistiocytosis).

The primary endpoint of this study was to characterize HEV infection prior to allo-HSCT. A case of HEV infection was considered as a patient with an HEV RNA positive sample and/or by showing both anti-HEV IgM and IgG positive sample. A case of past HEV infection was defined as anti-HEV IgG positive and anti-HEV IgM negative. A naive case was defined as HEV RNA negative and anti-HEV IgM and IgG negative. We have managed to classify the status of infection according to Table 3 and considering the HEV natural infection data (Nassim Kamar et al. 2017).

Table 3. Clinical interpretation according to biomarkers

Anti-IgM	Anti-IgG	HEV RNA	Interpretation
Neg	Neg	Neg	Naive
Neg	Pos	Neg	Past HEV infection
Pos	Pos	Neg	Recent/Active HEV infection
Neg	Neg	Pos	

The outcomes analyzed were GVHD development, disease recurrence, and mortality. Acute GVHD was defined as occurring during the 100 days after transplant, and chronic GVHD as occurring after 101 days from transplant. Progression free survival (PFS) was defined as the time between the day of HSCT and the day of relapse. Overall survival (OS) was defined as the time between the day of HSCT and the day of the last visit to hospital or the day of death.

1.6. Statistical Analysis

Statistical analysis was performed with IBM® SPSS Statistics 20 software (Statistical Package for Social Sciences version 24.0) for Mac. Chi-square ($\chi 2$) or Fisher's exact test was used to compare the categorical variables with a 5% significance level. Continuous variables were tested for normality with the Kolmogorov-Smirnov Test. Univariate and multivariate logistic regression models were used to estimate odds ratio (OR) and the corresponding 95% confidence intervals (CIs) as a measure of association between the categorical variables and the risk of HEV infection. Multivariate analyses were performed by logistic regression adjusting for covariates: gender, median age at allo-HSCT, stem cell source, conditioning regimen, and when applicable, GVHD and HEV infection. The Kaplan-Meier method, adjusted by log-Rank and Breslow tests, was used to calculate the association between HEV infection and overall survival.

2. RESULTS

2.1. Characterization of the population

Clinical variables collected from patients are summarized in Table 4. Briefly, in our study, 52% of patients who underwent allo-HSCT were male, and the median age at time of transplantation was 42±17.1 years (range: 0-68). Most patients underwent allo-HSCT for: acute leukemia (n=103, 52.6%); followed by chronic lymphoproliferative diseases (n=31, 15.8%) and myelodysplastic/myeloproliferative diseases (n=30, 15.3%); aplastic anemia (n=14, 7.1%); chronic myeloproliferative diseases (n=8, 4.1%); and other types of diseases (n=10, 5.10%). Peripheral blood was the first choice for stem cell source in 172 cases (87.8%), followed by bone marrow (n=23, 11.7%) and cord blood (n=1, 0.5%). Identical HLA was obtained in 144 (73.5%) allo-HSCT recipients, with 52 cases (26.5%) using non-identical HLA. One hundred and twenty-eight patients (65.3%) underwent a non-myeloablative/reduced-intensity conditioning regimen prior to transplantation, against sixty-eight (34.7%) recipients who underwent myeloablative conditioning regimen.

Table 4. Baseline characteristics of the study population

Characteristics	Total, n (%)
Gender	
Female	94 (48%)
Male	102 (52%)
Age	
<18 years old	24 (12.2%)
≥18 years old	172 (87.8%)
Underlying Disease	
Acute Leukemia	103 (52.6%)
Chronic Myeloproliferative Diseases	8 (4.1%)
Chronic Lymphoproliferative Diseases	31 (15.8%)
Myelodysplastic/Myeloproliferative Diseases	30 (15.3%)
Aplastic Anemia	14 (7.1%)
Others	10 (5.1%)
Stem Cell Source	
Bone Marrow	23 (11.7%)
Peripheral Blood	172 (87.8%)
Cord Blood	1 (0.5%)
HLA	
Matched/Related	144 (73.5%)
Mismatched/Unrelated	52 (26.5%)
Conditioning Regimen	
Myeloablative	68 (34.7%)
Non-Myeloablative/ Reduced Intensity	128 (65.3%)

2.2. HEV infections

From the 196 allo-HSCT recipients tested for HEV infection, 8 were considered positive cases for HEV infection (Table 5), with a prevalence of 4.1%. All samples were tested for HEV RNA ORF 2/3 overlapping region (protocol 1) and 6 were positive, giving an HEV RNA prevalence of 3.1%, however, anti-HEV IgM was negative in these patients. Simultaneously, all 196 samples were tested for the presence of anti-HEV IgG, and 39 were positive, giving an anti-HEV IgG prevalence of 19.9%. Of these, 2 patients were also IgM positive, giving an anti-HEV IgM prevalence of 5.13%, though HEV viremia could not be confirmed in these cases by RT-qPCR with both methods – Figure 12**.** Of the 6 HEV RNA positive cases, 2 were also anti-HEV IgG positive, which could mean the patients had a possible reinfection.

Regarding the HEV status according to the infection characteristics, we observed that the majority of allo-HSCT recipients had never come in contact with HEV (n=153, 78.1%), while 39 (19.9%) allo-HSCT recipients had a past HEV infection; 6 (3.1%) had a recent/active HEV infection, and 2 (1.0%) were suspected of reinfection.

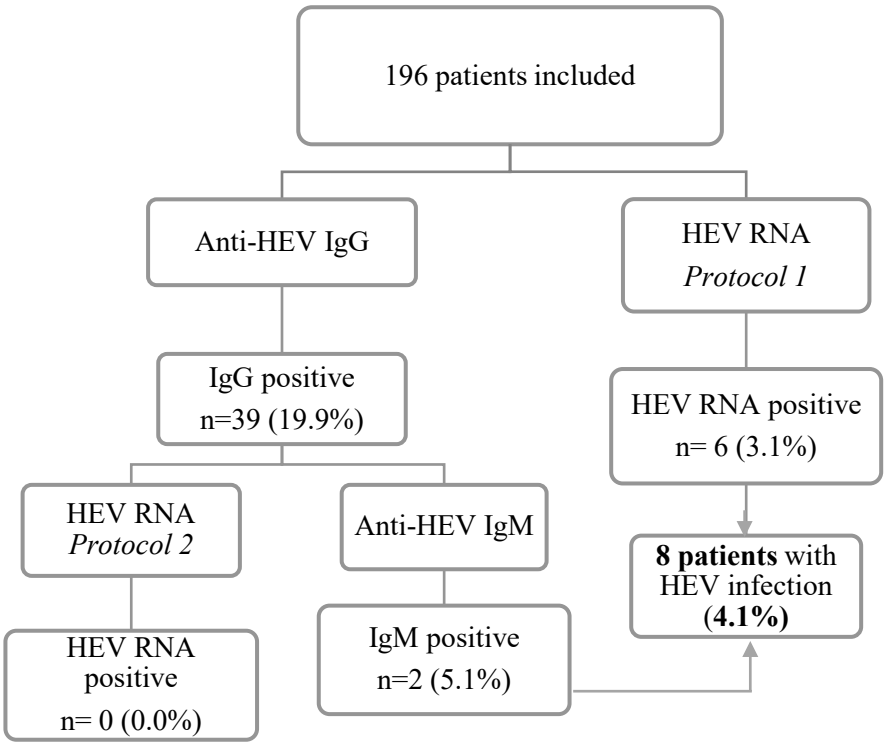

Figure 12. Overview of sample selection and study results

Table 5. Patient characteristics of hepatitis E confirmed patients (n=8)

Patient	Gender	Age, years	Underlying Disease	Stem cell source	Conditioning regimen	GVHD	At time of allo-HSCT			ALT * Median (range) U/L	ALT ** Median (range) U/L	Alive at end of follow-up
							HEV RNA	IgM	IgG			
A	F	59	Spinal cord aplasia	PB	RIC	None	-	+	+	69 (36-200)	37 (11-212)	No
B	M	50	Myelodysplastic/myeloproliferative syndrome	PB	RIC	Acute and Chronic	-	+	+	13 (11-24)	17 (5-104)	No
C	M	20	Spinal cord aplasia	PB	RIC	Acute and Chronic	+	-	+	21 (14-64)	30 (13-198)	Yes
D	M	30	Acute lymphoid leukemia	PB	RIC	Acute	+	-	-	22 (13-191)	18 (6-243)	Yes
E	M	51	Myelodysplastic/myeloproliferative syndrome	PB	Myeloablative	Acute	+	-	+	85 (51-186)	41 (26-146)	Yes
F	F	50	Acute myeloid leukemia	PB	RIC	Chronic	+	-	-	56 (14-108)	70 (12-5628)	Yes
G	M	21	Acute lymphoid leukemia	PB	RIC	None	+	-	-	17 (11-20)	26 (13-257)	Yes
H	F	8	Spinal cord aplasia/hypoplasia	CB	RIC	Acute and Chronic	+	-	-	36 (16-89)	55 (15-282)	Yes

+, positive; -, negative; ALT, Alanine Transaminase; CB, cord blood; F, female; M, male; PB, peripheral blood; RIC, reduced intensity regimen

* Median vale of ALT in the pre-transplant period

** Median vale of ALT in the post-transplant period

The median age of the 8 HEV infected patients was 40 years (range: 8-59) at time of transplantation, including 5 (62.5%) males and 3 (37.5%) females - Table 5. The stem cell source for 7 patients was peripheral blood (87.5%) except from patient H that used cord blood (12.5%), and 7 patients received a reduced-intensity regimen except for one patient that receive a myeloablative regimen. GVHD was present in 6 patients (75%). The patients that were both anti-HEV IgM and IgG positive died months after transplantation.

2.3. Patients Outcome

In our study, GVHD was observed in a total of 130 patients (66.3%), of which 113 (86.9%) had acute GVHD and 45 (34.6%) had chronic GVHD. The average time until the appearance of GVHD was 1.7 ±2.7 months (median:1.00; range: 0-16), with an average time until the appearance of chronic GVHD of 11.2 ±8.0 months (median: 8.00; range:3-30).

The overall PFS was of 16.8 ±12.4 months (median 12.0; range: 0-44) – Figure 13a. In addition, at the end of the follow-up period, 121 patients (61.7%) were still alive, with an OS of 17.6±12.2 months (median 14.50; range: 0-44) – Figure 13b.

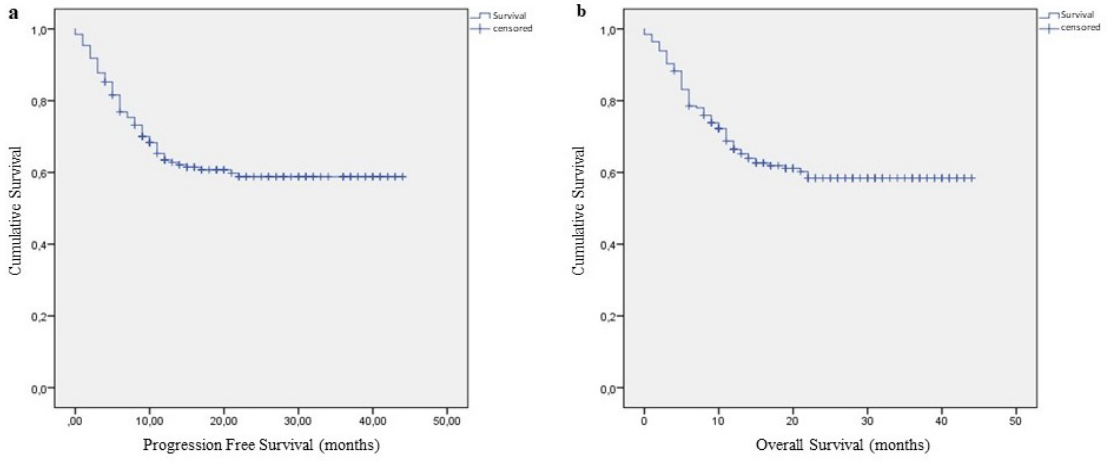

Figure 13. Graphics for progression free survival and overall survival in allo-HSCT patients. Kaplan–Meier plots with log-rank test estimate a) progression free survival and b) overall survival of allo-HSCT patients

2.4. Correlation between HEV infection status and patient characteristics

The statistical analysis revealed no significant differences in HEV infection prevalence according to gender, age, stem cell source, conditioning regimen, and presence of GVHD– Table 6. However, past infection is higher in adult males, with myeloablative regimen and in the absence of GVHD. Recent/active infection is slightly higher in men, and prevalent among adults with peripheral blood as stem cell source and matched HLA, and when GVHD is present. All recent/active infection cases were observed in non-myeloablative/reduced-intensity conditioning regimen, except for one case.

Table 6. Analysis of HEV infection among allo-HSCT recipients

	Naive n, (%)	**Past infection n, (%)**	**Recent/Active infection n, (%)**	***P***
Gender, n =196				
Female, n=94	75 (79.8%)	16 (17.0%)	3 (3.2%)	0.574
Male, n=102	78 (76.5%)	19 (18.6%)	5 (4.9%)	
Age, n=196				
<18 years old, n=24	22 (91.6%)	1 (4.2%)	1 (4.2%)	0.272
≥18 years old, n=172	131 (76.2%)	34 (19.8%)	7 (4.0%)	
Stem Cell Source, n=196				
Bone Marrow, n=23	19 (82.6%)	3 (13.1%)	1 (4.3%)	0.981
Peripheral Blood, n=172	133 (77.3%)	32 (18.6%)	7 (4.1%)	
Cord Blood, n=1	1 (100.0%)	0 (0.0%)	0 (0.0%)	
HLA, n=196				
Matched/Related, n=144	112 (77.8%)	27 (18.7%)	5 (3.5%)	0.812
Mismatched/Unrelated, n=52	41 (78.8%)	8 (15.3%)	3 (5.7%)	
Conditioning Regimen, n=196				
Myeloablative, n=68	52 (76.5%)	15 (22.0%)	1 (1.5%)	0.216
Non-Myeloablative/Reduced Intensity, n=128	101 (78.9%)	20 (15.6%)	7 (5.5%)	
GVHD, n=196				
Absence, n=66	48 (72.7%)	16 (24.3%)	2 (3.0%)	0.302
Present, n=130	105 (80.8%)	19 (14.6%)	6 (4.6%)	
Acute				0.809
Present, n=113	92 (81.4%)	16 (14.2%)	5 (4.4%)	
Absence, n=17	13 (76.5%)	3 (17.6%)	1 (5.9%)	
Chronic				0862
Present, n=45	35 (77.8%)	7 (15.6%)	3 (6.6%)	
Absence, n=85	70 (82.4%)	12 (14.1%)	3 (3.5%)	
Relapse, n=180				
Absence,n=161	127 (78.9%)	28 (17.4%)	6 (3.7%)	0.851
Present, n=19	16 (84.2%)	3 (15.8%)	0 (0.0%)	

3. DISCUSSION

There is much evidence that recipients of allo-HSCT are at increased risk of opportunistic bacterial, fungal, and viral infections. HEV infections are being suggested as one potential risk factor for allo-HSCT recipients, and the fact that most of the HEV-3 infections become chronic in immunocompromised hosts makes these patients an important group of study (Gyurkocza and Sandmaier 2014; Singh et al. 2018; W. Webb and Dalton 2019). To revise this topic, we have performed a retrospective study to understand the burden of HEV infection in a cohort of allo-HSCT recipients in a single center in Porto.

We have started by screening all patients in samples prior transplant. From the 196 allo-HSCT recipients tested for HEV infection, an HEV infection prevalence of 4.1% was obtained, with 6 positive cases for HEV RNA, the most robust marker of acute/active infection, and 2 positive cases for both anti-HEV IgM and IgG, concluding that these 8 patients were with a recent/active infection at time of transplantation. Although, the 2 positive cases for both anti-HEV IgM and IgG were not RNA positive, which can be explained by the different time window of the biomarkers. HEV RNA is detectable in the blood for 4 weeks (Wen et al. 2015), and anti-HEV IgM becomes detectable on average at the 8 week and persist for 6–9 months (S. Huang et al. 2010), with the anti-HEV IgG response four weeks later than IgM, and persists for several years after the infection (M. S. Khuroo et al. 1993; Nassim Kamar et al. 2017).

After follow-up, it was possible to assess information about the clinical course of these patients. Patient A was a 59 years old woman with spinal cord aplasia that died of allo-HSCT complications 3 months after transplantation, without evidence of GVHD. Patient B was a man with 50 years old with myelodysplastic/myeloproliferative syndrome that died 12 months after transplantation. The patient had chronic GVHD grade II with skin and hepatic involvement. Patient C was a young male diagnosed with spinal cord aplasia that developed acute skin GVHD grade II that evolved to chronic GVHD. Patient D was a 30 years old male with acute lymphoid leukemia that developed acute GVHD grade II with skin involvement. Patient E was a man of 51 years old diagnosed with myelodysplastic/myeloproliferative syndrome that developed acute skin GVHD grade II. Patient F was a 50 years old female with acute myeloid leukemia that developed chronic GVHD grade I/II with pulmonary, digestive and hepatic involvement. The patient did a liver biopsy and the morphological aspects described favor chronic GVHD. Patient G was

a 21 years old man with acute lymphoid leukemia that showed no evidence of development of GVHD at end of follow-up time. Patient H was an 8 years old girl diagnosed with spinal cord aplasia/hypoplasia that developed acute GVHD grade I with skin, digestive and hepatic involvement that evolved to chronic hepatic GVHD, which was confirmed by liver biopsy.

Of the 6 HEV RNA positive cases, 2 were also anti-HEV IgG positive, suggesting the patients had a possible reinfection, although we could not unequivocally demonstrate it. Reinfection with HEV is reported (Abravanel et al. 2014; S. A. Baylis et al. 2015; Schemmerer et al. 2017), being identified by a rapid increase in anti-HEV IgG levels, with HEV RNA becoming detectable, however infection is probably not symptomatic, since symptoms decrease during subsequent infections (Pischke et al. 2016; J. Zhang et al. 2014).

Two of the six allo-HSCT recipients that were positive by protocol 1, an RT-qPCR that targets the overlapping ORF2/3 region, were retested with protocol 2, an RT-qPCR that targets the ORF2 region that encodes the capsid protein and was negative by the protocol 2. This disagreement is not unusual and is consistent with previous reports that have shown that the sensitivity of real-time assays is highly dependent on the target region of the HEV genome, since ORF3 region is described as more conserved than ORF2, is therefore more sensitive and reliable for HEV RNA detection (Mokhtari et al. 2013; Ward et al. 2009). Despite this, a false positive result from the RT-qPCR for ORF2/3 overlapping region cannot be ruled out.

Despite our results, we are aware of some limitations in our study, as it was a single center, retrospective survey, that addressed only the prior-transplant period. However, the cohort of allo-HSCT patients from a Portuguese regional center provides a sound basis for estimating the risk and course of HEV in allo-HSCT recipients.

V. GENERAL DISCUSSION AND CONCLUSION

By comparing results from our study (Study II) with the data from the systematic-review (Study I), we observed that in our population there is a similar HEV RNA prevalence (3.1% vs 1.5%), and as far as anti-HEV IgM detection, a comparable value to the reported by the detection of HEV RNA was obtained (5.1%), which is expected since both are markers of acute infection.

An anti-HEV IgG prevalence of 19,9% was obtained, meaning that 19.9% of the allo-HSCT recipients had a past HEV infection prior transplantation. Recently, it has been reported that 16.3% of the general population in Portugal tested positive for anti-HEV IgG (Nascimento et al. 2018). The difference of HEV seroprevalence can be explained by the fact that the Portuguese survey was performed in healthy population, and our study was performed in an immunocompromised population, however it can also be explained by the different immunoassays used, since it is known that the commercially available assays for the detection of HEV-specific antibodies vary in sensitivity and specificity (Bendall et al. 2010; Pas et al. 2013; Avellon et al. 2015; Hartl et al. 2016). Results from the study I showed an overall anti-HEV IgG prevalence of 11.4% in HSCT patients. Again, these results are difficult to compare due to the different immunoassays used, however, two studies used the same immunoassay as we used and obtained an anti-HEV IgG prevalence of 12.5% and 36.4%, respectively (Versluis et al. 2013; Abravanel, Mansuy, et al. 2012).

This difference in seroprevalence rates can also be explained by food habits (World Health Organization 2010; Hartl et al. 2016) and differences in culinary practices between countries/regions (Hartl et al. 2016). Reported anti-HEV IgG seroprevalence varies between and within countries, with significant variance between regional areas (Hartl et al. 2016). Indeed, even seroprevalences in Europe are very difficult to compare in consequence of these serology limitations and very different rates have been reported across the continent, ranging from 1.3-52% (Lapa, Capobianchi, and Garbuglia 2015; Scotto et al. 2014; J.-M. Mansuy et al. 2011).

In conclusion, this study shows that recipients of allo-HSCT are at risk for HEV infection, although with relatively low risk, that could lead to chronic infection. Therefore, immunocompromised patients should be screened prior to transplantation, and during episodes of liver enzyme abnormalities post-transplantation. Thus, HEV RNA RT-PCR testing is the preferred diagnostic method in these immunocompromised patients. Moreover, HEV should be included in the differential diagnosis of liver GVHD and drug-

induced liver injury, because of the largely overlapping picture with respect to liver enzyme abnormalities. Over the last years, some attention has been given to this group of immunocompromised patients, but there is still a small number of studies, therefore, more studies are needed to increase our understanding of the epidemiology of HEV in HSCT recipients.

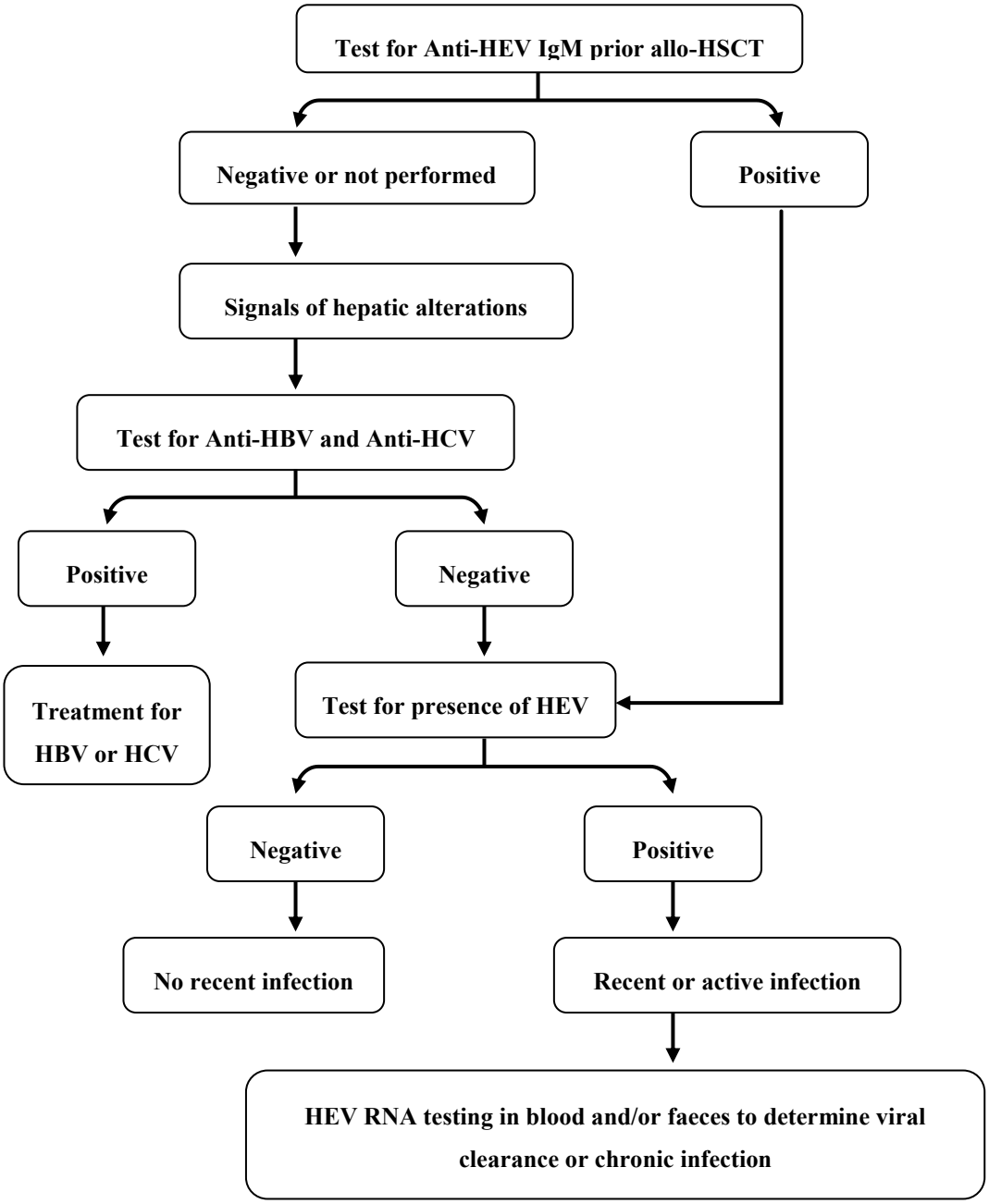

Figure 14. Consideration of possible screening strategies for identifying HEV in allo-HSCT

VI. FUTURE WORK

Considering the positive cases of HEV infection, thorough research of the allo-HSCT recipients' history is necessary to revise all clinical data, like times of liver enzyme abnormalities, so we can perform a proper follow-up of the patients. This follow-up is important to assess if the HEV RNA is still present in positive cases, with acute infections becoming chronic. A follow-up of the seropositive patients to determine if seroconversion occurred is also necessary.

We are currently doing a screening of HEV RNA in liver biopsies, when available, in the context of GVHD. A screening of HEV RNA in all patients at time of liver enzyme abnormalities should also be performed.

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APPENDIX

Appendix I.

Institution Ethical Committee

IPOPORTO
INSTITUTO PORTUGUÊS DE ONCOLOGIA DO PORTO FG, EPE

Exmo. Senhor,
Prof. Doutor Manuel Teixeira
Diretor do Centro Investigação
IPO Porto FG - EPE

Ref. CES. 129/019
Porto, 12 de abril de 2019
Assunto: **Avaliação de Pedido de Parecer**

Exmo. Sr. Prof. Dr. Manuel Teixeira,

Cumpre-me remeter a V/Exa. o pedido de parecer dirigido a esta CES sobre o pedido de realização de projeto de investigação intitulado ***"Hepatitis E virus (HEV) in hematopoietic stem cell transplant recipients"***, tendo como Investigador Principal a **Dr. Hugo Sousa,** foi avaliado em reunião ordinária da Comissão de Ética a 11 de abril de 2019, emitindo-se o parecer anexo.

Respeitosos cumprimentos,

Dr. Artur Lima Bastos
Presidente da CES – IPO Porto EPE

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Assunto: Avaliação do pedido realização de projeto investigação ***"Hepatitis E virus (HEV) in hematopoietic stem cell transplant recipients"***

Investigador Principal: **Dr. Hugo Sousa**

Data: 11 de abril de 2019

PARECER

É parecer desta CES, não existir impedimento de natureza ética ao desenvolvimento do referido estudo de Investigação, garantida a existência de Consentimento Informado para uso de amostras para fins de investigação.

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Appendix II. Journal Article – Study I

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Hepatitis E virus in hematopoietic stem cell transplant recipients: A systematic review

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ABSTRACT

Background: In developed countries, Hepatitis E virus (HEV) infections, especially by HEV-3, are frequently associated with asymptomatic infection or self-limiting acute hepatitis, although it has been described as a cause of chronic infection, especially in immunocompromised hots. Hematopoietic stem cell transplant (HSCT) recipients have been recognized as an important risk group for HEV infection due to their prolonged immunosuppression state.

Objectives: We aimed to perform a systematic review of published data to evaluate HEV infection prevalence among HSCT recipients.

Study Design: Literature search was performed concerning published manuscripts regarding 'hepatitis E virus AND stem cell transplantation' following the Preferred Reporting of Systematic Reviews and MetaAnalyses (PRISMA) guidelines. Statistical analysis was performed using the MetaXL software to estimate the overall prevalence of HEV infection according to the different diagnostic approaches (HEV RNA and anti-HEV IgM and/or IgG detection).

Results: A total of 7 manuscripts were included for data analysis, with 6 studies performed in Europe and 1 study in China. Regarding HEV RNA detection, the overall HEV infection prevalence was 1.50% (95% CI: 0.70–2.60). The overall anti-HEV IgM seroprevalence was 2.00% (95% CI: 0.30–4.50), and anti-HEV IgG was 11.4% (95% CI: 1.80–26.3).

Conclusions: This systematic review reveals that the overall prevalence of HEV infection in HSCT patients differ according to the diagnostic, thus emphasizing the need of more studies to increase the data regarding prevalence and incidence in HSCT recipients.

1. Background

Hepatitis E virus (HEV) was recently recognized as the most common cause of acute viral hepatitis worldwide, with the World Health Organization (WHO) estimating 20 million infections, > 3 million acute cases, and > 57,000 HEV-related deaths, annually [1,2]. In industrialized countries, hepatitis E was considered a rare disease until the discovery of the new HEV genotypes that turned this infection a concern of public health. In fact, four major HEV genotypes infect humans: genotypes 1 (HEV-1) and 2 (HEV-2), that are transmitted through the fecal-oral route via fecal contaminated water, being prevalent in areas of poor sanitation, such as in developing countries [3,4]; and genotypes 3 (HEV-3) and 4 (HEV-4) that are zoonotic viruses transmitted to humans mainly through undercooked pork and boar meat and

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contact with pigs [3–8], although, the transfusion of blood products has also been recently recognized as a risk factor [9–11]. HEV-3 is today recognized as the main cause of sporadic autochthonous cases in industrialized countries [3,4] and the numbers show that the incidence of reported cases has been increasing [12–17].

In Europe, HEV infections are mainly caused by HEV-3, a genotype that causes asymptomatic infection or self-limiting acute hepatitis in healthy individuals, although it can lead to chronic infection with rapidly progressive cirrhosis in immunosuppressed patients, such as individuals with HIV, hematological malignancies, or transplant-related patients [18–21]. Solid organ transplant (SOT) recipients and hematopoietic stem cell transplant (HSCT) recipients are an important group at risk for HEV infection due to their prolonged immunosuppression state, that increases the risk for developing chronic infection [19]. Patients undergoing allogeneic-HSCT (allo-HSCT) have in general a higher risk for viral infections and higher incidence of graft-versus-host disease (GVHD) than autologous-HSCT (auto-HSCT) [22,23]. In allo-HSCT recipients, progression to chronic infection may be favored by the severity of immunosuppression, which results in impaired immune reconstitution, including insufficient lymphocyte recovery, that are risk factors for post-transplantation infections [24–26]. Moreover, HSCT recipients have a high transfusion burden, which is a problem particularly in countries that have not introduced HEV screening in blood donations [22,27,28].

The evidence that most of the HEV-3 infections become chronic in immunocompromised hosts, especially those after transplantation, makes these patients an important group of study [29,30]. In SOT recipients, the reported acute HEV infection prevalence is 1–3%, with 47–83% of the patients developing chronic hepatitis [31–33], however, the prevalence and incidence of HEV in HSCT recipients is largely unknown.

2. Objectives

In the present study, we aim to summarize published data regarding HEV infections in HSCT recipients by performing a systematic review of the literature.

3. Study Design

3.1. Literature search and study selection

Preferred Reporting of Systematic Reviews and MetaAnalyses (PRISMA) guidelines were followed in the preparation of this systematic review. Different queries, including MeSH terms, were tested and the literature search was performed with the query that obtained more representative manuscripts: 'hepatitis E virus AND stem cell transplantation'. PubMed and Scopus databases were searched, independently by two of the authors (SC and CC), for published manuscripts on 31st January 2019 without restrictions on time period, sample size or population.

The eligibility criteria applied to studies were: 1) HEV infection (present or past) identified by the presence of HEV RNA and/or HEV-specific antibodies (IgM/IgG) in tested samples; 2) HSCT recipients; 3) provide prevalence data. The exclusion criteria applied were: 1) duplicate data; 2) other types of manuscripts (reviews, case reports, comments or letters to the editor); 3) no access to abstracts and/or full texts; and 4) other languages rather than English, Spanish or Portuguese. Manuscript titles and abstracts were screened according to the eligibility criteria and selected manuscripts were fully reviewed for data extraction (author, publication date, country, population, age range, type of HSCT, HEV detection methods and the number of positive and negative cases). All manuscripts were reviewed independently by two of the authors (SC and CC) with disagreements mediated by the senior researcher (HS).

3.2. Statistical analysis

All data was inserted in a database that was used for prevalence analysis and comparison between studies. Prevalence analysis was performed using the *MetaXL* program version 5.3 (EpiGear International, Sunrise Beach, Queensland, Australia). The overall prevalence of HEV infection was estimated using the different approaches of diagnosis (HEV RNA or anti-HEV IgM/IgG detection) in HSCT recipients pooling the study data using the random effects model. The random effects model was used since a considerable heterogeneity among studies was expected, due largely to the different settings (populations, types of patients, age, gender, diagnostic methods) in which studies were conducted. The double arcsine transformation method was used for variance stabilization [34] considering a 95% confidence interval and a 5% statistical significance level ($p < 0.050$).

4. Results

4.1. Study selection and description

The literature search retrieved a total of 73 manuscripts from both databases, and after duplicates removal, a total of 54 records were screened (Fig. 1). After applying inclusion/exclusion criteria, 41 records were excluded: language (n = 1), reviews (n = 18), other types of articles such as Case Reports and Letter to the Editor (n = 9), and studies not related to HSCT patients or HEV detection (n = 13). A total of 13 full-text articles were assessed for full-reading of which 6 were excluded: 1 review, 4 case reports and 1 was not performed in HSCT patients. The bibliography of the selected manuscripts was reviewed to identify any new publications and no other article was added to the analysis.

After the full revision process, we included 7 manuscripts for data analysis [18,21,35–39] (Table 1). Overall, these 7 studies included a total of 1178 HSCT patients from different countries: six studies were performed in Europe (United Kingdom, Netherlands, France, and Germany) [18,21,36,36,37,38,39] and one study in China 35]. These studies evaluated the prevalence of HEV infection based on the detection of HEV RNA (n = 7) and/or the presence of anti-HEV IgM (n = 3)/IgG (n = 4). Phylogenetic analysis was performed in only three of these seven studies, revealing only HEV-3 genotype [21,37,39].

Here, we briefly resume the data from all included studies. In China, a study evaluated HEV infection in 177 haploidentical-HSCT recipients that presented unexplained elevated transaminases after transplant, identifying 7 patients with an acute/current HEV infection (2 positives for HEV RNA and 5 positives for anti-HEV IgM/IgG) [35]. In the United Kingdom, two studies identified a total of 4 recipients with an acute HEV infection based in the presence of HEV RNA: in one study, 259 HSCT recipients (111 allo-HSCT, 145 auto-HSCT, and 3 CD34 top-up procedures) were evaluated and only 1 HEV RNA positive patient was identified [39]; while the other study analyzed 144 allo-HSCT recipients and 3 patients were positive for HEV RNA [21]. In the Netherlands, two studies analysed HEV infection in HSCT: one study was performed in 130 allo-HSCT recipients with elevated alanine aminotransferase (ALT), identifying 5 HEV RNA positive patients [36]; the other study analyzed 328 allo-HSCT recipients transplanted over a 5-year period, identifying a total of 10 patients with a current infection (8 were positive for HEV RNA and 2 were positive for anti-HEV IgM) and 41 patients were seropositive for anti-HEV IgG [37]. In France, a study with 88 HSCT recipients (72 allo-HSCT and 16 auto-HSCT) revealed that none patient tested positive for HEV RNA, while 3 were positive for anti-HEV IgM [18]. Interestingly, this study found a seroprevalence of anti-HEV IgG of 125% (11/88) and 364% (32/88) in the same group of HSCT recipients when using two different enzyme immunoassays (EIA) methods. In the study from Germany with 52 allo-HSCT recipients with elevated ALT, but without any specific cause of hepatitis, none of them tested positive for HEV RNA, while 3 were positive for anti-HEV IgG

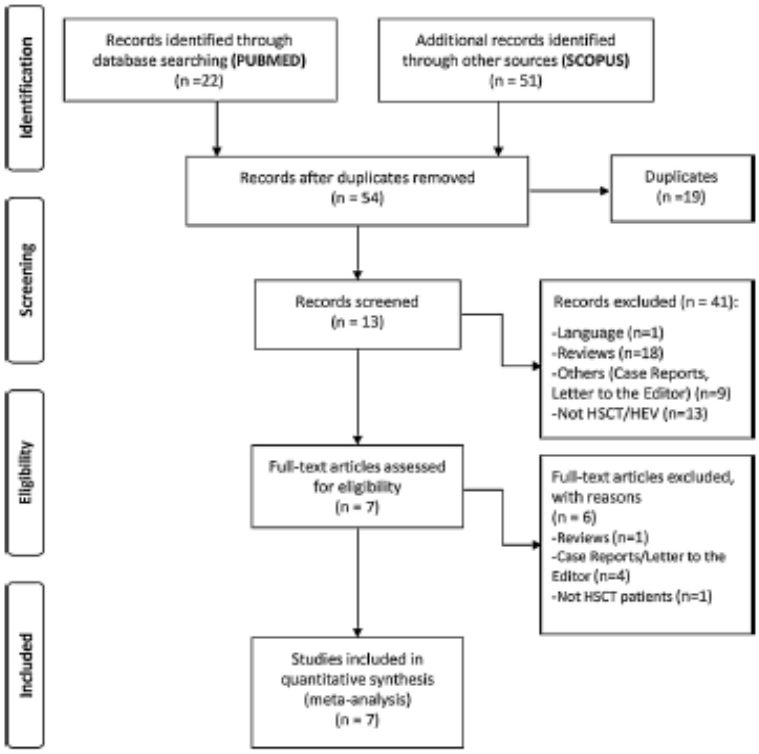

Fig. 1. PRISMA flow diagram.
Abbreviations: HEV, Hepatitis E Virus; HSCT, Hematopoietic stem cell transplantation.

[38].

4.2. HEV infection prevalence analysis

We have performed an analysis of the prevalence of HEV infection in HSCT recipients according to the different approaches of diagnosis. The diagnosis of HEV positive cases is based on the identification of a patient with an HEV RNA positive sample (serum, plasma, blood or feces) or both anti-HEV IgM and IgG positive sample, nevertheless, the seroprevalence of the infection is referred to the detection of anti-HEV IgG only.

Considering the analysis of HEV RNA detection, a total of 1178 HSCT patients were studied and only 19 were positive, which gives an overall prevalence of 1.50% (95% CI: 0.70–2.60) with no significant difference observed between the different studies ($p = 0.090$; Fig. 2).

The analysis of anti-HEV IgM and IgG was performed separately: the detection of anti-HEV IgM as marker of acute infection was tested in a total of 593 HSCT patients with only 10 positive cases, giving an overall anti-HEV IgM prevalence of 2.00% (95% CI: 0.30–4.50; $p = 0.060$; Fig. 3); while the detection of anti-HEV IgG, a marker of past infection, was described in a total of 645 samples, 82 HSCT recipients were found positive to anti-HEV IgG giving an overall IgG seroprevalence of 11.4% (95% CI: 1.80–26.3), with statistically significant differences between the studies ($p < 0.001$; Fig. 4).

Table 1
Details of the studies reporting HEV infection in HSCT recipients used in data analysis.

First author (country, year)	Patients, n	Type of HSCT	HEV Diagnostic Methods	Assay	Positive, n	Prevalence
Tang FF et al. (China, 2019)	177	Allo-HSCT	Anti-HEV IgM	EIA (MP Diagnostics *)	5	2.82%
			Anti-HEV IgG			
			HEV RNA	Commercial RT-qPCR	2	1.13%
Reekie J, et al. (England, 2018)	259	Allo/Auto-HSCT	HEV RNA	In-house RT-qPCR	1	0.39%
Ankcorn MJ, et al. (United Kingdom, 2018)	144	Allo-HSCT	HEV RNA	In-house RT-qPCR	3	2.08%
Willemse SB et al. (Netherlands, 2017)	130	Allo-HSCT	HEV RNA	Commercial RT-qPCR	5	3.85%
Verhuis J, et al. (Netherlands, 2013)	328	Allo-HSCT	Anti-HEV IgM	EIA (Wantai *)	2	0.61%
			Anti-HEV IgG		41	12.5%
			HEV RNA	In-house RT-qPCR	8	2.44%
Abravanel F, et al. (France, 2012)	88	Allo/Auto-HSCT	Anti-HEV IgM	EIA (Adaltis *)	3	3.41%
			Anti-HEV IgG		11	12.5%
			Anti-HEV IgG	EIA (Wantai *)	32	36.4%
			HEV RNA	In-house RT-qPCR	0	0.00%
Kamps C, et al. (Germany, 2012)	52	Allo-HSCT	Anti-HEV IgG	EIA (Abbott *)	3	5.77%
			HEV RNA	In-house Nested RT-PCR	0	0.00%

Abbreviations: EIA, Enzyme Immunoassay; HEV, Hepatitis E Virus; HSCT, Hematopoietic stem cell transplantation; IgG, Immunoglobulin G; IgM, Immunoglobulin M; n, sample size; Nested RT-PCR, Nested Reverse Transcription-Polymerase Chain Reaction; RT-qPCR, Reverse Transcription-quantitative Polymerase Chain Reaction.

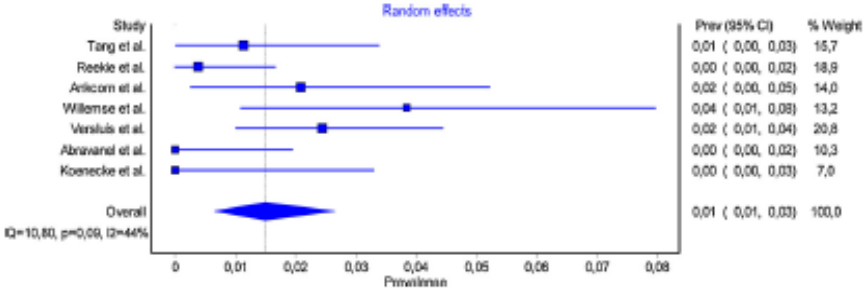

Fig. 2. Forest plot of the overall prevalence based on the detection of HEV RNA.
Abbreviations: p, *p*-value; Prev (95%CI), Prevalence 95% Confidence Interval.

5. Discussion

The evidence that most of the HEV-3 infections become chronic in immunocompromised hosts, especially those after transplantation, makes these patients an important group of study [29,30]. Since the prevalence and incidence of HEV in HSCT recipients is largely unknown, we have performed a systematic review to understand the burden of HEV infection in these group of immunocompromised patients.

We found that there is a wide variation in HEV infection definition in literature, although in accordance with a recent surveillance report by the *European Centre for Disease Prevention and Control* (ECDC), concerning hepatitis E virus in Europe, a positive case is considered in a patient with an HEV RNA positive sample (serum, plasma, blood or feces) or both anti-HEV IgM and IgG positive sample [12]. HEV RNA is detected usually between 2 and 8 weeks when viremia reaches its peak before strong declining, then, around 3 weeks after clinical symptoms, HEV RNA becomes undetectable, with the virus continuing to be shed in the stool for another 1–2 weeks. On the other hand, the anti-HEV IgM immune response remains detectable for 3–12 months and the IgG response reaches its peak four weeks later than IgM, remaining detectable for several years, although the exact duration of this response remains uncertain [20,30].

The fact that there is no standardized case definition, in addition to the heterogeneity in the analytical sensitivity of the commercial HEV assays, makes the comparison of studies more difficult. In this systematic review, all the seven studies performed the detection of HEV RNA, the most robust marker of acute/active infection [18,21,35–39], while four performed also the detection of anti-HEV IgM/IgG [18,35,37,38]. The studies that performed HEV RNA detection as the diagnostic method for HEV infection in HSCT recipients revealed similar results between them, with an overall prevalence of HEV infection of 1.5%. Furthermore, we found that the overall prevalence of anti-HEV IgM was of 2.0%, a comparable value to the reported by the detection of HEV RNA, which is expected since both are markers of acute infection.

The analysis of anti-HEV IgG showed an overall seroprevalence of 11.4% in HSCT patients, with significant differences between the results of the different studies, mainly due to the study from France which revealed a much higher prevalence when compared to the others studies [18]. These results are difficult to compare due to the differences in the sensitivities and specificities of anti-HEV IgG commercial immunoassays [40–43]. Moreover, seroprevalence is greatly influenced by food habits [5,43] and to differences in culinary practices between countries/regions 43]. Reported anti-HEV IgG seroprevalence not only varies between countries but also within countries, with significant variance between regional areas 43]. Indeed, even seroprevalences in Europe are very difficult to compare in consequence of these serology limitations and very different rates have been reported across the continent, ranging from 1.3 to 52% [44–46]. In Finland, anti-HEV IgG seroprevalence was found to be 27.6% in general population [47], while in Norway, Germany and Portugal reported seroprevalences were 11.4%, 16.8% and 16.3%, respectively [48–50]. Furthermore, several countries such as Netherlands, Spain, France, and Southwest England have performed these studies in healthy blood donors reporting an anti-HEV IgG prevalence ranging from 16.0 to 26.7% [51–54]. These facts contribute to the assumption that the anti-HEV IgG prevalence does not reflect the spread of HEV in the HSCT population, which reinforces the importance of better characterization in this group of patients.

HSCT recipients are at higher risk of HEV infection, that could lead to chronic infection, and the diagnosis of HEV infection is highly recommended. Over the last years, some attention has been given to this group of immunocompromised patients, but there is still a small number of studies in HSCT as demonstrated in the present systematic review. Therefore, more studies are needed to increase our understanding of the epidemiology of HEV in HSCT recipients.

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Author contributions

SC, acquisition of data, analysis and interpretation of data, drafting the article, final approval; CC, acquisition of data, final approval; CC and AT final approval; RM, conception and design of the study, final approval; MSJN, interpretation of data, revising the article, final

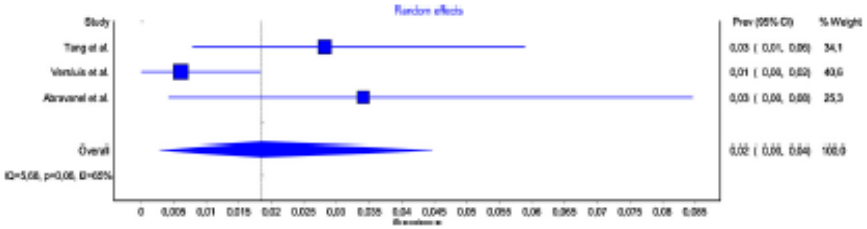

Fig. 3. Forest plot of the overall prevalence based on the detection of IgM anti-HEV.
Abbreviations: p, *p*-value; Prev (95%CI), Prevalence 95% Confidence Interval.

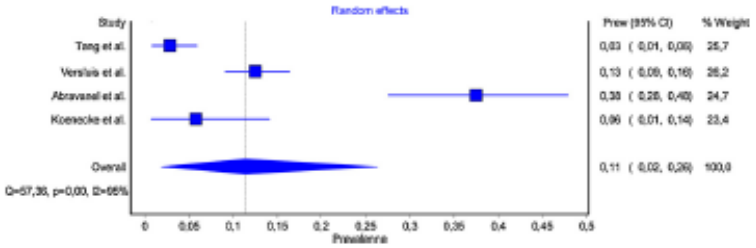

Fig. 4. Forest plot of the overall prevalence based on the detection of IgG anti-HEV.
Abbreviations: p, *p*-value; Prev (95%CI), Prevalence 95% Confidence Interval.

approval; HS, analysis and interpretation of data, conception and design of the study, revising the article, final approval.

Declaration of Competing Interest

The authors declare they have no conflict of interest.

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Appendix III. Poster Presentation for European Society for Clinical Virology 2019

HEPATITIS E VIRUS IN HEMATOPOIETIC STEM CELL TRANSPLANT RECIPIENTS: A SYSTEMATIC REVIEW* updated

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Introduction

Hepatitis E virus (HEV) is a cause of acute viral hepatitis worldwide, with the World Health Organization estimating 20 million infections, >3 million acute cases, and >57,000 HEV-related deaths, annually[1]. Four major HEV genotypes infect humans: genotypes 1 (HEV-1) and 2 (HEV-2) are transmitted through the fecal-oral route via contaminated water, prevalent in developing countries; and genotypes 3 (HEV-3) and 4 (HEV-4) are zoonotic viruses transmitted mainly through consumption and contacted with pigs[2].

Figure 1. Schematic representation of the distribution of the different HEV genotypes in the world[3].

In Europe, HEV-3 infections causes asymptomatic infection or self-limiting acute hepatitis in healthy individuals, although it can lead to chronic infection with rapidly progressive cirrhosis in immunosuppressed patients, such as transplant-related patients[2–4].

Hematopoietic stem cell transplant (HSCT) recipients are an important risk group for HEV infection due to their prolonged immunosuppression state[2]. The evidence that most of the HEV-3 infections become chronic in immunocompromised hosts, especially those after transplantation, makes these patients an important group of study[5]. The prevalence and incidence of HEV in HSCT recipients is largely unknown.

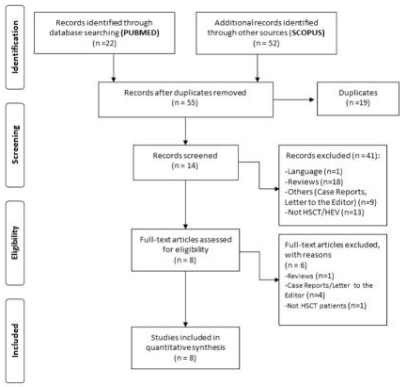
Aim

In the present study, we aimmed to summarize published data regarding HEV infections in HSCT recipients by performing a systematic review of the literature.

Methods

Study Design

Literature search was performed concerning published manuscripts regarding 'hepatitis E virus AND stem cell transplantation' following the Preferred Reporting of Systematic Reviews and MetaAnalyses (PRISMA) guidelines.



Statistical Analysis

Statistical analysis was performed using the MetaXL software to estimate the overall prevalence of HEV infection according to the different diagnostic approaches (HEV RNA and anti-HEV IgM and/ or IgG detection).

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Results

A total of 8 manuscripts were included for data analysis, with 7 studies performed in Europe and 1 study in China. These studies evaluated the prevalence of HEV infection based on the detection of HEV RNA (n=8) and/or the presence of anti-HEV IgM (n=4)/IgG (n=5).

Table 1. Details of the studies reporting HEV infection in HSCT recipients used in data analysis.

First author (country, year)	Patients, n	Type of HSCT	HEV Diagnostic Methods	Assay	Positive, n	Prevalence
Cruz S, et al. (Portugal, 2019)	196	Allo-HSCT	Anti-HEV IgM Anti-HEV IgG	P/A (Wantai *) P/A (Wantai *)	2 39	1.13% 28.9%
			HEV RNA	In-house RT-qPCR	4	2.04%
Tong FF, et al. (China, 2018)[6]	177	Allo HSCT	Anti-HEV IgM Anti-HEV IgG	P/A (MP Diagnostics *) P/A (MP Diagnostics *)	6	3.67%
			HEV RNA	Commercial RT-qPCR	2	1.13%
Reckle I, et al. (England, 2018)[7]	259	Allo/Auto HSCT	HEV RNA	In-house RT-qPCR	1	0.39%
Ankcorn MJ, et al. (United Kingdom, 2018)[8]	144	Allo-HSCT	HEV RNA	In-house RT-qPCR	3	2.08%
Willemse SB et al. (Netherlands, 2017)[9]	130	Allo-HSCT	HEV RNA	Commercial RT-qPCR	5	3.85%
Verlaet J, et al. (Netherlands, 2015)[10]	328	Allo-HSCT	Anti-HEV IgM Anti-HEV IgG	EIA (Wantai *) EIA (Wantai *)	2 41	0.61% 12.5%
			HEV RNA	In-house RT-qPCR	8	2.44%
Abravanel F, et al. (France, 2012)[4]	88	Allo/Auto HSCT	Anti-HEV IgM Anti-HEV IgG Anti-HEV IgG	P/A (Adaltis *) P/A (Adaltis *) P/A (Wantai *)	1 11 32	0.11% 12.5% 36.4%
			HEV RNA	In-house RT-qPCR	0	0.00%
Kraemer S, et al. (Germany, 2017)[11]	52	Allo-HSCT	Anti-HEV IgG	P/A (Abbott *)	3	5.77%
			HEV RNA	In-house Nested RT-PCR	0	0.00%

Regarding HEV RNA detection, a total of 1374 HSCT patients were studied and 23 were positive, which gives and overall HEV infection prevalence of 1,6% (95% CI: 0.80–2.60).

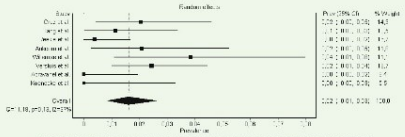

Figure 4. Forest plot of the overall prevalence based on the detection of HEV RNA. Abbreviations: p, p-value; Prev (95%CI), Prevalence 95% Confidence Interval.

Detection of anti-HEV IgM as marker of acute infection was tested in a total of 632 HSCT patients with 12 positive cases, giving an overall anti-HEV IgM prevalence of 1,7% (95% CI: 0.60–3,30).

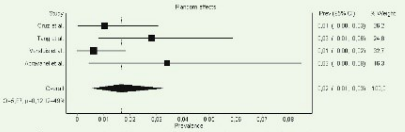

Figure 5. Forest plot of the overall prevalence based on the detection of IgM anti-HEV. Abbreviations: p, p-value; Prev (95%CI), Prevalence 95% Confidence Interval.

Detection of anti-HEV IgG, a marker of past infection, was described in a total of 841 HSCT patients with 121 positive cases, giving an overall anti-HEV IgG seroprevalence of 13,3% (95% CI: 4.70–25.0).

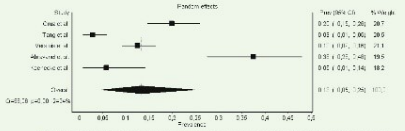

Figure 6. Forest plot of the overall prevalence based on the detection of IgG anti-HEV. Abbreviations: p, p-value; Prev (95%CI), Prevalence 95% Confidence Interval.

Conclusion

HSCT recipients are at higher risk of HEV infection, that could lead to chronic infection, and the diagnosis of HEV infection is highly recommended. Over the last years, some attention has been given to this group of immunocompromised patients, but there is still a small number of studies in HSCT as demonstrated in the present systematic review. Therefore, more studies are needed to increase our understanding of the epidemiology of HEV in HSCT recipients.

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