A Euro-Egyptian initiative to fight Hepatitis C infections

HEPACUTE

مبادرة أوروبية-مصرية لمكافحة عدوى الالتهاب الكبدي C

SEVENTH FRAMEWORK PROGRAMME

PROJECT FINAL REPORT

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1. Final Publishable Summary Report

1.1 Executive Summary

With more than 170 million chronically infected individuals hepatitis C is one of the most common chronic viral diseases Worldwide. Prevalence rates range from 0.5% in Northern European countries to 28% in some areas of Egypt. While blood products today play a minor role in the transmission of hepatitis C virus, community acquired hepatitis C is the leading cause of transmission in high prevalence countries such as Egypt.

To improve detection and management of acute hepatitis C in Egypt a local network of acute care hospitals was established and linked with large centres such as the National Liver Institute (NLI), National Research Centre (NRC) and Vacsera. A case definition for acute hepatitis C was established within the HepaCute consortium and a management consensus was established. For those patients requiring antiviral treatment, access to PEG-interferon and ribavirin was provided.

Despite the changes caused by the Egyptian revolution and an unavoidable delay in the recruitment of patients and collection of samples for the studies planned between the HepaCute partners, a very close collaborative network between European and North African partners was established and with a 6-month-extension of the project we could successfully address the major objectives of HepaCute.

A comprehensive analysis of host and viral markers predicting the outcome of acute hepatitis C was performed, including host genetics, viral pathogenesis, innate as well as adaptive cellular and humoral immunity, proteomics and micro-RNAs. We could clearly show that the outcome of acute hepatitis C is influenced by host genetic polymorphisms, most notably within the IL28B/IFNL4 gene region, and this is true for both European and North African patients. For the first time, the transcriptome of dendritic cell subsets was studied during acute hepatitis C and a clearly discriminative gene expression pattern was defined that distinguishes patients with self-limited from patients with chronically evolving acute hepatitis C. For natural killer (NK) cells, relevant genetic polymorphisms have been identified that are linked with viral clearance. A comprehensive analysis of the transcriptome of virus specific CD8+ T cells from patients with acute hepatitis C was performed and the phenotype and function of virus specific CD4+ T cells was elucidated, revealing highly predictive regulatory genes such as *t-bet*. Viral sequencing of acute phase samples with extended virological tools showed that in acute hepatitis C the majority of viral sequences are incorporated in defective particles. This unsuspected finding may have dramatic consequences for our understanding of the pathogenesis of acute hepatitis C and for the interpretation of laboratory results of HCV-RNA quantification. Viral sequence data in combination with humoral immune responses led to a series of discoveries regarding the viral entry mechanisms, the understanding of which is key for the development of preventive vaccines.

While the above described patterns of innate and adaptive immune responses predictive of viral clearance have been characterized by complex technologies including comprehensive transcriptome analysis and proteomics, it has been possible to identify footprints of the diverging immune responses in more clinically available assays such as cytokine or miRNA analysis in patient sera and proteomics in urine. There are a number of candidate signatures that can be readily developed for routine clinical testing.

Intensive training activities and collaborative research activities between EC countries, Egypt, and Morocco have set up a strong research community with sustained activities beyond the duration of the HepaCute project.

The results of HepaCute have to be interpreted in the light of evolving new therapies which offer cure in >95% of patients but are associated with high treatment costs, even if discounts for developing countries can be expected. Nevertheless, even very active treatments first require the identification of affected patients and a medical and scientific infrastructure that ensures appropriate patient selection and management of treatment. The local hepatitis C network that has been built by HepaCute has the competence to provide this expertise to a large area in Egypt which has the potential, depending on the political development in Egypt, to further growth. The sustained collaboration with the European partners will further enhance the quality of care for hepatitis C patients in Egypt and North Africa and will ensure access of the Egyptian partners to knowledge and expertise of the European partners as well as the continuing support of public and scientific organizations such as EASL and the German Liver Foundation.

1.2 Summary description of project context and objectives

More than 170,000,000 patients world-wide are chronically infected with hepatitis C virus (HCV) with prevalence rates ranging from 0.5% in Northern European countries to 28% in some areas of Egypt (source: WHO). Chronic hepatitis C is thus a leading cause of acute and chronic liver disease including fibrosis, cirrhosis and hepatocellular carcinoma. HCV persists in 50-85% of acutely infected patients and once chronic infection is established, spontaneous clearance is rare. Prior to the discovery of HCV and the implementation of routine screening of blood products, HCV was the leading cause of transfusion transmitted hepatitis. Although HCV transmission by blood products is rare in most developed countries, new cases of HCV infection still occur with up to 4,000,000 new cases every year (source: WHO). The main routes of transmission in industrialized countries are intravenous (IV) drug use whereas in many poor countries, unscreened blood products and non-sterilized needles are the major cause. A special situation is seen in Egypt where high prevalence rates of 15% lead to incidence rates of 6.8 per 1,000 person years.

Acute hepatitis C is a mild or asymptomatic disease and goes undiagnosed in 70-80% of cases. Early treatment with interferon-alpha during the acute phase of infection can resolve infection in 95% of cases and abrogate viral persistence whereas the same regimen is successful in only 40-50% of patients. Thus, treating HCV infected patients during the acute phase of infection provides a unique opportunity to prevent new cases of chronic hepatitis C. However, early interferon-alpha treatment can be avoided in the 40-50% of symptomatic patients who resolve infection spontaneously, obviating the need to treat patients with an expensive drug (estimated one year treatment of 20,000 €) that has a high frequency of adverse effects. **Development of a biomarker that predicts viral clearance or persistence would provide optimal patient management and ensure appropriate targeting of drugs to the patient population most likely to benefit. A combined strategy of increased acute hepatitis C detection and efficient patient management could dramatically decrease the number of new cases of chronic hepatitis C and reduce treatment-related morbidities.**

During the lifetime of HepaCute we have seen an enormous progress in the development of antiviral drugs which has to be considered in the evaluation of the objectives of HepaCute. During the first half of the project the approval of the protease inhibitors telaprevir and boceprevir has dramatically increased the cure rates for chronic genotype 1 infection. The lack of approval for the treatment of genotype 4, the additional costs and side-effects, however, prevented a major impact for the management of hepatitis C in Egypt. In contrast, the approval of sofosbuvir in January 2014 and recently of simeprevir has now opened an era of well tolerated and highly efficient antiviral drugs, that soon promise an interferon-free antiviral treatment with >95% rates of viral clearance. The astronomic costs (>100,000 € per treatment) in Western countries will be ameliorated with special access programs for Egypt and probably other developing countries with discounts of up to 99%, which, however, still imposes a significant financial burden on these countries. **Thus, while chronic hepatitis C will soon be a readily curable disease, many of the objectives of HepaCute are still valid beyond the lifetime of the project, given the importance of prevention of infection, early detection of acute hepatitis C and selecting patients for antiviral treatment.**

In this context, the HepaCute project's major objectives are:

Objective 1. To increase acute hepatitis C detection rate(s) in European and Egyptian model regions.

Objective 2. To understand host genetic factors and their functional impact on innate and adaptive antiviral immune responses that control acute HCV replication.

Objective 3. To understand the role of viral fitness and evolution in relation to adaptive humoral and cellular immunity.

Objective 4. To identify biomarkers of spontaneous acute hepatitis C resolution.

Objective 5. To improve clinical care of patients with acute hepatitis C in Europe and Mediterranean Partner Countries (MPC).

Broken down into eight individual workpackages, the objectives were addressed as follows:

WP1: Management and Coordination
The Project Management Team (PMT) had the major objective to set up an effective management framework to ensure a high quality management and coordination of the project's work programme, that all work and tasks are performed on time, an adequate reporting procedure and a constant flow of information in close interaction within the HepaCute consortium and with the European Commission.

A common objective for WP2-8 is the collection of patient samples for genetic, virological, and immunological studies. Samples from previously characterized cohorts as well as recruitment of new patients from all different European and North African regions represented in the consortium is intended.

WP2: Host genetics
The major objective of WP2 was to identify a comprehensive set of host genes that are associated with spontaneous resolution of HCV infection across ethnically diverse populations using Caucasian and North African populations.

WP3: Innate Immunity
Characterization of molecules differentially expressed by cells of the innate immune system in correlation with clinical outcome was the major WP3 objective.

WP4: Viral tropism and adaptive humoral responses
The major objective of WP4 was extensive analysis of the adaptive humoral response in relation to viral tropism in samples from patients with acute hepatitis C and diverse clinical outcomes.

WP5: Specific Cellular Immune Response
The major objective of WP5 was the identification of up- or down-regulated genes encoding costimulatory or apoptotic molecules by transcriptome analysis of HCV-specific CD4+ and CD8+ T cells in correlation with the outcome of acute hepatitis C.

WP6: Viral Genetics
The major objective of WP6 was the isolation of HCV genomes from acute and chronic phase samples in order to investigate sequence diversity and its correlation with functionality in cell entry, receptor dependence and immune evasion assays.

WP7: Molecular and metabolic markers for viral clearance
The major objective of WP7 was the identification of protein and miRNA markers in serum and urine that would help predict the course of an acute hepatitis C infection, and also give insights as to the outcome of a treatment in a given patient.

WP8: EU/MPC Research Collaboration in Hepatitis Research and capacity building in Egypt, Morocco and Europe
The objectives of WP8were to exploit synergies between EU and Egyptian hepatitis research programmes, to establish appropriate operational tools for programme partnerships, to develop perspectives for sustained programme cooperation, to establish criteria for the early diagnosis of patients with acute hepatitis C, to develop and disseminate a consensus for the management of newly diagnosed acute hepatitis C cases, to build research capacity in Egypt, Mediterranean countries and Europe and to establish an Egyptian model for a network/data base for acute hepatitis C cases.

1.3 A description of the main S&T results/foregrounds

The results of the HepaCute project provide a comprehensive analysis of host and viral markers for the outcome of acute hepatitis C. We could clearly show that the outcome of acute hepatitis C is influenced by host genetics, most notably by the IL28B/IFNL4 gene region, innate immune responses exemplified by dendritic cells and NK cells, as well as virus specific humoral and cellular immune responses. While patterns of specific immune

responses predictive of viral clearance have been characterized by complex technologies including comprehensive transcriptome analysis and proteomics, footprints of the diverging immune responses have been confirmed in standard assays in patient sera and urine that can be readily developed for routine clinical testing.
Acute hepatitis C is a global health problem with a particular high incidence in Egypt. HepaCute achieved the building of a strong network, consensus definitions of acute hepatitis C and management and treatment guidelines to be implemented in areas with high hepatitis C prevalence. Intensive training activities and collaborative research activities between EC countries, Egypt, and Morocco have set up a strong research community with sustained activities beyond the duration of the HepaCute project.

With regard to the individual workpackages, the following results have attained:

WP1: Management and Coordination
The Project Management Team (PMT) has kept an effective management framework to ensure a high quality management and coordination of the project's work programme, an adequate reporting procedure and a constant flow of information. A management handbook has been provided to each partner, presenting FP7 and consortium rules. The Work Plan has been kept up to date by a constant monitoring of the project's progress through regular Executive Council (ExCom) meetings. The knowledge management and the information within the Consortium have been backed by an online web-platform with restricted access and personal access codes. For the dissemination and communication around the project's activities, the PMT has provided communication tools, including chart, public website, posters, slides, leaflets and newsletters on HepaCute as well as corporate rules to the partners for disseminating and publishing on the project.
The PMT has brought support to the organisation of the three consortium general meetings, the Executive council meetings and the 2 HepaCute training workshops. For each meeting, minutes have been produced and distributed to the whole Consortium through the online platform.

WP2: Host genetics
Initial work focused on the role of the interleukin (IL) 28B associated SNP rs12979860 and other SNPs within this gene on the outcome of HCV infection in North African cohorts.
At the beginning of 2013, the publication of SNP ss469415590 which is in high linkage disequilibrium with rs12979860 in IL28B, but introduces a frame shift mutation leading to the production of IFNL4, showed that this SNP is more strongly associated with impaired HCV clearance in individuals of African ancestry (Prokunina-Olsson et al, 2013). It was therefore decided to explore the predictive value of this genetic marker in North African populations. The role of other genetic markers was, also, studied, including KIR, HLA-C, L-Sign and PNPLA3. KIR:HLA-C genes have been shown to influence the outcome of HCV infection in Caucasians. L-SIGN represents a liver-specific receptor for HCV, and may play an important role in HCV infection and immunity. Genetic variation in PNPLA3 has previously been reported to confer susceptibility to non alcoholic fatty liver disease.

Task 2.a: Enrolment of Caucasian, Egyptian and Moroccan populations into genetic studies
DNA samples of North African ethnicity (420 Egyptian and 438 Moroccan samples) have allowed studying the effect of host genes which affect the outcome of HCV infection in this cohort, including the influence of variants of interleukin (IL)28B, interferon lambda 4 (IFNL4), killer cell immunoglobulin-like receptor (KIR), Liver/lymph node-specific intercellular adhesion molecule-3-grabbing integrin (L-SIGN)-Sign, and patatin-like phospholipase domain containing 3 (PNPLA3).
In detail, a total of 420 Egyptian subjects/samples were collected from Partner12 (VACSERA), 13 (NLI) and 14 (NRC). Most Egyptian patients are infected with HCV genotype 4. The Egyptian sample set included 414 samples collected from patients during the acute phase of HCV infection. Their outcome of HCV infection was monitored throughout the duration of the work program. 438 Moroccan samples were collected by partner 17 (IPM). 70 % of the Moroccan HCV patients had been infected with HCV genotype 1b, 30% had been infected with genotype 2 (Ezzikouri et al., PLOS One, 2013).
The IFNL4.ss49615590 genotype of 312 Egyptian samples (77 resolvers, 108 chronically infected, 127 healthy controls) and the KIR:HLA-C genotypes of 170 Egyptian samples (51 Resolvers, 77 chronic, 2 acute, 40 healthy controls) were determined. For 11 acute and 27 chronic Egyptian cases, the KIR:HLA genotype using Luminex technology was determined

In 438 Moroccan samples (232 chronically infected patients, 68 resolved subjects and 138 healthy subjects) the influence of the IL28B.rs12979860 and IL28B.rs8099917 polymorphism on the outcome of HCV infection was studied.

The impact of L-SIGN neck region length variation was studied in 322 subjects (150 chronically infected patients, 63 resolvers and 109 healthy subjects). It was assessed whether the PNPLA3 rs738409 (I148M) polymorphism may also affect the resolution and/or the progression of hepatitis C in 437 Moroccan individuals (230 patients with chronic infection of which 101 had hepatocellular carcinoma (HCC), 75 resolvers and 132 healthy subjects).

1303 Caucasian patients from two cohorts, the Swiss Hepatitis C Cohort Study (n=1231, of whom 89 clearers) and a cohort of 72 persons (all spontaneous HCV clearers) enrolled through a collaboration with Dr A. Mangia (Ospedale Casa Sollievo della Sofferenza, San Giovanni Rotondo, Italy; all HIV-negative) were enrolled. The whole study population was revised, eliminating doubtful results: several patients previously classified as spontaneous clearers had to be reassigned to the chronic group, as they had eliminated HCV as a result of a course of antiviral therapy; the details of which were missing in the original database. Thus, the assignment of all patients to each case definition was verified. The final study population included 1290 chronically infected patients and 160 clearers. Overall, this is the largest cohort of spontaneous HCV clearers ever put together. In our population, the minor allele frequencies of CCR5 Δ32 and IFNL3 rs12979860 were 8.6% and 36.8%, respectively. Considering also that 15.1% of patients were heterozygous for CCR5 Δ32 deletion and 1.1% homozygous, they can affirm that this cohort is representative of the distribution of this allele in a Caucasian population.
The final results showed that the carriage of the CCR5 Δ32 allele occurs less frequently in spontaneous clearers (i.e. 11%) compared to 17% of chronically infected patients (OR=0.59, 95% CI = 0.35-0.99, P=0.047). Carriage of this allele also tended to be observed more frequently among patients with liver inflammation (19%) compared to those without inflammation (15%, OR=1.38, 95% CI = 0.99-1.95, P=0.06). The CCR5 Δ32 was not associated with sustained viral response (P=0.6), fibrosis stage (P=0.8), fibrosis progression rate (P=0.4), or steatosis (P=0.8).

Task 2.b: Comprehensive analysis of IL28B in ethnically diverse populations

Single-nucleotide polymorphisms (SNPs) around IL28B, and IFNL4 are associated with spontaneous clearance of HCV genotypes 1 and 3 in white and African-American populations. Initial work of WP2 focused on the role of the IL28B associated SNP rs12979860 and other SNPs within this gene in the outcome of HCV infection in North African cohorts. Since the publication of the IFNL4.ss469415590 marker, the influence of this variant on the outcome of HCV infection was explored in the Egyptian cohort, and is under way in the Moroccan samples.
It was investigated to which extend the variants *IL28B.rs12979860* and *IFNL4.ss469615590* are associated with spontaneous clearance of HCV genotype 4, in 185 Egyptian subjects (78 with spontaneous clearance including 18 from a recent acute infection; and 108 chronically infected patients). For overview, please see Table 1. A manuscript that contains these data has been prepared for publication.
The minor allele frequency for *IL28B.rs12979860 (T)* and *IFNL4.ss469415590 (dG)* in 126 healthy control samples of the Egyptian cohort was 0.39 and 0.32 respectively, compared to 0.38 for both polymorphisms in the Caucasian population as reported previously (Bibert et al, JEM, 2013).
The CC genotype was found more frequently in 60 individuals with spontaneous clearance (without the resolvers from recent acute cases) (54% vs 26%; OR=3.25; 95% confidence interval [CI]=1.68–6.28; p=0.0005) compared to individuals with chronic infection. The protective C allele of *IL28B.rs12979860* was more common in those with spontaneous clearance compared to those with chronic infection (75% vs 54%; OR=2.6 (95% CI, 1.59-4.25); p=0 .0001).
The TT genotype of *IFNL4.ss469415590* was found more frequently in individuals with clearance (50% vs 20%; OR=3.86; 95% CI=1.93-7.70; p=0.0001). The T-allele of *IFNL4.ss469415590* was more common in subjects with spontaneous clearance (74 % vs 49%; OR=2.98; 95% 1.83-4.86; p< 0 .0001).
Thus, the polymorphisms of *IL28B.rs12979860* and *IFNL4.ss469415590* have a comparable influence on the natural resolution (clearance) of HCV genotype 4 in the Egyptian population, with the IFNL4 variant having a slightly higher influence (OR=3.86 vs OR=3.25), see Table 2and Figure 1.

The frequency of genotypes and alleles for *IL28B.rs12979860* and *IFNL4.ss469415590* in 17 spontaneous resolvers of acute infection was comparable to the frequency of genotypes and alleles in subjects of spontaneous response

to HCV infection. We, therefore, combined the spontaneous resolvers with the resolvers from acute infection and obtained only slight changes in the strength of association for both variants with clearance (resolution) of HCV infection.

	Total	Male (%)	Female (%)	Av Age (years)
Resolver	77	45 (58)	32 (42)	42
	60 from previous infection	42 (70)	18 (30)	
	17 from acute	3 (18)	14 (82)	36
Chronic	108	69 (64)	39 (36)	44
	107 from prev infection	69 (64)	39 (36)	44
	1 from acute	1 (100)		
Healthy controls	127	49 (39)	78 (61)	34

Table 1: Egyptian samples genotyped for *IL28B.rs12979860+IFNL4.ss469415590*

	Genotype/allele frequency Resolvers (%)	Genotype/allele frequency Chronics (%)	Genotype/allele frequency healthy (%)	Comparison	OR (95% CI)	P-value
IL28B.rs12979860	**N=78/61**	**N=110/109**	**N=123**			
CC genotype						
+ acutes	44:34 (56)	29:81 (26)		CC vs CT+TT	3.61 (1.965-6.70)	<0.0001
- acutes	33:28 (54)	29:80 (26)			3.25 (1.68-6.28)	0.0005
- acutes	33:28 (54)		60:63 (49)		1.24 (0.67-2.29)	0.53
C allele						
+ acutes	119:37 (76)	119:101 (54)		C vs T	2.73 (1.73-4.30)	<0.0001
- acutes	92:30 (75)	118:100 (54)			2.60 (1.59-4.25)	0.0001
- acutes	92:30 (75)		174:72 (71) MAF 0.29		1.27 (0.77-2.08)	0.39
IFNL4.ss46945590	**N=60/77**	**N=107/108**	**N=126**			
TT genotype						
+acutes	38:39 (50)	22:86 (20)		TT vs TdG+dGdG	3.81 (1.99-7.28)	<0.0001
- acutes	30:30 (50)	22:85 (20)			3.86 (1.93-7.70)	0.0001
- acutes	30:30 (50)		59:67 (47)		1.11 (0.63-1.95)	0.77
T allele						
+ acutes	112/42 (73)	106:110 (49)		T vs dG	2.77 (1.77-4.31)	<0.0001
- acutes	89:31 (74)	105:109 (49)			2.98(1.83-4.86)	<0.0001
- acutes	89:31 (74)		172:80 (68) MAF 0.32		1.34 (0.82-2.17)	0.27

Table 2: Influence of *IL28B.rs12979860* and *IFNL4.ss469415590* genotype/allele on resolution of HCV genotype 4 in the Egyptian cohort.

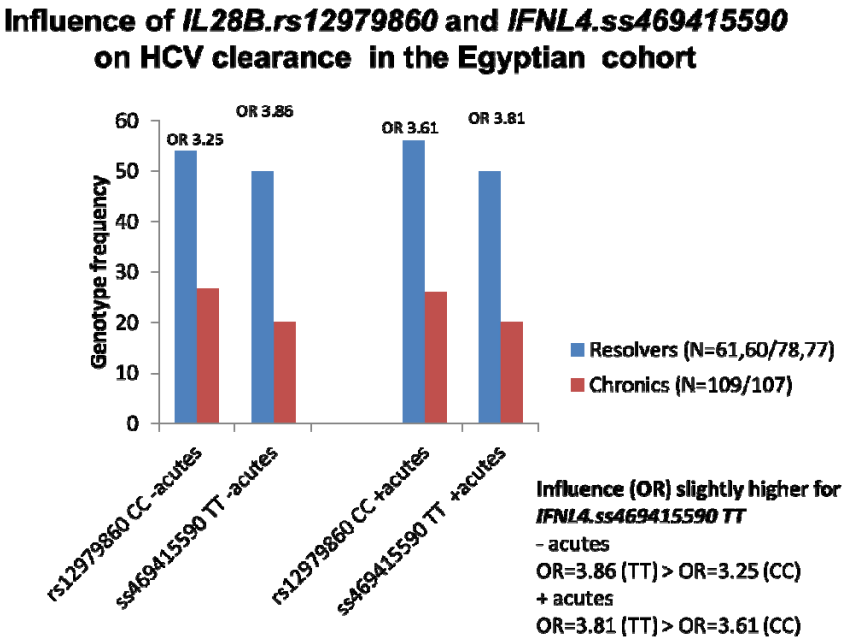


Figure 1. 438 Egyptian samples (232 chronically infected patients, of whom 115 patients had mild chronic hepatitis and 117 had advanced liver disease (cirrhosis and hepatocellular carcinoma), 68 resolved subjects and 138 healthy subjects for *IL28B rs12979860* and *rs8099917* polymorphism.
The protective rs12979860-C and rs8099917-T alleles were more common in subjects with spontaneous clearance. Individuals with clearance of HCV infection were 4.69 times more likely to have the C/C genotype for *rs12979860* polymorphism and 3.55 times more likely to have the T/T genotype at *rs8099917*. Patients with advanced liver disease carried the rs12979860- T/T genotype more frequently than patients with mild chronic hepatitis C and this risk was even more pronounced when we compared them with healthy controls. The *rs8099917-G* allele was also associated with advanced liver disease. In the Moroccan population, polymorphisms near the IL28B gene play a role both in spontaneous clearance and progression of HCV infection. The data are now published (Ezzikouri et al, PLoS One, 2013) and also illustrated below.

Task 2.c: Epistasis of IL28B receptors and other innate immune receptors

KIR:HLA: the full KIR genotype profile combined with a HLA C1/C2 determination was established for 170 Egyptian and 93 Moroccan samples.

Partner 9 has carried out KIR genotyping (in collaboration with Dr James Traherne from the laboratory of Professor John Trowsdale in Cambridge, UK) in the 170 Egyptian and 93 Moroccan samples using RT-PCR technologies, which has a higher resolution than conventional KIR typing, including allowing the detection of copy number variation (CNV) (Jiang et al, Genome Research 2012). As activating and inhibitory KIR receptors exert their function on NK cells after interacting with HLA-C ligands, KIR:HLA–C genotypes have also been determined in those samples. The table below gives an overview of the number of samples in each outcome group.

Preliminary results show a difference in the representation of KIR genes between Caucasian, Egyptian and Moroccan samples sets, see below. The KIR2DL3homozygocity, protective in the Caucasian population (especially in combination with HLA-C1 homozygocity), is present with different frequency in the Egyptian and Moroccan population. Due to low numbers, only trends can be observed and no statistical significance was reached. More samples would be needed in order to produce results reaching statistical significance.
The genotyping of the Egyptian samples has been completed for KIR and HLAC1/2, but due to the original delay in the availability of samples, followed by a technical problem with the genotyping facility (which has now been resolved), and the complexity of the dataset which also needs to be combined with other genetic information in WP2, like IL28B and IFNL4, the detailed analysis is still in progress. Preliminary data show interesting trends, which would require a higher number of samples to be KIR:HLA-C genotyped in order to produce results with statistical significance.

Task 2.d: Functional correlation of IL28 polymorphisms and IFN-α related genes

As biopsies are a limited resource and were not available in the North African cohort, the original suggested deliverable was replaced by the establishment of an *in vitro* system. An allele-specific and IL28B specific quantitative PCR assay was developed to investigate if the genetic association of IL28B polymorphisms is due to a variation of allele-specific expression in specific cell populations. Partner 9 developed the assay which is able to distinguish between IL28B and IL28A (1) (2) and at the same time discriminates between expressed allelic variants of IL28B (rs4803217). This assay was able to detect a difference in the allele specific expression levels of IL28B specific transcript of a Raji B-cell line. This suggests that the allele specific effect of the IL28B gene could possibly be context/tissue specific and might vary between individuals. Indeed, a variation of the C allele specific expression was observed in 6 healthy individuals heterozygous for the IL28B gene. A manuscript is under review at BMC (Knapp et al, Detection of allele specific difference of IL28B mRNA expression, BioMed Central, MS: 1640113478106385, Technical advance).

Task 2.e: Novel genetic targets in HCV

A GWAS analysis was carried out in the North African cohort to find new candidate genes, which are associated with the outcome of HCV infection.

Partner 15 carried out a GWAS of North African samples. No new candidate genes were detected, but the results of the work progress are described and illustrated below.

Mapping genetic markers of HCV clearance other than previously identified IFNL3 by GWAs.

The genotyping was successful with a very high quality data. The missingless rate was low. SNPs were excluded with a MAF<1% (because the power to detect an association is low), a call rate <98% (standard QC) and a HWE p-value >1E-5.

Association analyses with spontaneous clearers versus chronic individuals were run, both correcting for age and gender and uncorrected. The two associations gave very similar results. No inflation is observed in the QQ-plot, suggesting that no confounding factor was left unaccounted for.

No genome-wide significant signal emerged from the data. The IFNL3 polymorphism replicated with comparable effect size as in the Swiss Hepatitis C Cohort Study:

Morocco	rs8099917-T	beta=0.6719	SE=0.4281	P=1.1650e-01
SCCS	rs8099917-T	beta=0.8315	SE=0.1437	P=7.1795e-09

The meta-analysis of the Swiss Hepatitis C Cohort Study and the Moroccan/Egyptian samples did not yield any new genome-wide significant hit, but strengthened the best association (rs8099917), lowering the association P-value to 2.16E-9.

Hurdles and rescue plans:

As no new targets were discovered using GWAS, it was agreed within the consortium that exome sequencing to detect new variants should be undertaken, to be completed by September 2014. The work program is described below.

Exome sequencing:

The analyses of exome sequence data are ongoing. It consists of four, sequentially ordered steps:

1) Alignment of sample reads to the human reference assembly;

2) Determination of nucleotide differences between the reference and the sample at single positions (single nucleotide variants) or involving multiple positions (insertion or deletions, copy-number variants), and comparison with known polymorphic sites (dbSNP, 1000 genomes, internal databases, etc.);

3) Location of these nucleotide differences relative to current gene models (termed annotation) and determination of their effect on open reading frames (non synonymous changes, gain or loss of start and stop codons, alteration of splice sites);

4) Prediction of how a given change will affect protein function. Several algorithms have been developed for alignment (Burrows-Wheeler2, Smith-Waterman3, Needleman-Wunsch4, Hirschberg5), variant calling

(implemented in the Genome Analysis ToolKit6, or SAMtools7), annotation (Annovar8) and prediction of altered protein function (SIFT9, Polyphen-210).

The challenge is to select and combine these algorithms into an efficient processing pipeline in which the output of the preceding analysis is rendered compatible with the input for the following step, and where the parameters for each algorithm are optimised for our local sequence data characteristics. We have developed a pipeline over the past 12 months that satisfies these criteria and can efficiently process a typical exome experiment comprising 90 million paired end reads in about 20 CPU hours, to produce a list of annotated variants.
The next stage will be to prioritise likely causative germ-line variants (or somatic mutations) based on their clinical relevance. These discoveries will be then carried forward for further validation; for example, sequencing/genotyping of additional samples, various functional experiments, etc.
Partner 17 investigated the influence of the PNPLA3 polymorphism in the Moroccan cohort.

The aim of the study was to examine the role of PNPLA3 I148M (gene identified in metabolic disorders) in Moroccan patients and its association with spontaneous clearance and HCC outcomes. No significant difference in association with the MM genotype of rs738409 was observed in patients with spontaneous resolution or persistence of HCV infection. The I148M variant was found to be associated with the progression of chronic infection to hepatocellular carcinoma. The risk of developing HCC is associated exclusively with the MM homozygous genotype, as it also has been reported previously in a Caucasian cohort (Romeo et al, 2008). The results are now published (Ezzikouri et al; Infection, Genetics and Evolution, 2013).

WP3: Innate Immunity
During the first half of the project, patient samples have been collected by the different European partners and Morocco. However, due to the political situation in Egypt, Egyptian samples (those in whom most of the work was relying on) have not been available for European partners, mainly for partner 6, involved in dendritic cell (DC) analysis. Concerning this last issue, after the decision taken on the use of frozen samples for DC analysis, new protocols based on the use of this type of material have been established: they include thawing of samples, purification of DC, stimulation and analysis by RT-PCR of representative genes related to their activation.
Initial purification process involved the use of anti-BDCA-1 and anti-BDCA-2-labelled magnetic beads for isolation of myeloid (mDC) and plasmacytoid (pDC), respectively. Purity after this step corresponded to 50-60 %. In order to increase the purity, a second purification step based on cell sorting by flow cytometry (FACSAria) was carried out reaching a cell purity of 98 %. To measure DC activation, cells were cultured with different amounts of the synthetic agonists of TLR3 and TLR7 poly (I:C) and Imiquimod, respectively. For mDC activation, cells were stimulated with poly (I:C) alone or in combination with DEAE-dextran or lipofectamine to establish the best stimulation conditions. pDC were stimulated with Imiquimod alone. mRNA from untreated and stimulated cells was extracted at different time points. Quality and quantity of mRNA obtained were in the range of those needed to carry out microarray experiments. To confirm DC activation, expression of representative genes was analyzed. These experiments have led to define the best conditions for sample handling and DC stimulation, in order to start with analysis of gene expression in DC from patients (task 3.b).
For the transcriptome analysis of dendritic cell (DC) subsets, mRNA from purified DC populations was obtained from 6 patients and 8 controls. A list of differentially expressed genes between patients with acute HCV infection, corresponding to a broad array of cellular functions and molecules has been generated, indicating that pDC have clear differences irrespective of their stimulation status, whereas for mDC, these differences become more evident after cell stimulation. Genetic analysis of KIR molecules in NK cells from similar patients has been also performed.
The second half recruitment of patients with acute HCV infection has continued and now also included patients from Egypt. Patients have been followed up by corresponding partners and clinical outcome has been determined. Two types of samples have been used. For DC studies, blood samples corresponding to early time-points of infection have been obtained, PBMC have been purified, frozen and stored for shipping to partner 6 for DC transcriptomic analysis. After processing by partner 6, the following samples have been selected for transcriptomic analyses: patients resolving HCV infection (AR; n=2) and non-resolving infection (ANR; n=4). In a similar manner, patients with chronic HCV infection (CHR; n=4) and healthy seronegative individuals (CTRL; n=4) have been included as controls. For NK genetic studies, a different set of samples has been collected.

DC have been purified from 48 samples collected along the project, yielding only 6 samples suitable for transcriptomic analyses in the group of patients with acute HCV infection out of almost 50 samples processed. Purified pDC and mDC, have been stimulated with TLR7 and TLR3 ligands, respectively, or left unstimulated. mRNA was obtained and amplified. Hybridization experiments with specific probes have determined expression of different genes in the groups of patients analyzed.
In general, there are a higher number of upregulated genes in AR patients than in ANR. The number of differentially expressed genes is higher in pDC than in mDC when studying unstimulated cells. However, for stimulated DC, a high number of differentially expressed genes is found in both DC populations. Genes found in these groups belong to different families and molecular functions, not only related to immune functions but also to more general processes, such as metabolism, enzymatic activity, transcription factors, etc.
Regarding NK cell genetic studies, their KIR gene repertoire, consisting of inhibitory and activating NK cell receptors, has been determined. Although studies have been completed, analysis of results is still ongoing.

Task 3.a: Collection of samples from patients with acute hepatitis C and different clinical outcomes
After approval by their respective IRBs, partners indicated above who are involved in patient recruitment and sample collection have carried out this task. Previously established criteria within the consortium have been used for recruitment and follow-up. Clinical characterization has been recorded for patient classification according to their outcome. Different samples have been collected, and for the purposes of WP3 studies, PBMC have been purified from blood samples, frozen and stored for further analysis. Forty eight samples obtained from different partners have been sent in an initial shipment to partner 6 for DC studies.
Although this task has been achieved in terms of patient recruitment, follow-up and sample collection, only a first shipment has been completed. Additional samples corresponding to new patients already characterized are to be shipped for future studies.

Task 3.b: Microarray experiments using RNA from DC samples: Analysis and validation by RT-PCR
Samples (48) obtained from WP3 partners have been processed, and suitable material has been obtained from 4 ANR patients and 2 AR patients. As controls, patients who already had chronic infection (CHR) and healthy individuals (CTRL) have been also recruited and included. mRNA was obtained from non-stimulated (NST) and stimulated (ST) purified pDC and mDC populations, after treatment with Imiquimod and poly(I:C) TLR ligands, respectively, and gene expression analyses were carried out after mRNA amplification. These studies have allowed the characterization of differentially expressed genes between groups in all conditions. These results have shown that for patients with acute HCV infection, in the case of pDC, a high number of genes are differentially expressed between AR and ANR patients, both in NST (196 genes) and ST (140 genes) conditions. For mDC, a low number of differentially expressed genes were found when considering NST cells (50 genes), which clearly increased in the group of ST samples (185 genes). In most cases, these differences corresponded to up-regulated genes in AR patients. Regarding comparisons with control groups, no clear general gene clustering has been observed, since it depended on the cell type and the stimulatory status. When analyzing the type of genes (e.g. functions and molecules encoded), a high number of them is associated with catalytic activity and metabolism, although other minor groups, such as immune-related genes, receptors and transporters, are statistically enriched in these comparisons. These results show that there are clear differences between DC from AR and ANR patients, corresponding to a broad type of molecules and functions. These differences are perceptible for pDC irrespective of their stimulation status, but are only evident for mDC upon cell stimulation.

Task 3.c: Characterization of differential expression of proteins: phenotypic and functional assays
PBMC samples received in the first shipment have been used for Task 3.b. After analysis of results obtained in that Task, selected genes are to be confirmed at the protein expression level. For these experiments, samples corresponding to a second shipment will be used.

A new set of samples has been collected for this task, but they have not been shipped yet. This will be achieved by Month 50.

Task 3.d: Phenotypic and functional experiments on NK cells

As a consequence of the departure of Professor Salim Khakoo from Imperial (Partner 9) in October 2011, a different approach was undertaken to analyze NK cells. Instead of functional experiments, a genetic analysis has been carried out to characterize NK cells from individuals with acute HCV, comparing those spontaneously clearing HCV infection with those becoming chronically infected. Egyptian and Moroccan DNA samples were genotyped with regards to the composition of their KIR gene repertoire, consisting of inhibitory and activating NK cell receptors. We used an RT-PCR method which has the potential to discover a higher complexity compared to conventional KIR genotyping (Jing et al, Genome Research 2012), including the detection of copy number variation (CNV). 170 Egyptian samples were analysed:

- 51 spontaneous resolvers, 10 from acute infection;
- 2 acute NRC samples;
- 74 chronic (one from acute infection);
- 3 treated resolved from acute;
- 40 hospital workers (14 with reported needle stick injuries without consequent HCV infection).

Using this method we, also, determined the full repertoire of KIR genes in 46 Moroccan Resolvers and 47 Moroccan Chronics (11 undergoing treatment). The genotype for the HLA-C ligand (C1/C2) was determined in the Egyptian and the Moroccan cohorts. The results will enable correlation of the presence of specific NK cell receptors with functional data and outcome of infection.
Status: The genotyping of the Egyptian samples has been completed for KIR and HLA-C1/2, but due to the original delay in the availability of samples, followed by a technical problem with the genotyping facility (which has now been resolved), and the complexity of the dataset, which also needs to be combined with other genetic information in WP2, like IL28B and IFNL4, the detailed analysis is still in progress.

Task 3.e: Co-culture experiments using NK cells and autologous dendritic cells

As a consequence of the lack of available samples for these studies and the departure of Professor Salim Khakoo from Imperial (Partner 9) in October 2011, no contributions to tasks 3e (co-culture of NK and DC) was made.

Hurdles and rescue plans:

Although some tasks have been completed, there are still some deviations from the program. These deviations are mainly due to the political situation in Egypt (which was proposed as the main source of samples), leading to delay in sample collection and shipment. However, European and Moroccan partners have recruited a number of patients, allowing initial shipment of PBMC samples for experiments by European partners. Thus, although delayed, Tasks 3a, 3b and 3d have been completed. The main impact of this delay has been that results from Task 3.b were necessary to progress on Tasks 3.c and 3.e. However, with results of Task 3.b available at this moment, we can continue with remaining tasks and finish them on month 50, i.e. September 2014.
A second deviation of the program is related to NK cell analysis. After departure of Prof. Khakoo from Imperial (partner 9), instead of functional experiments, which were under his expertise, a genetic analysis has been carried out to characterize NK cells. These studies have focused on KIR molecules, responsible for interaction with other immune cells, including DC. Although with a delay, analysis of these results, together with the presence of related genes found in DC studies, will allow to carry out experiments of Task 3.e, involving co-culture of DC and NK cells.

WP4: Viral tropism and adaptive humoral responses

Ongoing problems in Egypt with sampling have precluded completion of the prime tasks of this WP. Given the lack of material to work with, we re-assessed the primary objectives of WP4 and formulated a rescue plan which has solved many of the scientific objectives using an alternative approach. These changes in methodology (see below) do not lend themselves readily to simple categorizations against the WP tasks and milestones. However using the funding, we have succeeded in addressing many of the prime objectives elucidated for this workpackage in the original HepaCute application, including a firmer understanding of the processes which drive the immune response to early infection.

Rather than explore the biology of primary infection using Egyptian HCV samples, we have sought to model the primary infection of the liver using alternative model systems available locally (see below). In addition, we focused on the primary objectives; which were not dependent on external clinical resources. We have explored how differences in HCV-receptor engagement define viral transmission routes and how sensitivity to neutralizing antibodies (nAbs) define viral persistence in the acute phase of infection (Task 4a and 4d). HepaCute funding has supported the WP4 related work reported in nine peer reviewed publications (see the Dissemination report).

Task 4.a: Serum recognition of autologous and heterologous HCV gps

We generated a diverse panel of HCV E1E2 expression plasmids which express these proteins and can be recognized by heterologous sera from chronically infected subjects by ELISA. All of the control reagents for these studies were validated and ready to receive clinically characterized serum samples for screening.

Task 4.b Serum antibody neutralization of heterologous virus strains

We generated a panel of HCV E1E2 expression plasmids that generate infectious pseudoparticles that can be neutralized by heterologous sera from chronically infected subjects. In summary, all of the control reagents for these studies are validated.

Task 4.c Serum antibody neutralization of autologous virus strains

The genesis of autologous virus sequences has been delayed by the lack of available samples. The panel of neutralizing antibodies and antisera to be used in this phase of the work is available.

Task 4.d: Receptor tropism of acute virus and escape from immune surveillance during early infection

UoB have published several papers characterizing HCV-receptor engagement (see dissemination report) and have developed systems to rapidly characterize the receptor tropism properties of diverse HCV variants.

Hurdles and rescue plans:

Hurdles

The problems in Egypt and hence the lack of material from acute seroconversion cases has been a source of concern throughout this grant, though that situation may ease in the future. The major funding required under WP4 was at UoB. UoB intentionally delayed the recruitment of staff for HepaCute until January 2012 when samples were first expected to be available. In 2012, we were able to recruit Dr. Michelle Farquhar to carry out work related to the objectives of WP4 and her vast experience has been invaluable in generating a large body of data related to WP4 despite the lack of available clinical material.

Rescue Plan

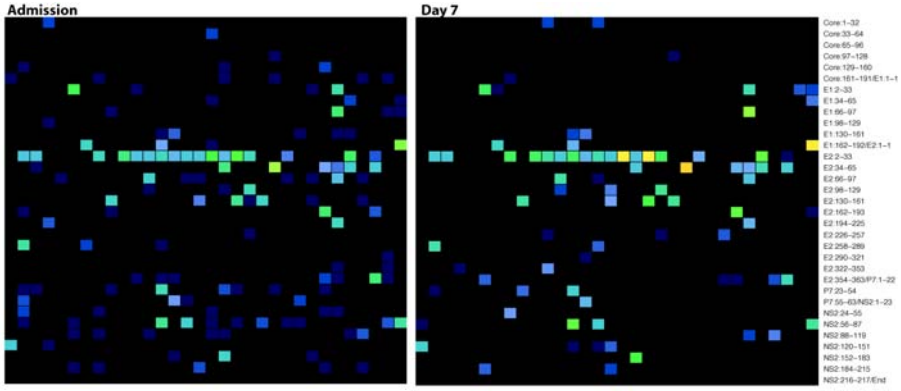
Dr. Farquhar's initial work sought to address the primary interaction of HCV with the hepatocyte. This study showed that primary engagement of the cell by HCV resulted in co-internalization of the virus, CD81 and Claudin-1 to an early endosomal compartment. Interestingly, the work, also, showed that antibodies specific for CD81 could neutralize infection long after the virus was internalized, suggesting intracellular trafficking of antibodies to sites of viral infection. Although published only 2 years ago (April 2012), this paper has already attracted 20 citations. A similar high impact paper, also, partially supported by HEPACUTE funding was published at the same time, exploring the susceptibility of non-liver cells to HCV. This paper demonstrated that brain cells bear all of the requisite receptors for HCV entry and can support infection *in vitro*. This work addresses directly the objectives of WP4 and especially deliverables 4b, c and d.

A second objective of WP4 was to elucidate the interplay between HCV and its receptors. To this end, we have published a series of studies partially funded by HEPACUTE which address this area. Publications include studies

on the dynamics of the CD81 and claudin-1 interplay at the cell surface, the distribution of claudin-1 in human liver and the role of polarization in the dynamics of HCV infection. An exploration of the first hours of infection has shown that HCV exhibits surprisingly low infectivity *in vitro*. In addition, we have elucidated the role of TNF-α and HIF-1 α in infection and the role of CD81 in metastatic disease.

The lack of primary seroconversion cases and paucity of clinical material available for study led us to develop an alternative approach to studying the events associated with primary infection. As part of a larger clinical trial, we have been able to collect detailed clinical sample from 23 patients with end stage liver disease who received transplants. Blood samples were obtained on the day of transplant, every 4-8 hours for 48 hours, daily thereafter until 7 days and weekly thereafter, a total of 20 time points. In addition, large scale sampling of the explanted liver was performed at 8 sites representing each of the major lobes. To date, we have completed Ultra-Deep Pyrosequencing (UDP) of the HCV structural region (core-E1E2) from 17 patients before transplant and at 7 days post-transplant. These sample pairs allow us to describe in unprecedented detail the genetic events occurring in the virus during primary infection (See Figure 2). In addition; to date; we have amplified 164 single molecules of RNA from these patients (WP4b). These samples will allow us to perform detailed serological and virological characterization of HCV in the future.

A



B

Patient 20	Admission	Day 7
VTYTTGGAAAQTTAGLASFLTPGPKQD	50.00% (129)	48.40% (91)
A.....F.R.F.....	22.87% (59)	6.91% (13)
.....K.....	6.59% (17)	0% (0)
.....K...F..L.....	4.65% (12)	0% (0)
N.....I.R..G.I.GLFS..A...	4.65% (12)	1.06% (2)
N.....I.R..G.I.G.VS..A...	4.26% (11)	3.19% (6)
N.....V.R..G.I.G.VS..A...	2.33% (6)	12.77% (24)
N.....V.R..G.I.GLFS..A...	2.33% (6)	13.83% (26)
A.....F.R.....	1.16% (3)	0% (0)
A.....	1.16% (3)	1.06% (2)
A.....A...F.R.F.....	0% (0)	12.77% (24)

Figure 2: An example of primary infection in a post-transplant setting. UDP of plasma samples obtained on the day of admission to hospital and 7 days post transplant A). Heatmap representation of sequence diversity across core-E1-E2 in samples at 0 and 7 days. B) HVR sequences detected in plasma samples, 258 sequence fragments encoding the entire HVR were detected in the day 0 pool and 188 at day 7. Although the predominant sequence was preserved over 7 days, there were changes in relative abundance of the different members of the quasispecies and some new variants were seen to emerge.

Albeit problems with the collection of patient samples from Egypt precluded completion of the primary work plan of this WP a very successful rescue plan was implemented and important insights were gained in the understanding of viral tropisms and virus specific humoral immune responses, resulting in series of associated publications.

WP5: Specific Cellular Immune Response

Transcriptome analysis of HCV-specific CD4+ and CD8+ T cells. Since the minute amounts of RNA which can be obtained from very low-frequency T cells represent a major hurdle, initial experiments were devoted to set up effective procedures for the isolation of virus-specific T cells (>95% pure) and to the retrieval/amplification of adequate amounts of mRNA. While transcriptome analysis of HCV specific CD4+ T cells is still in progress and results will be available in 3-6 months, HCV-specific CD8 cell analysis has been completed on samples from two sequential time points of the acute stage of infections with self-limited or chronic evolution. Microarray analysis of HCV- and flu-specific CD8 cells by moderated *t*-test revealed a list of candidate genes (549) differentially regulated in HCV-specific CD8 cells from the acute phase of patients with a different evolution of infection, as a gene expression signature of infection outcome. The most represented, differentially perturbed pathways are involved in regulation of apoptosis, proliferation, response to stress and are related to the cellular metabolism, in particular the metabolism and transport of vitamins and cofactors. Among the most differently expressed genes from this list, which was confirmed also by Gene Set Enrichment Analysis (GSEA), we were able to identify 5 genes that are exclusively expressed in one group of acute patients but not in the other one. This restricted gene list is assumed to contain candidate bio-predictors of infection evolution because of their totally different expression in relation to the outcome. Validation experiments at the protein level are now in progress.
In a second part of the study, virus-specific CD8 cells from 8 patients with a long history of an established chronic hepatitis C and from 4 patients in the resolution phase of infection were analyzed, in order to better define the

features of chronic evolution of infection. By comparing CD8 cells from chronic patients with those of patients in the resolution phase of infection by *t* test and pathway analysis, followed by GSEA, many altered biological processes emerged. Specifically, cytokine and regulatory pathways, such as TGF-beta signaling, IL-1, IL-2, IL-4, IL-5, IL-6 and TNF-alpha, appeared to be dysregulated; in addition, apoptosis and senescence pathway genes were up-regulated.

In order to further identify genes crucial for infection outcome, we then compared by the *t*-test of all samples collected at early, intermediate and late time points from acute self-limited cases on one hand and chronically evolving cases on the other. Among an initial list of 250 differentially regulated genes, 21 genes with an expression profile markedly different in the two groups were selected as potential signatures of chronic evolution or virus clearance in HCV infection to be used as targets for functional experiments aimed at correcting the exhausted T cell function in chronic patients.

Phenotype and function of HCV-specific T cells. An assay to quantify and characterize polyfunctional HCV-specific CD4+ T cells was established and validated. Studying PBMC from AHC patients, we were able to show that HCV-specific CD4+ T-cells initially display a polyfunctional cytokine response (IFN-gamma, TNF-alpha and IL-2), which is gradually lost over time in chronically evolving infections. This analysis, however, could not provide prognostic information at early time points for prediction of viral clearance because during the early acute phase of infection there was no significant difference in functionality between subjects who resolved disease or progressed to chronicity.

Analysis of HLA-B27-restricted HCV-specific CD8+ T cell responses has allowed to identify a unique memory-like CD8+ T cell phenotype of HLA-B27 restricted HCV-specific CD8+ T cells characterized by high expression of CD127 and PD-1 but low expression of other inhibitory markers, such as 2B4, CD160, KLRG1, and Tim-3, that contributes to viral control despite the emergence of clustered viral escape mutations in the targeted epitope.

Role of inhibitory receptors in T cell exhaustion. No correlation was found between expression of inhibitory receptors (PD-1, CTLA-4 and TIM-3) and clinical outcome of acute HCV infection. Moreover, *in vitro* manipulation of PD-1, CTLA-4 and CD137 pathways had only a partial efficacy on functional HCV-specific CD8 T cell restoration.

Genotype 4 epitope mapping. 8 HCV genotype 4 specific CD8+ T cell epitopes have been identified and 4 of them have been deeply characterized in terms of fine mapping and HLA restriction. Additional mapping analysis is in progress on acute patients in partner 12 lab and results will be shortly available.

Task 5.a: Transcriptome analysis and polyfunctionality of HCV-specific CD4+ and CD8+ T cells

Transcriptome analysis of HCV-specific CD8 cells has been completed ***(achieved).*** On the other hand, transcriptome analysis of HCV-specific CD4 cells is still in progress and results of the microarray assays will be available in 3-6 months ***(delayed)***. Analysis of polyfunctional HCV-specific CD4+ T-cells (able to produce IFN-gamma, TNF-alpha and IL-2) has been completed ***(achieved).***

By transcriptome analysis of HCV-specific CD8 cells, 5 genes expressed exclusively in the acute phase of either self-limited or chronically evolving HCV infections have been identified as candidate bio-predictors of infection evolution that are under evaluation in acute HCV patients using antibodies against the correspondent gene encoded proteins. Also, 21 genes with an expression profile markedly different throughout all time points analyzed in self-limited and chronically evolving infections have been identified as candidate targets for functional experiments of T cell functional reconstitution.

Polyfunctional HCV-specific CD4+ T-cells (able to produce IFN-gamma, TNF-alpha and IL-2) were detected in the acute stage of hepatitis C. They were gradually lost over time in chronic evolving infections while they were maintained in self-limited AHC. Since a Th1 differentiation of HCV specific CD4+ T cells is associated with viral clearance and the transcription factors T-bet and Eomesodermin are critical regulators of Th1 differentiation, the expression of T-bet was studied in virus specific CD4+ and CD8+ cells in AHC and in acute hepatitis B, as a control. High expression of T-bet in virus-specific CD8+ T-cells during acute HBV and HCV infection was followed by spontaneous resolution, while T-bet deficiency characterized chronic evolving HCV infections, identifying T-bet expression in HCV-specific CD8+ T cells as a strong early predictor of spontaneous viral clearance.

By analyzing protective HLA-B27 restricted HCV-specific CD8+ T cell responses in the chronic phase of infection, we could demonstrate that elevated *ex vivo* frequencies of HLA-B27 epitope-specific CD8+ T-cells correlated with low viral loads. A similar correlation was not observed for less protective HLA-A02 restricted responses. Viral mutations were detected within the HLA-B27 epitope in all tested patients. HLA-B27 epitope-specific CD8+ T-cells

showed high expression of CD127 and low expression of the inhibitory markers 2B4, CD160, KLRG1, and Tim-3. Despite this memory-like phenotype, HLA-B27 restricted CD8+ T-cells displayed very high PD-1 levels. Their proliferative capacity, however, did not increase after PD-1 blockade, indicating that PD-1 was an activation rather than an exhaustion marker in this context. Thus, the immunodominant HLA-B27 epitope is not only associated with spontaneous clearance of acute HCV infection, but also with low viral loads in persistent infection.

Task 5.b: The role of inhibitory receptors for maintenance or loss of HCV specific CD4+ and CD8+ T cell responses in HCV infection

Work description and progress towards WP objectives for this task:

Analysis of inhibitory receptors (PD-1, CTLA-4, TIM-3) showed that PD-1 and CTLA-4 are universally up-regulated on activated CD4+ T-cells with no significant correlation between their expression in the early acute phase and later outcomes. PD-1 and CTLA-4 expression persisted when infection was not controlled. TIM-3 showed no significant expression in acute or chronic infection and thus seems not to be of critical importance for disease outcome.

PD-1, CTLA-4 and CD137 have been targeted with specific antibodies and ligands for reconstitution of the CD8 cell function in patients with chronically evolving HCV infection. The best efficacy on HCV-specific CD8 function was displayed by the association of anti-PDL1 and CD137L; no further improvement was observed adding anti-CTLA4 antibody.

Task 5.c: T cell epitope mapping in HCV genotype 4 and search for HLA class I alleles associated with spontaneous viral clearance

Work description and progress towards WP objectives for this task:

To date, no HCV genotype 4-specific CD8+ T cell epitopes have been reported in the literature. Partner 4 analyzed a total of 21 HCV genotype 4 infected patients (1 acute and 20 chronics) for HCV genotype 4 specific CD8+ T cell responses. Epitope mapping was done by two different experimental approaches: the first one HLA-independent, based on the use of overlapping peptides spanning the complete HCV genotype 4 proteome; the second one based on epitope prediction depending on the HLA class I haplotype of individual patients. A total of 8 HCV genotype 4 specific CD8+ T cell epitopes were identified; fine mapping and HLA restriction experiments were successfully performed for 4 of these epitopes, 2 HLA-A24.02, 1 HLA-B18.01, and 1 HLA-B35.01 restricted.

Partner 12 enrolled 23 acute HCV patients. PBMCs were obtained at weeks 0, 2, 4, 8, 12, 16, 20, 24 from the acute phase and stimulated with 9 pools of overlapping 15-mer peptides representing the whole HCV genotype 4 proteome. Frequency of HCV specific-T-cells was determined by an IFN-gamma ELISpot assay. Higher strength and frequency of T cell responses were detected in patients with acute self-limited hepatitis compared to patients with chronic evolution either undergoing successful (SVR) or unsuccessful (NR) interferon treatment. Epitope mapping has led to the identification of several epitopes mainly contained in nonstructural HCV proteins.

Hurdles and rescue plans:

- Transcriptome analysis of CD8 cells has been completed; functional and molecular validation experiments are in progress with HCV-specific CD8 cells of acute HCV patients with different outcome of infection and a first descriptive manuscript is in preparation.
- For transcriptome analysis of HCV-specific CD4 cells, the recruitment of suitable patients was delayed and a number of technical problems had to be overcome. The amplified RNA is now awaiting microarray analysis pending the recruitment of appropriate controls (EBV and influenza specific CD4+ T cells in healthy controls) that have been more difficult to recruit than expected.
- Epitope mapping in genotype 4 infected patients has already led to the identification of several new epitopes but this part of the project has not been finished yet and the relevance of the identified epitopes with respect to control of infection requires further validation; mapping will be completed in a few weeks time frame in order to be disseminated in scientific publications.

Transcriptome analysis of HCV specific CD8+ T cells allowed the identification of five genes that are differentially expressed during early acute hepatitis C in patients with diverse clinical oucomes. Twenty-one additional genes were differentially expressed at later time points of acute HCV infection. Analysis of T cell regulatory factiors identified T-bet expression in HCV specific CD8+ T cells as a strong predictor of spontaneous viral clearance. Polyfunctional CD4+ T cell responses at an early stage of acute HCV infection are a strong predictor of viral

clearance, whereas the analysis of inhibitory receptors on HCV specific CD4+ T cells failed to discriminate between diverse clinical outcomes.
Fine-mapping and HLA restriction of the first HCV genotype 4 specific CD8+ T cell epitopes was achieved.

WP6: Viral Genetics

To analyse quasispecies formation in acute phase of hepatitis C infection, 6 kb HCV amplicons have been isolated by partner 10 from acute and chronic phase HCV sera that were provided by partners 1, 5, 12, 13, 14 and 17. Recovery of HCV sequences has proven more difficult in acute compared to chronic phase samples. One possible reason was presence of degraded viral RNA, which may be due to inflammation in the acute phase. Because of the presence of degraded RNA, characterization of quasispecies evolution was performed by cloning and sanger sequencing only and not by ultra deep sequencing. A total of 31 full length and 259 partial contigs have been obtained from acute and chronic phase samples and are currently being processed for bioinformatics analysis.

Task 6.a: Genesis of HCV quasispecies cDNA from acute samples

HCV genomes halves have been isolated by partner 10 from acute and chronic phase HCV sera that were provided by partners 1, 5, 12, 13, 14 and 17. Recovery of HCV sequences has been considerably more difficult and less efficient in acute compared to chronic phase samples. We think that this is due to the presence of degraded viral RNA, which we could detect by density gradient analysis. Degradation of viral RNA in acute phase sera may be due to inflammation. We do not think that viral RNA degradation was due to quality of serum conservation, as similar observations were made with acute and chronic phase sera contributed from different partners. Because of the presence of degraded RNA, characterization of quasispecies evolution was continued by cloning and sanger sequencing while ultra deep sequencing has been put on hold. To optimize sanger sequencing degenerate pan-genotypic oligos were developed to allow for efficient sanger sequencing in a genotype independent manner. Using this method, a total of 31 full length and 259 partial contigs have been obtained from acute and chronic phase HCV samples and additional clones are being processed. Further sequencing is currently ongoing in order to complete all partial contigs.

Task 6.b: Sequence analysis of acute phase HCV

Bioinformatic analysis of this set of sequence information has started but will take up to another 6 months, and will be performed by Dr. C Combe, an expert on HCV genetics.

Task 6.c: The role of quasispecies evolution in virus persistence

As mentioned above, a total of 31 full length and 259 partial contigs have been obtained from acute and chronic phase samples. This collection contains sequence information from acute phase and chronic phase sera of the same patient in several cases (including genotype 4 and 3 patients). Besides, we have obtained sequence information of chronic and acute phase sera from genotype 4 infections which will allow a comparative analysis. Bioinformatic analysis of quasispecies evolution in viral persistence will take up to another 6 months, and will also be performed by Dr. C Combe, an expert on HCV genetics.

Task 6.d: Analysis of HCV core sequences that stabilize HLA-E, the ligand of an inhibitory NK cell receptor

The role of WP6 was to sequence the core region of HCV in serum samples where WP2 (Prof. S. Khakoo, Partner 9; Imperial College) had planned to analyse NK cell activity. This task has been abandoned with the departure of S. Khakoo from Imperial College.

Task 6.e Scientific and Technical Training in screening for functional HCV envelope sequences

Training has been successfully provided by Partner 10 to all partners implied. Two lab members from partner 14 (W. El Senousy and M Nar Fathi) visited Partner 10 in France from 16.10.2011 to 02.11.2011. Also, a student (F Zohra Fakhir) from partner 17 spent 6 weeks (01.06.2011 to 15.07.2011) in the laboratory of partner 10. In addition, technology transfer to partners 14 and 17 concerning the isolation and genetic as well as functional characterization of HCV genomes has been successful. Partner 10 has, furthermore, very significantly contributed to the set up of molecular and genetic techniques that were accessible at the Casablanca Summer School to all HepaCute and SPHINX members and several lab members of partner 10 have helped to run the Casablanca summer school.

Hurdles and rescue plans:

Amplification of HCV amplicons from acute phase sera has proven much more difficult than from chronic phase sera. This was an unexpected finding, and WP6 has obtained evidence that this may be due to HCV RNA degradation, possibly induced by inflammation by performing density gradient analysis followed by HCV-specific one step RTqPCR. For that reason, we have based our studies on clonal sequencing analysis, and put the ultra deep sequencing approach on hold. Using standard cloning and sequencing approaches, we have been able to amass an important amount of contigs from genotype 4 sera (acute and chronic) and to obtain HCV genome clones from acute and chronic phase samples in the same patient. The latter type of samples is derived from genotype 3 and 4 patients that became chronic. Furthermore, we were able to amplify and clone sequences from a number of acute resolvers. The bioinformatics analysis is delayed due to the late access to sera, but only a matter of time as the sequencing data have been produced. HCV genomes halves have been isolated from acute and chronic phase. Recovery of HCV sequences has proven much more difficult in acute compared to chronic phase samples. The underlying reason is likely to be degradation of viral RNA due to inflammation in the acute phase. This is a very new and unexpected finding which may have very important implications for the further study of virological samples during acute hepatitis C including the interpretation of viral load measurements. Because of presence of degraded RNA, characterization of quasispecies evolution was performed by subcloning and sanger sequencing while ultra deep sequencing has been put on hold. A total of 31 full length and 259 partial contigs have been obtained to date from acute and chronic phase samples and are currently being processed for bioinformatics analysis.

WP7: Molecular and metabolic markers for viral clearance

The identification of characteristic patterns of proteins in this project is mainly divided into four parts. The analysis of proteins in urine, the cytokine pattern is the serum and the measurement of IDO and miRNA in the serum.

Part 1 urine: Endogenous peptides for HCV in general and acute HCV in particular could be identified by CE-MS in the urinary proteome of patients from North Africa and Europe. When combined to multidimensional marker panels, these peptides allowed for accurate differentiation of chronic and acute HCV case groups from the respective reference groups, including normal subjects without signs of viral infection and patients who cleared the virus spontaneously or after IFNα-therapy. The good performance of the chronic and acute HCV classifier was demonstrated by an area under the curve (AUC) value >0.9 in receiver operating characteristic (ROC) analysis.

Part 2 cytokines: In this part of the analysis, we were able to identify cytokines which divide acute baseline patients with spontaneous clearance of the virus from those who relapsed. Furthermore patients who do not clear the virus within the first 12 weeks show also different cytokine pattern at baseline. In the second part of the analysis with the Egyptian samples, we were able to show specific cytokines which differentiate acute from chronic HCV patients.

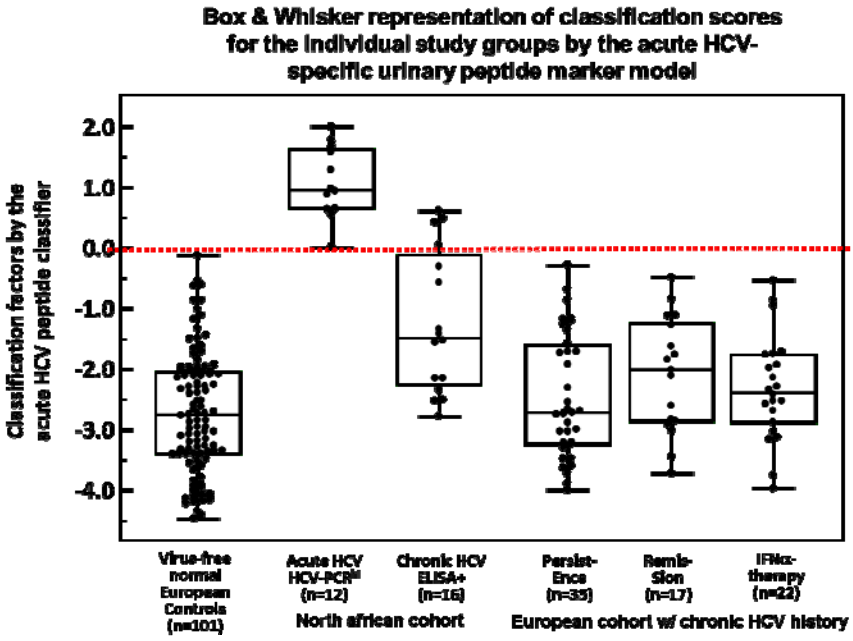
Part 3 IDO analysis: We have found that patients with self-limited infection (n=10) have higher IDO activity during the first two months after first visit (HCV diagnosis) than those who do not clear HCV infection spontaneously (n=9). These differences are maintained at all time-points between diagnosis and week 8 and are lost thereafter.

Part 4 miRNA: Analysis of miR-122 confirmed preliminary results obtained in the first period and showed the following: miR-122 levels are higher when the period of chronic HCV infection is reached, than during the phase of acute hepatitis (no significant data). Although in the first four weeks of the onset of disease, miR-122-levels are higher in patients, who recover. After 4 weeks of onset of AHC infection, patients, who develop later chronic hepatitis, have significantly higher miR-122 levels than patients with spontaneous recovery (p=0.026).

Task 7.a: Identification of biomarkers in urine being associated with spontaneous clearance from acute hepatitis C or chronic evolution

The aim of this task was to establish a multidimensional proteome classifier consisting of urinary peptides indicative for the presence of an acute or chronic HCV infection and sensitive for spontaneous HCV remission and viral clearance by IFN therapy. For this purpose, capillary electrophoresis-mass spectrometry (CE-MS) spectra of urinary samples from (i) 12 North Africans with acute HCV and high virus load, as revealed by high virus titers in PCR, (ii) 16 North Africans with persistent chronic HCV, as revealed by high OD's in a HCV-specific ELISA and persistent viremia and (iii) 35 Europeans with persistent chronic HCV were compared with those from (j) 101

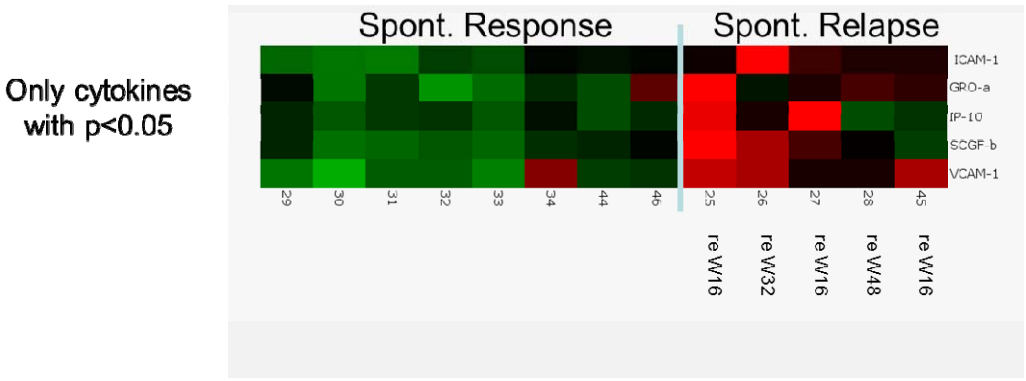
normal Europeans without any known infectious disease, (jj) 17 Europeans after remission of HCV and (jjj) 22 Europeans being virus-free after interferon alpha treatment. A total of 45 peptides with good discriminatory ability in a statistical comparison of the case groups to normal controls ("(i)-(iii) versus (j)") and independently to the HCV resolving reference groups ("(i)-(iii) versus (jj)+(jjj)") were selected for construction of the multidimensional support vector machine-based classification model.



Task 7.b: Identification of distinct serum cytokine/chemokine profiles being associated with spontaneous clearance of acute hepatitis C

The aim of this task was to identify characteristic cytokine patters in sera from patients with AHC and to characterize easy to apply biomarkers to predict the outcome of HCV infection. We analyzed serum with the Biorad Multiplex Cytokine Assay leading to results for in total 50 cytokines and chemokine's.

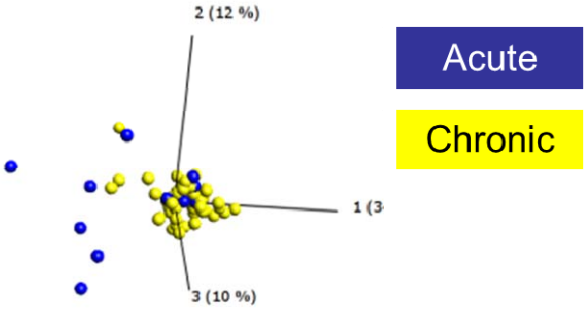
a) Samples from the acute HCV-III trial of the German network of competence on viral hepatitis were analyzed at different time points as well as samples collected by Egyptian partners (acute and chronic samples). We analyzed samples from 26 patients (not treated but observed until week 12) from some of those who spontaneous cleared the infection (8), some did not cleared the infection until week 12 (13) and some relapsed after week 12 (5). Using a principal component analysis we were able to separate typical cytokine/chemokine pattern in the serum samples from baseline (see figure).



b) In collaboration with the German Network of Competence of Viral Hepatitis, we started in August 2011 a new patient's registry for patients with AHC. This registry is still ongoing and until the end of the reporting period, 16 patients have been recruited.

Task 7.c: Identification of distinct serum cytokine/chemokine profiles being associated with treatment response in acute hepatitis C

We analysed the samples from 12 patients with acute HCV (VACSERA) at 8 different time points as well from 50 chronic patients at one time point. We presented in London the first results of these analysis.The aim of this study was to differentiate acute HCV infections from first diagnosis of chronic infections.



Task 7.d: Analysis of IDO activity measured as tryptophan metabolites in the serum of patients with acute hepatitis C

IDO activities (measured as tryptophan metabolites) have been studied in serial serum samples from 19 patients during acute HCV infection. IDO values have been analysed in association with clinical outcome and other clinical and biochemical parameters. We have found that patients with self-limited infection (n=10) have higher IDO activity during the first two months after the first visit (HCV diagnosis) than those who do not clear HCV infection spontaneously (n=9). These differences are maintained at all time-points between diagnosis and week 8 and were lost thereafter. By using a cut-off value of 0.26 (Kynurenine/tryptophan; Kyn/Trp ratio), we have found that 80% of resolver patients have at least a time-point above this cut-off. By contrast, only 11 % of non-resolver reached this value at any time-point (Figure 4). Regarding other clinical parameters, we have found a positive correlation of IDO activity with transaminases and bilirubin, which might suggest a link with inflammation/damage.

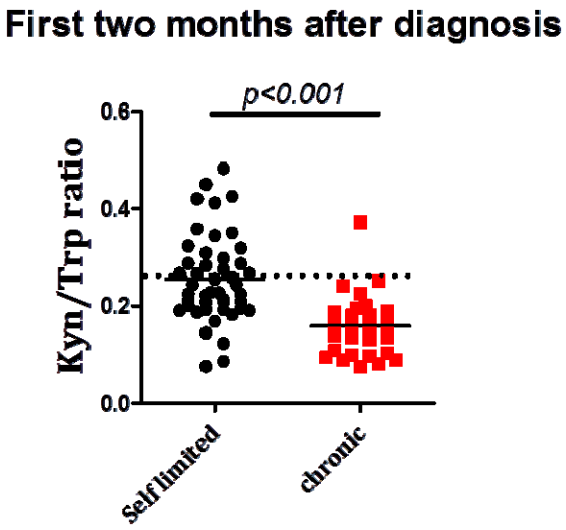


Figure 4: Kynurenine/tryptophan ratio values in sera from patients with acute hepatitis C during the first two months after HCV diagnosis. Dotted line corresponds to a cut-off value of 0.26.

Task 7.e: The role of microRNAs (miRNA) in acute hepatitis C virus infection

From Egyptian Partners, we got a total of 214 plasma samples. 108 samples were derived from patients with chronic hepatitis and 106 samples originated from 16 patients with acute hepatitis C infection, taken at different time points of the first months after the onset of disease. The follow up of these patients has shown that 12 patients recovered and four developed chronic hepatitis. On all samples, we analysed miR-122 and miR-34a,

miR-100 and miR-886-5p, which were selected to be measured on a 2nd cohort according to hierarchical cluster analyses of miRNA profiles.
Analysis of miR-122 confirmed preliminary results obtained in the first period and showed the following:
- miR-122 levels are higher when the period of chronic HCV infection is reached, than during the phase of acute hepatitis (no significant differences).
- although in the first four weeks of the onset of disease, miR-122-levels are higher in patients, who recover,
- after 4 weeks of onset of acute hepatitis infection, patients, who develop later chronic hepatitis, have significantly higher miR-122 levels than patients with spontaneous recovery (p=0.026).
- during the acute phase, the correlation of miR-122 with ALT level was low (rSp =3.7**) in patients developing chronic hepatitis.
The increase of a cluster of circulating miRNAs such as miR-34a, miR-100, miR-29, and miR-886-5b identified by array analyses as potential predictors of chronic hepatitis was confirmed by our studies on the Egyptian cohort, but significant data of outcome prediction were not yet achieved.

Hurdles and rescue plans:
The major hurdle has been the problem that shipment of human samples out of Egypt was not possible within the first period of this project. This led to the delay of the whole project activities in Europe. Partner 3 contacted German authorities to clarify import regulation and provided documents to VACSERA. After clarification partner 12 and partner 3 coordinated the shipment of the samples and the distribution within the consortium.
Urinary peptide markers that are differentially regulated between acute selflimited and chronicall evolving acute hepatitis C were analysed by mass spectrometry. A total of 45 peptides with good discriminatory ability in a statistical comparison of the case groups were selected for construction of the multidimensional support vector machine-based classification model.Strongly predictive serum cytokine patterns could be identified at baseline of patients who cleared acute HCV infection.
IDO values have been analysed in association with clinical outcome and other clinical and biochemical parameters. Higher IDO activity during the first two months of acute hepatitis C predicted spontaneous viral clearance.
In serum form patients with acute hepatitis C miR-122 and miR-34a, miR-100 and miR-886-5p were analyzed. miR-122 levels are increasing in patients developing chronic hepatitis C virus failed to discriminate between viral clearance and persistence at an early time point.

WP8: EU/MPC Research Collaboration in Hepatitis Research" and capacity building in Egypt, Morocco and Europe
WP8 has nine tasks in the HepaCute project. The first task was Joint meetings of the EU-funded SPHINX and HepaCute, together with the Egyptian STDF projects. The second task was Identification International Symposium on the subject of acute hepatitis C in EU and MPC. These two tasks were achieved by conducting joint kick off meetings of both projects in Cairo in 2010. Also, three training workshops were jointly conducted (2 by HepaCute and one by SPHINX). In addition, two EU/STDF/HepaCute/SPHINX program level cooperation meetings were held (one in Cairo in 2010 and one in Barcelona in 2012). Moreover, three scientific workshops were conducted by HepaCute and SPHINX along the last three EASL Congresses, between 2012 and 2014. To achieve the third task, training of MPC researchers at EU institutions, five members from Egypt and Morocco were trained at the EU Labs of the HepaCute participants. Development of consensus guidelines for the diagnosis and management of AHC cases achieved the fourth and fifth tasks of the WP. The sixth task was achieved by enrolling 72 AHC patients, 520 chronic subjects, 128 resolvers and 315 normal subjects. Also, 1630 archived samples were included in the analysis. An Egyptian model of a national data base for AHC cases was developed and contains the demographic, clinical and laboratory data of the study subjects. This achieved the seventh task and the eighth task was performed by the development of a notification system for acute HCV cases. The last objective was to engage in a policy dialogue to translate HepaCute evidence into policy. To achieve this goal, Egyptian Partners (12, 13 and 14) have been in touch with the MOH officials and National HCV treatment committee for implementing the notification system and to adopt/create a registry for HCV cases at the MOH level and to change the treatment guidelines to include AHC cases. Also, IPM (partner 17) has participated in several meetings organized by the Directorate of Epidemiology (Moroccan Ministry of Health) for better management of HCV patients. Updated HepaCute results will be conveyed to the Egyptian MOH for implementation.

Task 8.a: Joint meetings of the EU-funded SPHINX and HepaCute, together with Egyptian STDF projects

After the parallel kick-off and general meetings for the EU-sponsored consortia in November 2010, in Cairo, a joint workshop (HepaCute/SPHINX/STDF) was organized and aimed at highlighting the interactions between EU and Egyptian partners in their shared effort to address public health concerns for the Egyptians and other MPC. These meetings were open to the public and included representatives of the EU funding bodies and Egyptian STDF and Health ministries. Another EU/STDF/HepaCute/SPHINX program level cooperation meeting was held in Barcelona in April 2012. In addition, three scientific workshops were conducted by HepaCute and SPHINX along the last three EASL Congresses, from 2012 to 2014. Moreover, three training open workshops were jointly conducted, two by HepaCute (Nov 2011 and Jan 2014) and one by SPHINX (June 2012).

Task 8.b: Identification International Symposium on the subject of acute hepatitis C in EU and MPC

During the course of the project, HepaCute and SPHINX Consortia organized three open scientific workshops during the last three EASL International Liver conferences (2012-2014). Also, the EU-sponsored consortia (SPHINX and HepaCute) organized, together with STDF projects, two satellite meetings associated with the kick off meeting (2010) and the annual 2012 EASL meetings. These symposia included members of the EU and Egyptian STDF projects as invited speakers and provided a forum for disseminating information and insight learned during the course of sponsored research. Also, high-level epidemiology presentations were presented to inform the community of the public health situation in Egypt and MPC.

Task 8.c: Training of MPC researchers at EU institutions

To achieve this task, five members from Egypt and Morocco were trained at the EU Labs of the HepaCute participants. Also, training programs were organized in a joint effort between SPHINX and HepaCute. One workshop was organized by IPM (partner 17) on molecular techniques and genetics, 25-27 November, 2011, in Casablanca, Morocco. More than 50 participants attended the theoretical day. About 24 young researchers (including four researchers from the SPHINX consortium) participated to the event and had the opportunity to be trained on molecular virology and molecular genetics techniques that are used in the HepaCute activities. One of the workshop activities (production of HCV pseudo-particles) offered the participants the opportunity to work in a biosafety level 3 (BSL-3) lab. The protocols related to the Workshop activities have been made available to all the HepaCute partners on the extranet of the website. Another workshop was held by SPHINX in June 2012 in Tunis, Tunisia, on hepatitis C epidemiology, public health, and molecular markers of viral clearance. A third workshop was organized by HepaCute on advances in immunological techniques in Parma, Italy, in January 2014. Researchers had the chance to work on sorting and characterization of HCV-specific cells using flow cytometry and elispot techniques. Partners 9 and 10 trained individuals from partners 12, 14 and 17 at their labs in the EU on basic viral and host genetic techniques for building research capacities in MPC.

Task 8.d: Assessment of existing local AHC diagnosis-treatment algorithms, collection of epidemiological data and review of ongoing control programmes

This task consisted of listing and evaluating existing diagnosis-treatment algorithms in the MPC and European partners' countries for patients with AHC. International guidelines (e.g. EASL and WHO) were reviewed. Existing epidemiological data and ongoing control programmes were gathered for each of the participating countries. Partner 12 led the task with the contributions of all other partners in this WP. This has contributed to the development of a consensus for management of AHC cases (Task8.e).

Task 8.e: Development of a consensus for the management of newly diagnosed AHC cases

Task 8.e was a follow up on task 8.d which consisted of listing and evaluating all existing diagnosis-treatment algorithms in the MPC and European partners' countries for patients with AHC. During the meetings in Cairo and Casablanca, all partners exchanged their experience with the different management protocols and proposed common guidelines to improve the care of patients with AHC. The HepaCute partners agreed on the diagnostic criteria for AHC and defined an algorithm for the clinical management that includes the timing of blood tests and recommendations for antiviral treatment. Local treatment algorithms were developed in Germany, Italy, Egypt and Morocco. A consensus was regularly discussed and revised according to results generated by the HepaCute partners during the course of the project. The developed algorithm will be exchanged with the SPHINX

Consortium to establish a consensus for the management of newly diagnosed AHC. The consensus that will be reached within this task will be broadly communicated to health care providers, risk groups, policy makers, and others not directly involved in patient care.

Task 8.f: Improving detection rate of new cases of acute hepatitis C

It was crucial for HepaCute to develop strategies to increase the detection rate of acute hepatitis C. In this regard, approximately, 20% of AHC cases are detected as the majority of patients have mild or non-specific symptoms and if no blood test is performed, the diagnosis and thus the opportunity to efficiently prevent chronic hepatitis C is missed. Egyptian, Moroccan and European partners collaborated to enroll a reasonable number of symptomatic AHC cases using several networks and existing cohorts. This work was achieved in close collaboration with existing national networks (HepNet, Swiss Hepatitis C Cohort Study, and STDF in Egypt). High risk groups included: IV drug users, healthcare workers, patients subjected to repeated blood transfusion due to haematological disorders, haemodialysis patients and persons having close contacts with HCV infected patients. A total of 79 AHC cases, 520 chronic patients, 128 HCV resolvers and 315 normal subjects were enrolled into the study. Also, 1630 archived samples were included.

Task 8.g: Establishment of a pilot Egyptian model for a national network/database for acute HCV cases

This task aimed at establishing a network or data base to identify, analyze and monitor acute HCV cases for further immunological, molecular and virological testing in collaboration with the Egyptian and Moroccan Ministries of Health. This network can facilitate clinical care and epidemiological studies, lead to early identification and management of AHC and their follow up, assist adopting a protocol for regular check up for high risk groups, increase patients and family awareness about risk factors of transmission, and improve the medical awareness of general practitioners to use the proper differential diagnosis comprising HCV for diagnosing a case of acute hepatitis. Documentation of our experience in this small network may be used as a model for community or health system-based registries of all patients with diagnosed hepatitis C at the National level. To this end, VACSERA, in collaboration with other Egyptian partners, developed a pilot model for an Egyptian database for AHC cases that was based on the unified clinical/demographic and laboratory data base and sheets that are used throughout by the Egyptian participants in the project. These forms were also made available to partner 17 (IPM). The data base contains the demographic, clinical and laboratory data of the study subjects and is accessible through the internet; only with secure user ID and password (http://hcw.umbegypt.com/HepaCute.htm).

Task 8.h: Development of a notification system for acute HCV cases

The objective of this task was to establish a notification system for reporting AHC cases as a preliminary step for reporting all cases of HCV infection in Egypt in the future following the model of polio and measles reporting. This is because there are some discrepancies in the exact incidence and prevalence of HCV in Egypt. A CRF was prepared and is under trial in certain MOH directorates in Egypt. This objective was reached in collaboration with the Egyptian Ministry of Health through achieving the following specific objectives:

- Identify the current policies and status of reporting HCV cases in Egypt;
- Evaluate the knowledge, attitudes, and practices of healthcare providers on reporting confirmed cases;
- Enforce reporting of AHC cases.

Task 8.i: Engage in a policy dialogue to translate HepaCute evidence into policy

To allow for the field adaptation and the subsequent scaling-up, the pilot study approach developed in task 8.g has been followed, in which the new and validated algorithm (task 8.e) was first piloted on a trial basis in the listed health centres involved in the HepaCute research effort. The results of these pilots have been discussed with stakeholders, to allow them to adopt them for scaling up if results are satisfactory. This pilot approach was developed by partner 12 and will have to be adapted specifically to all sites taking into consideration the country context. To allow for easy training and uptake of the messages, a tool kit with several training materials will be developed for the frontline healthcare workers, and quality assurance manuals and guidelines for programme managers. The adoption of HepaCute recommendations in policy and practice is the responsibility of all partners in different EU and MPC.

Hurdles and rescue plans:

- The kick off meeting took place on November 9-10th 2010, in Cairo, Egypt and two months later the political events took place in Egypt and the work was postponed consequently. The IRB approval by the MOH was also granted in late May 2011. This has been followed up by training of the Fever hospital staff involved in enrolling patients. Therefore, there was a delay in enrolling Egyptian patients into the study.

  Political events continued in Egypt for the duration of the project and certainly affected the enrolment. We enrolled 44 AHC cases in Egypt and 3 cases dropped out.
- The MOH IRB approval of the study mandated no shipment of biological samples outside of the country unless the security officials approve the shipment, which means approval will not be given by the National Security Officials. We found a legal alternative and shipped the samples to the EU partners.

1.4Potential Impact

The major objective of HepaCute as indicated in the call text was the identification of markers of spontaneous viral clearance in acute hepatitis C. The data presented in this final report provide the first comprehensive analysis of genetic, virological, and immunological factors that are associated with viral clearance during acute hepatitis C. As suggested in the call, we have employed state of the art genomics, transcriptomics, and proteomics approaches in addition to advanced immunological and virological technologies including miRNA analysis and deep sequencing. In addition to the basic scientific aspects, a strong focus was on societal and public health issues, particularly addressing the huge burden and unique epidemiology of hepatitis C infection in Egypt.

When the project started, chronic hepatitis C, particularly infection by genotypes 1 and 4, was difficult to treat with rates of sustained viral eradication of below 50%. Treatment was expensive, of long duration and poorly tolerated by the patients. Importantly, success rates of antiviral treatment were dramatically higher in patients with acute hepatitis C, who may have a rate of 30-50% of spontaneous viral clearance, but would clear with IFN treatment in almost 100% of cases. Two goals thus seemed imminent, first, detection of cases of acute hepatitis, which is frequently clinically silent or presents with non-specific symptoms, and second, initiation of early treatment for those patients at risk of developing chronic hepatitis C. A marker of spontaneous viral clearance that could reliably identify, at the time of diagnosis, those patients that will run a self-limited course of infection would direct resources to the patients who need treatment and at the same time would avoid potentially dangerous side effects in those that will clear spontaneously. Due to the high prevalence of chronic hepatitis C in Egypt, there is also a unique epidemiology of acute hepatitis C in this country. Whereas in Western countries, transmission by blood products has virtually been eliminated and the major source of transmission is IV drug abuse, community acquired and also health care associated hepatitis C is still a major problem in Egypt. The optimization of the management of acute hepatitis C would particularly be desirable in Egypt where the incidence is high and the resources are limited.

During the lifetime of HepaCute, both Egypt and the hepatitis C field have faced revolutionary changes. The political changes in Egypt that started shortly after the kick-off meeting of HepaCute in Cairo initially led to a delay in the recruitment of Egyptian patients and several scientific approaches had to be adapted, relying more of European patients. Only in the second half of the project, samples from Egypt could be studied so that the strong focus on genotype 4, the most common hepatitis C virus subtype in Egypt, could not be maintained. Instead, a broader view on the entire spectrum of HCV subtypes as observed in Europe and North Africa formed the basis for our analyses. The major revolution in the hepatitis C field was the development of direct acting antiviral drugs which started by the first generation of protease inhibitors in 2012 but reached a new dimension with the approval of sofosbuvir and simeprevir this year and the already expected approval of daclatasvir and ledipasvir. The combination of two of these drugs, most importantly without interferon, will cure >95% of patients with chronic hepatitis C with an 8-12 week course of well tolerated oral drugs. The major issue of course are the costs which can be as high as 100,000 € per treatment course. However, there are already special programs for developing countries such as Egypt that will receive a discount of up to 99%.

Although it thus seems that the medical problem of hepatitis C is principally solved on the individual patient level, there are important issues that need to be addressed to solve the hepatitis C problem as a whole and most importantly in high-prevalence countries such as Egypt. The results of HepaCute have a significant impact on several aspects of the hepatitis C problem, namely (i) setting up clinical and scientific networks for the identification of patients with acute and chronic hepatitis C, definition of treatment indication and treatment

protocols, (ii) understanding the pathogenesis of acute hepatitis C transmission and infection, (iii) understanding mechanisms of chronic viral persistence, (iv) identification of markers of spontaneous viral clearance, (v) providing a rational basis for the development of preventive hepatitis C vaccines. In addition, hepatitis C is a unique disease model that allows the characterization of immunological and virological mechanisms of spontaneous viral clearance versus chronic viral persistence. It can therefore be expected that the results of the comprehensive studies in HepaCute will also (vi) enhance our understanding of virus-host interaction in general and will support (vii) the development of preventive and therapeutic strategies against other chronic infectious disease.
The impact of HepaCute can thus be summarized as follows:

(i) setting up clinical and scientific networks for the identification of patients with acute and chronic hepatitis C, definition of treatment indication and treatment protocols

In a collaborative effort between Egyptian partners and European research groups a case definition of acute hepatitis C was achieved which will be useful in the future for the identification and subsequently appropriate management of patients with acute hepatitis C in Egypt. The Egyptian partners have developed a network for notification of new cases of hepatitis C virus infection and have established a network between community hospitals and academic centres for the treatment of patients with acute hepatitis C. These networks will be important in the future when rapidly changing treatment strategies for hepatitis C become available and when it will be extremely important to identify patients with special access programs.

(ii) understanding the pathogenesis of acute hepatitis C transmission and infection

Infection of hepatocytes by hepatitis C virus is a very complex event which relies on a series of receptors that have been identified during the last decade. Understanding the humoral immune response that is able to block viral entry and would thus provide sterilizing immunity would be a big step forward towards a successful hepatitis C vaccine. The understanding of the dynamics of the CD81 and claudin-1 interplay at the cell surface, the distribution of claudin-1 in human liver and the role of polarization in the dynamics of HCV infection are key findings of our project. An exploration of the first few hours of infection has shown that HCV exhibits surprisingly low infectivity in vitro. The model of graft reinfection following liver transplantation has helped to elucidate the virus host interaction during the first few hours following infection which may be critical to understand viral tropism and the relevance of viral heterogeneity for primary infection. These are the events that should be prevented be sterilizing immunity and these results will therefore play an important role in vaccine development.
The development of highly effective antiviral drugs will dampen the interest in vaccine development in Western countries. The high costs of antiviral treatment and the high risk of community acquired hepatitis C in high prevalence countries such as Egypt however create a different scenario for these countries. It is highly likely that vaccine development for genotype 4 infection in Egypt will be further pursued since this will eventually be the only way of impacting the hepatitis C epidemic in Egypt.

(iii) understanding mechanisms of chronic viral persistence

While prevention of infection is primarily mediated by the humoral immune response which has the potential to provide sterilizing immunity, clearance of infection depends on virus specific T cell responses. Induction of specific T cells is mediated by a complex interplay of members of the innate immune response such as dendritic cells and NK cells with naïve T cells. Subsequently, virus specific CD4+ T cells are induced which again are indispensable for the induction and maintenance of virus specific CD8 T cells. This scenario can be observed in both acute and chronic infectious diseases as well as in malignancy. For both, infectious disease and malignancy, therapeutic induction of disease specific T cells holds great promise for new treatment strategies and is among the most vigorously pursued strategies in biomedicine today. Hepatitis C is a unique disease in a way that both successful viral clearance as well as chronic viral persistence can be observed in patients with acute hepatitis C. The studies within HepaCute for the first time provide a comprehensive transcriptome analysis of virus specific CD8+ T cells in clinically relevant diverging scenarios (which for example is not possible in HIV infection since spontaneous viral clearance is not observed). These findings will have an enormous impact on our understanding of successful cellular immune responses versus a failing immune response allowing viral persistence, or in the case of

malignancy, tumour progression. The full potential of the clinical use of disease specific T cells will not be available for several years but the studies in acute and chronic hepatitis C provide unique insights to further develop these strategies. While immunotherapy for chronic hepatitis C is unlikely to be further pursued due to the highly effective new antiviral drugs, a very closely related disease, chronic hepatitis B, may dramatically benefit from these insights gained in hepatitis C. Chronic hepatitis B affects 350 million patients worldwide and is the most prevalent chronic viral infection and a leading cause of cirrhosis and hepatocellular carcinoma. For chronic hepatitis B, usually lifelong treatment with expensive antiviral drugs is required and it is a major goal in the biotechnology field at the moment to combine antiviral drugs with hepatitis B virus specific immunotherapies to achieve sustained viral clearance. This would be of paramount importance for developing countries where resources do not allow long term drug treatment. But even in Western countries, long term compliance with antiviral drugs is an issue and carries the risk of treatment failure and resistance development which can potentially jeopardize a seemingly successful treatment strategy. The characterization of gene patterns of successful and failing T cell responses in hepatitis C will guide similar studies in hepatitis B and will thus foster the development of immunotherapies in this related disease.

(iv) identification of markers of spontaneous viral clearance

While important marker of spontaneous viral clearance have been obtained by high-end technologies such as transcriptome analysis of very rare FACS-sorted dendritic cells or antigen specific T cells, clinically useful candidates predicting viral clearance have been identified using cytokine analysis in peripheral blood of urine proteomics. Instead of finding a single parameter, these approaches identified patterns of cytokines or proteins that yield the strong positive predictive values for spontaneous viral clearance. These results can be readily transformed into clinically applicable test that can in addition to genetic markers identify those patients with a high likelihood of viral clearance.

(v) enhance our understanding of virus-host interaction in general and development of preventive and therapeutic strategies against other chronic infectious disease.

Hepatitis C virus infection and in particular acute hepatitis C provides a unique model of disease that has different outcomes, namely spontaneous viral clearance and chronic viral persistence. The comprehensive analysis of genetic, viral and immunological factors involved in the disease outcome therefore provides a unique data set leading to a better understanding of the pathogenesis of chronic viral infection. It is amazing that we found strong predictive factors for spontaneous viral clearance at every level of virus host interaction. Starting at the host genetics, polymorphisms in the IL-28B/IFNL3 gene region are strongly predictive of disease outcome. While these results become available during the application phase of HepaCute, studies within HepaCute could extend these findings to North African populations. At the next level, the transcriptome analysis of dendritic cell subsets displayed a surprisingly clear pattern of gene expression that is related to viral clearance. Similar findings were obtained with the polymorphisms on the NK cell receptors. Induction of virus specific T cell responses is the next hierarchical step in the antiviral immune response and again, the transcriptome analysis yielded a distinct pattern of gene expression that was associated with viral clearance. It has thus for the first time become obvious that successful clearance of a potentially chronic viral infection depends on every single step in the hierarchy of antiviral immune responses. These findings will play an important role in the comparative analysis of other chronic infections as well as malignant disease. The gene expression profiles identified in dendritic cells and T cells may provide new targets for immunotherapeutic interventions.

(vi) Training of students from Egypt and Morocco

Two workshops have been organized by HepaCute, in Casablanca, Morocco and Parma, Italy. In addition, due to the collaboration with SPHINX, partners from HepaCute had access also to a workshop organized by SHINX in Tunisia. Several researches were exchanged between laboratories within HepaCute and this has led to excellent training and acquisition of expertise in students and researchers from Egypt and Morocco.

(vii) Building a European-Egyptian network for hepatitis C research

The collaboration between European and Egyptian research groups, the exchange of patient material and personal, the joint workshops and regular meeting at the level of the Executive Committee, International Conferences and HepaCute meeting has established a strong network on both a scientific and personal level that promises a sustainable scientific collaboration. Several efforts have been made at a national level in Egypt, involving the Minister of Health, which was hampered by the political instability in Egypt. Despite these obstacles, the links between the individual research groups are strong and new sources for common research funding are actively pursued.

1.5 Public website and contact

http://www.hepacute.eu/

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Logo:

A Euro-Egyptian initiative
to fight Hepatitis C infections
HEPACUTE
مبادرة أوروبية-مصرية
لمكافحة عدوى الالتهاب الكبدي C

2. Use and Dissemination of foreground

Section A (Public)

LIST OF SCIENTIFIC (PEER REVIEWED) PUBLICATIONS										
Nº	**Title**	**Main Author**	**Title of the periodical or the series**	**Number, date or frequency**	**Publisher**	**Place of publication**	**Year of publication**	**Relevant pages**	**Permanent identifier** (if available)	**Open access** (Yes / No)
2012										
1	A genetic validation study reveals a role of vitamin D metabolism in the response to interferon-alfa-based therapy of chronic hepatitis C.	Lange CM	PLoS One				2012		PMID: 22808108	Yes
2	Hepatitis C virus induces CD81 and claudin-1 endocytosis.	Farquhar MJ, Hu K, Harris HJ, Davis C, Brimacombe CL, Fletcher SJ, Baumert TF, Rappoport JZ, Balfe P, McKeating JA.	Journal of Virology	1	ASM	USA	2012	86: 4305-16		Yes
3	Hepatitis C virus infects the endothelial cells of the blood-brain barrier.	Fletcher NF, Wilson GK, Murray J, Hu K, Lewis A, Reynolds GM, Stamataki Z, Meredith LW, Rowe IA, Luo	Gastroenterology	1	AASLD	USA	2012	142: 634-643		Yes

		G, Lopez-Ramirez MA, Baumert TF, Weksler B, Couraud PO, Kim KS, Romero IA, Jopling C, Morgello S, Balfe P, McKeating JA.								
4	In silico directed mutagenesis identifies the CD81/claudin-1 hepatitis C virus receptor interface.	Davis C, Harris HJ, Hu K, Drummer HE, McKeating JA, Mullins JG, Balfe P.	Cellular Microbiology	1	Elsevier	USA	2012	14: 1892-903		Yes
5	A dual role for hypoxia inducible factor-1α in the hepatitis C virus lifecycle and hepatoma migration.	Wilson GK, Brimacombe CL, Rowe IA, Reynolds GM, Fletcher NF, Stamataki Z, Bhogal RH, Simões ML, Ashcroft M, Afford SC, Mitry RR, Dhawan A, Mee CJ, Hübscher SG, Balfe P, McKeating JA.	Journal of Hepatology	1			2012	56: 803-9		Yes
2013										
6	IL28B polymorphisms predict response to therapy among chronic hepatitis C patients with HCV genotype 4.	Antaki N	J. Viral Hepat.	January			2013	59-64	PMID: 23231085	
7	IL28B expression depends on	Bibert S	J. Exp. Med.	June			2013	1109-16	PMID:	

	a novel TT/-G polymorphism which improves HCV clearance prediction.								23712427	
8	Comparative genetic analyses point to HCP5 as susceptibility locus for HCV-associated hepatocellular carcinoma.	Lange CM	J. Hepatol.	September			2013	504-9	PMID: 23665287	
9	Genetic Variation in the Interleukin-28B Gene Is Associated with Spontaneous Clearance and Progression of Hepatitis C Virus in Moroccan Patients	Sayeh Ezzikouri, Rhimou Alaoui, Khadija Rebbani, Ikram Brahim, Fatima-Zohra Fakhir,Salwa Nadir, Helmut Diepolder, Salim I. Khakoo, Mark Thursz, Soumaya Benjelloun	PLOS ONE	January 2013 | Volume 8 | Issue 1 | e54793	Dr. Yong-Gang Yao		2013			yes
10	Lack of variant specific CD8+ T-cell response against mutant and pre-existing variants leads to outgrowth of particular clones in acute hepatitis C	Ulsenheimer A, Paranhos-Baccalà G, Komurian-Pradel F, Raziorrouh B, Kurktschiev P, Diepolder HM, Zachoval R, Spannagl M, Jung MC, Gruener NH				Virol J	2013 Sep	28;10:295		

11	Hep-Net Acute HCV-III Study Group. Delayed versus immediate treatment for patients with acute hepatitis C: a randomised controlled non-inferiority trial	Deterding K, Grüner N, Buggisch P, Wiegand J, Galle PR, Spengler U, Hinrichsen H, Berg T, Potthoff A, Malek N, Großhennig A, Koch A, Diepolder H, Lüth S, Feyerabend S, Jung MC, Rogalska-Taranta M, Schlaphoff V, Cornberg M, Manns MP, Wedemeyer H				Lancet Infect Dis.	2013 June	13(6):497-506		
12	Influence of hepatitis C virus infection and high virus serum load on biliary complications in liver transplantation	Horster S, Bäuerlein FJ, Mandel P, Raziorrouh B, Hopf C, Stemmler HJ, Guba M, Angele M, Stangl M, Rentsch M, Frey L, Kaspar M, Kaczmarek I, Eberle J, Nickel T, Gruener N, Zachoval R, Diepolder H				Transpl Infect Dis	2013 June	15(3):306-13		

13	Therapeutic DNA vaccination using in vivo electroporation followed by standard of care therapy in patients with genotype 1 chronic hepatitis C	Weiland O, Ahlén G, Diepolder H, Jung MC, Levander S, Fons M, Mathiesen I, Sardesai NY, Vahlne A, Frelin L, Sällberg M				Mol Ther	Sept 2013	21(9):1796-805		
14	The allele 4 of neck region liver-lymph node-specific ICAM-3-grabbing integrin variant is associated with spontaneous clearance of hepatitis C virus and decrease of viral loads	S. Ezzikouri, K. Rebbani, F.-Z. Fakhir, R. Alaoui, S. Nadir, H. Diepolder, M. Thursz, S. I. Khakoo and S. Benjelloun	Clin Microbiol Infect	6 NOV 2013 DOI: 10.1111/1469-0691.12403			2013			No
15	Urine proteomic analysis differentiates cholangiocarcinoma from primary sclerosing cholangitis and other benign biliary disorders.	Metzger J	Gut	62(1)	British Society of Gastroenterology	http://gut.bmj.com/content/62/1/122.long	2013	Pp 122-130	PMID: 22580416	No
16	Heterogeneous claudin-1 expression in human liver.	Harris HJ, Wilson GK, Hübscher SG, McKeating JA.	Hepatology	1		Europe	2013	57: 854-5		Yes
17	Hepatoma polarization limits CD81 and hepatitis C virus dynamics.	Harris HJ, Clerte C, Farquhar MJ, Goodall M, Hu K, Rassam P, Dosset P, Wilson GK, Balfe P, Ijzendoorn SC,	Cellular microbiology	1			2013	15: 430-45		Yes

		Milhiet PE, McKeating JA.								
18	Early infection events highlight the limited transmissibility of hepatitis C virus in vitro.	Meredith LW, Harris HJ, Wilson GK, Fletcher NF, Balfe P, McKeating JA.	Journal of Hepatology	1			2013	58: 1074-80		Yes
19	High Spontaneous Clearance of Symptomatic Iatrogenic Acute Hepatitis C Genotype 4 Infection	**M. Hashem**, H. Zaghla, **Z.Zakaria, W. Ramadan, N.N. Mikhail, M.Sobhy, G. Galal, I. Galal, S. F. Abdelwahab,** and I. Waked	AASLD meeting (USA)	1 poster	AASLD	USA	2013	AASLD meeting abstract book The poster is #1458, page 915A http://onlinelibrary.wiley.com/store/10.1002/hep.26727/asset/hep26727.pdf?v=1&t=hvsgbubx&s=e4fd211b3a77f03aed0a42380bbdaf75ab1bea1f		

20	Detection of allele specific difference of IL28B mRNA expression	Knapp et al		BMC (BioMed Central)						Under review
2014										
21	The Adiponutrin I148M variant is a risk factor for HCV-associated Liver Cancer in North-African Patients	Sayeh Ezzikouri, Rhimou Alaoui, Sana Tazi, Salwa Nadir, Naima Elmdaghri, Pascal Pineau, Soumaya Benjelloun	Infection, Genetics and Evolution	Jan;21:179-83. doi: 10.1016			2014			No
22	Clearance of HBV markers and HCC risk: who is safe?	Diepolder HM				Gut.	2014 Jan 7			
23	Activated macrophages promote hepatitis C virus entry in a tumor necrosis factor-dependent manner.	Fletcher NF, Sutaria R, Jo J, Barnes A, Blahova M, Meredith LW, Cosset FL, Curbishley SM, Adams DH, Bertoletti A, McKeating JA.	Hepatology	1			2014	59: 1320-30		Yes
24	A role for CD81 and hepatitis C virus in hepatoma mobility.	Brimacombe CL, Wilson GK, Hübscher SG, McKeating JA, Farquhar MJ.	Viruses	1			2014	6: 1454-72		Yes
25	Clinical significance of the CCR5delta32 allele in hepatitis	Isabelle Morard	(currently submitted to		plos.org, California					YES

	C		PLoS One)		(US) corporation #C2354500, based in San Francisco					
26	IL28B in HCV genotype 4 in Egyptian cohort	Knapp et al								Manuscript in preparation
27	KIR in North African cohort	Traherne and Knapp								Manuscript in preparation
28	EASL Clinical Practice Guidelines: management of hepatitis C virus infection.	Mutimer D, Aghemo A, Diepolder H, Negro F, Robaeys G, Ryder S, Zoulim F, Peck M, Craxi A, Fried M, Zeuzem S.			J Hepatol		2014 Feb	60(2):392-420		
229	Preparation of the publication of our data entitled UK-FR: 1) Circulating miRNA profiles of patients with acute hepatitis C infection. 2) High miR-122 levels in the acute phase of hepatitis C infection indicate the progression of chronic liver disease.	Odenthal ,.M	/	/	Planned in: Wiley Online Library	Planned in: Hepatology	2014	not determined yet	/	yes
30	Publication (in preparation)	Martin et al.	A protective	This paper				internati		

			HLA-B*27 epitope contributes to HCV control in chronic infection despite presence of viral escape mutations and is characterized by a unique T-cell phenotype	will be submitted soon to a high-ranking international journal				onal		
31	Publication (in preparation)	Grass et al.	Characterization of the hepatitis C virus genotype 4 specific CD8+ T cell response	This paper will be submitted to an international journal				internati onal		
32	Publication (in preparation): Genomic expression analysis of HCV-specific CD8 T cells in acute HCV infection reveals an early outcome-related gene signature	Barili et al.	The paper will be submitted soon to a high-ranking international journal							

A2: LIST OF DISSEMINATION ACTIVITIES

Nº	Type of activities	Main leader	Title	Date	Place	Type of audience	Size of audience	Countries addressed
1	invited speaker	Mc Keating	Symposium on Viral hepatitis & Liver Cancer	20/06/2012	Beijing	Academic/Clinical	200	International
2	invited speaker	Mc Keating	ISVHLD	22/06/2012	Shanghai	Academic	1500	International
3	Invited seminar	Mc Keating	Novartis Global Vaccines, Siena	09/07/2012	Italy	Academic	50	International
4	invited chair	Mc Keating	19th International HCV Meeting	05/10/2012	Italy	Academic/Clinical	2500	International
5	Invited chair	Mc Keating	New frontiers in microbiology and infection	08/10/2012	UK	Academic	200	International
6	invited seminar	Mc Keating	Sun Yat Sen University	20/10/2012	China	Academic	500	International
7	Lecture	Odenthal M.	Extracellular miRNA: Putative biomarker of acute and chronic liver disease.	December/ 04/2012	Vienna	Hepatologists , Virologists	50	Austria
8	Presentation (oral)	Barili V et al	Transcriptome profile of HCV-specific CD8 cells in early HCV infection	December 5 2012	Milano, Italy (Joint AISF-SIICA Meeting)	Physicians, Scientists	Congress attended by approximately 300 participants	International
9	invited speaker	Mc	AISF-SIICA Liver Immunology	05/12/2012	Rome	Academic	200	Internation

[1] A permanent identifier should be a persistent link to the published version full text if open access or abstract if article is pay per view) or to the final manuscript accepted for publication (link to article in repository).

[2] Open Access is defined as free of charge access for anyone via Internet. Please answer "yes" if the open access to the publication is already established and also if the embargo period for open access is not yet over but you intend to establish open access afterwards

A2: LIST OF DISSEMINATION ACTIVITIES

Nº	Type of activities	Main leader	Title	Date	Place	Type of audience	Size of audience	Countries addressed
		Keating						al
10	invited speaker	Mc Keating	HCV Research UK Meeting	21/01/2013	Glasgow	Academic	150	UK
11	invited seminar	Mc Keating	Institute of Virology	08/04/2013	Munich	Academic	50	International
12	invited seminar	Mc Keating	UCL, Division of Immunity & Infection	15/04/2013	London	Academic	50	International
13	EASL-HepaCute & SPHINX Workshop	H. Diepolder and D. Duffy	EASL-HepaCute & SPHINX Workshop	24 April 2013	Amsterdam	EASL participants, HepaCute and SPHINX participants	~35	GENERAL
14	invited seminar	Mc Keating	CIIC, University of Birmingham	24/05/2013	UK	Academic	50	UK
15	invited seminar	Mc Keating	Duke UOS, Emerging Pathogens	05/06/2013	Singapore	Academic/Clinical	200	International
16	invited speaker	Mc Keating	APASL Liver Week	07/06/2013	Singapore	Academic/Clinical	500	International
17	invited speaker	Mc Keating	Duke NUS	08/06/2013	Singapore	Academic	500	International
18	invited speaker	Mc Keating	STOP-HCV Launch, Oxford	17/06/2013	UK	Academic	50	International
19	Presentation	Helmut Diepolder	Presentation at the UEGW (European Association for Gastroenterology) in Berlin, October 15th 2013. Session: "Horizon 2020". Title of presentation: HepaCute – a successful FP7 project	Oct 15th 2013	Berlin, Germany	Gastroenterologists	50	International, but mainly European
20	invited	Mc	Fudan University, Shanghai	24/10/2013	China	Academic	50	Internation

A2: LIST OF DISSEMINATION ACTIVITIES

Nº	Type of activities	Main leader	Title	Date	Place	Type of audience	Size of audience	Countries addressed
	seminar	Keating						al
21	invited seminar	Mc Keating	WIIM, Oxford University	06/11/2013	UK	Academic	50	UK
22	Lecture	Odenthal M.	microRNA: Fine Tuners of Altered Gene Expression & Indicators of Disease	November/ 15/2013	Lyon	Basic Scientists	50-100	France
23	Presentation (oral)	Barili et al.	Genomic expression of HCV-specific CD8 T cells in acute and chronic HCV infection	March 13, 2014	Padova, Italy (Liver Gymnasium meeting)	Physicians, Scientists	Congress attended by approximately 100 participants	National
24	Lecture	Odenthal M.	microRNA: Fine Tuners of Altered Gene Expression & Indicators of Disease	March/21/ 2014	Zurich	Pathologists	50	Switzerland
25	Joint meeting HepaCute/SPHINX	H. Diepolder and D. Duffy	Joint meeting HepaCute/SPHINX	April 9th, 2014	London	EASL participants as well as HepaCute and SPHINX participants	~75	GENERAL
26	Presentation (oral)	Martin et al.	A protective HLA-B*27 epitope contributes to HCV control in chronic infection despite presence of viral escape mutations and is characterized by a unique T-cell phenotype	April 12, 2014	London	Physicians, Scientists	Congress attended by 10600 participants	international

Section B (Confidential)

Part B1: List of applications for Patents, Trademarks, registered designs

B1: LIST OF APPLICATIONS FOR PATENTS, TRADEMARKS, REGISTERED DESIGNS								
Type of IP Rights	**Application reference(s) (e.g. EP123456)**	**Subject or title of application**	**Confidential [2] (Yes / No)**	**Foreseen embargo date** (dd/mm/yyyy)	**Applicant(s) (as on the application)**	**URL of application**	**Status**	**Actions**

Part B2:

Type of exploitable foreground	**Exploitable Foreground (description)**	**Confidential YES/NO**	**Foreseen embargo date** (dd/mm/yyyy)	**Exploitable product(s) or measure(s)**	**Sector(s) of application**	**Timetable for commercial use or any other use**	**Patents or other IPR exploitation (licenses)**	**Owner & Other Beneficiary(s) involved**	**Status**	**Actions**
Exploitation of results through (social) innovation										
General advancement of knowledge										
General advancement of knowledge										

[2] Note to be confused with the "EU CONFIDENTIAL" classification for some security research projects.

In addition to the table, please provide a text to explain the exploitable foreground (= General advancement of knowledge, commercial exploitation of R&D results, exploitation of R&D results via standards, exploitation of results through EU policies or exploitation of results through (social) innovation), in particular:

- Its purpose
- How the foreground might be exploited, when and by whom
- IPR exploitable measures taken or intended
- Further research necessary, if any
- Potential/expected impact (quantify where possible)

Exploitable Foreground 1 :	
Exploitable Foreground 2 :	
Exploitable Foreground 3 :	
Exploitable Foreground 4 :	

3. Report on Societal implications

See the report online.