28 April 2016
EMA/CHMP/566359/2015
Committee for Medicinal Products for Human Use (CHMP)

Assessment report

Pandemrix

Common name: pandemic influenza vaccine (H1N1) (split virion, inactivated, adjuvanted) A/California/7/2009 (H1N1)v like strain (X-179A)

Procedure No. EMEA/H/C/000832/II/0079

Note

Variation assessment report as adopted by the CHMP with all information of a commercially confidential nature deleted.

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Assessment Timetable / Steps taken for the assessment

Timetable	Dates
Start of procedure:	24 August 2015
CHMP Rapporteur Assessment Report	22 September 2015
PRAC Rapporteur Assessment Report	22 September 2015
PRAC Outcome	8 September 2015
CHMP members comments	n/a
Request for supplementary information (RSI)	22 October 2015
CHMP Rapporteur Assessment Report	23 March 2016
PRAC Rapporteur Assessment Report	23 March 2016
PRAC members comments	n/a
Updated PRAC Rapporteur Assessment Report	n/a
PRAC Outcome	14 April 2016
CHMP members comments	n/a
Updated CHMP Rapporteur Assessment Report	n/a
Opinion	28 April 2016

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1. Background information on the procedure

1.1. Requested type II variation

Pursuant to Article 16 of Commission Regulation (EC) No 1234/2008, GlaxoSmithKline Biologicals submitted to the European Medicines Agency on 5 August 2015 an application for a variation.

The following changes were proposed:

Variation requested		**Type**	**Annexes affected**
C.I.11.b	C.I.11.b - Introduction of, or change(s) to, the obligations and conditions of a marketing authorisation, including the RMP - Implementation of change(s) which require to be further substantiated by new additional data to be submitted by the MAH where significant assessment is required	Type II	I, II, IIIA and IIIB

Update of annex II of the marketing authorisation regarding the conduct of post-authorisation studies. In addition, the opportunity was taken to correct minor editorial errors in the Product information and to bring the PI in line with the latest QRD template version 9.1.

The requested variation proposed amendments to the Summary of Product Characteristics, Annex II, Labelling and Package Leaflet and to the Risk Management Plan (RMP).

The enclosed data package also includes the MAH's position on Ahmed and co-authors recent publication (EMEA/H/C/000832/MEA/119).

1.2. Rationale for the proposed change

The present variation addresses the Post-Authorization Measure ANX121 linked to the MAH narcolepsy research plan. The following final commitments are provided in this data package:

- Identify T cell signature from narcoleptic patients by deep sequencing of total CD4 T cells obtained from narcolepsy patients and DQ0602-matched non-vaccinated healthy subjects and, if identified, verify if signature is found in CD4 T cells from healthy subjects after vaccination with Pandemrix or non-adjuvanted H1N1v vaccine.
- Verify influenza-specificity of hypocretin-specific CD4 T cells from narcoleptic patients by complementary assays and verify if cross-reactive CD4 T cells are found among influenza specific CD4 T cells from healthy subjects after vaccination with Pandemrix or non-adjuvanted H1N1v vaccine.
- Phenotypic characterization of hypocretin and influenza specific T cells after stimulation with hypocretin or influenza peptides.

As mentioned in the GSK letter of outstanding commitments (submitted to EMA on 23 July 2015), some elements of the research activities were ongoing at the time of the initial submission for variation II-79. The MAH obtained the remaining set of data (see listed below) by October 2015 and submitted these data within the Request for Supplementary Information (RSI) during the course of the procedure:

- TCR sequence analysis of CD45RO- CD4 T cells from 9 DQB1*0602+ subjects immunized with Pandemrix, before/after vaccination (Pr. Mignot);
- Single cell TCR sequencing from DQB1*0602 tetramer sorted cells from 7 pairs of samples (narcoleptic patients and controls);
- PCR-based immunophenotyping from DQB1*0602 tetramer sorted cells from 11 pairs of samples (narcoleptic patients and controls).

The MAH proposed to amend the SmPC via the present variation. The proposed changes in the SmPC are mainly in Annex II (amending Sections C & D), and other minor amendments in Annex III (Section labelling).

The dossier also contained an updated Pandemrix Risk Management Plan (RMP version 20) which included an update of the activities from the narcolepsy research plan, removal of solid organ transplant rejection as potential risk, and the addition of the EPI-FLU-H1N1-014 post-authorisation safety study to investigate signal for multiple sclerosis and neuritis.

2. Overall conclusion and impact on the benefit/risk balance

During an Article 20 procedure, the MAH made a commitment to conduct epidemiological and non-clinical (including mechanistic) studies in order to evaluate the association between Pandemrix and narcolepsy. Following EMA scientific advice (EMEA/H/SA/2289/1/2012/III) the company submitted a Type II variation (EMEA/H/C/000832/II/0061) containing a non-clinical research plan consisting of a series of in vitro and in vivo experiments, aiming to assess three hypotheses regarding the potential association between Pandemrix and narcolepsy induction: molecular mimicry, bystander activation and inflammation/damage to the hypothalamus. Translation of the research plan led to a set of objectives that was endorsed by the CHMP on 25 July 2013 and included in the Annex II of the Pandemrix SmPC. This submission represents the final outstanding commitments from this plan and includes the evaluation of the mimicry and bystander activation hypotheses.

Analysis of TCR sequences pre/post Pandemrix vaccination

The tight link between narcolepsy and HLA II haplotype as well as polymorphisms in the T-cell receptor α locus suggest that a specific interaction between the TCR and peptides presented through HLA class II could be important in inducing disease. As part of the planned research objectives, the MAH has been attempting to identify potentially autoreactive and/or disease-associated CD4 T cells by sequence analysis of their T cell receptors, using a new technology known as massive parallel ('deep') sequencing in collaboration with Stanford University. A total of 59 narcolepsy cases and 47 DQB-0602-matched healthy controls were studied. In addition 12 cases versus 5 controls before and after vaccination with a trivalent inactivated influenza vaccine (TIV) were analysed.

The probability of detecting a narcolepsy specific CD4 signature in a small number of patients is low if the frequency and prevalence of corresponding T-cells is low. Initial results for the TCR repertoire analysis were delayed as the technology was optimised to solve sequence error correction issues and to remove naïve CD4 cells from the analysis as a potential source of background noise. The resulting data has been analysed for both V and J chain segment usage and individual clone usage as represented by the hypervariable CDR3 region sequence. Using this technology no statistically significant effects (after Bonferroni correction for multiple comparisons) in terms of TCR repertoire were observed after TIV vaccination of 17 subjects. However, a number of changes in TCR usage approaching statistical significance were found and a larger sample would be needed to confirm these observations.

Analysis of TCR repertoires between narcolepsy patients and DQ0602-matched controls for TCR gene segment usage identified several differences that were nominally significant with some being close to reaching Bonferroni significance. A single TCR gene segment (TRBJ1-3*01) was found to be significantly different between narcoleptic patients and controls after Bonferroni correction. On the individual clone level, no Bonferroni significant differences were observed although a number of clones were differentially present in narcolepsy cases and controls. Again the lack of significance could be due to the small sample size.

Further analysis of TCR sequences pre/post Pandemrix vaccination has been completed and reported following the RSI. Analysis of the new data did not reveal significant differences comparing TCR repertoires before and after Pandemrix vaccination or when all post influenza vaccination samples (TIV and Pandemrix) were pooled.

Therefore, the original conclusion that a single Bonferroni-significant difference between narcolepsy cases and DQB1*0602 matched controls was identified and that there were no detectable differences in TCR repertoire after TIV vaccination, remains unchanged by the current pre/post Pandemrix repertoire data.

It is therefore concluded that either TCR deep sequence analysis of total CD4 T cells is insufficiently sensitive to detect differences because any such differences may be very small (due to low frequencies of relevant T cells in narcolepsy cases and controls) or not relevant (because T cell presence may not correlate with disease).

Molecular mimicry

To assess the mimicry hypothesis a parallel DQB1*0602 CD4 T cell epitope mapping approach was used to study the role of potential cross-reactive CD4 T cell responses focussing on the HA, NA and PB1 proteins from H1N1 and hypocretin. PB1, NA and HA are the only proteins from H1N1v that are present in the reassortant viruses that are used to generate the split influenza vaccine preparations. Hypocretin is the signature protein produced by the hypocretin neurons that regulate wakefulness and low levels of the protein are associated with narcolepsy. The original plan proposed to include a study of cross-reactive responses to set of proteins that are enriched in hypocretin-secreting neurons. However, following early positive results from the peptide binding analysis the research has focused on cross-reactivity to hypocretin only, which although a strong candidate for auto-reactive epitopes remains a potential limitation.

The first step in DQB1*0602 CD4 T cell epitope mapping was to measure binding of overlapping peptides spanning the HA, NA, PB1 and HCRT proteins to DQB1*0602. The analysis revealed multiple binding peptides in each protein. To evaluate the immunological significance of DQB1*0602 binding, CD4 T cell responses to each peptide were measured by IFN-γ ELISPOT. This approach initially produced promising results (De la Herrán-Arita et al, 2013) but IFN-γ ELISPOT data was not reproducible and led to the retraction of the publication by Prof Mignot and co-workers (De la Herrán-Arita et al, 2014) and the need to re-define the research objectives.

Using a complimentary approach Prof Mellins' research team, also from Stanford University, used soluble DQ-0602 HLA tetramers and provided preliminary evidence that the epitopes HA275-287, HCRT56-68 and HCRT87-99 were recognized by DQB1*0602-restricted CD4 T cells from a small number of narcoleptic patients. Unlike the initial IFN-γ ELISPOT data, the signals detected with the tetramer reagents are relatively weak, and require a period of T2/DQB1*0602/epitope co-culture and expansion to be detected, presumably reflecting the low frequencies of such CD4 T cells. Using cross culture experiments they were able to demonstrate cross reactive HRCT-specific CD4 T cells after stimulation with the HA epitope, suggesting the possibility that CD4 T cells that encountered H1N1 influenza epitopes could develop specificity for the HCRT epitopes.

These initial findings supported the hypothesis of CD4 T cell cross-reactivity but it was important to determine if the hypocretin-specific T cells were uniquely present in narcoleptic patients or also found in healthy subjects and how this was affected by vaccination. A study of a further 9 samples (4 narcoleptic patient samples and 5 controls) has shown that HA/HCRT cross-reactive CD4 T cells are also detectable in DQB1*0602+ healthy subjects. This implies that the presence of HA/HCRT cross-reactive CD4 T cells is not sufficient to cause narcolepsy and that these cross-reactive CD4 T cells cannot serve as a biomarker of narcolepsy. Still, these HA/HCRT cross-reactive T cells might be required for disease development but might not be sufficient for induction of narcolepsy. An additional co-factor may be required to induce a pathogenic phenotype that for example changes its migration properties into the CNS. Another explanation may be that cross reactive T cells have a different phenotype in narcoleptic patients and controls, with a more regulatory T cell phenotype in the control group. Alternatively, these cells might not be associated with narcolepsy.

If cross reactive HRCT-specific CD4 T cells are detected after stimulation with the HA epitope, in a DQB1*0602 restricted manner, then it is important to study the behaviour of such cells after vaccination with Pandemrix. PBMC samples from DQB1*0602+ subjects immunized with Pandemrix demonstrated that 1 out of the 5 analysed patients exhibits a cross reactive response against HCRT1 that was already present before vaccination. The signal was competed out by the presence of the HA tetramer but not with a control EBV tetramer, highlighting the specificity of the potential HA cross reactivity. No expansion of these cross reactive cells was seen after vaccination with Pandemrix which might have been expected and the MAH were requested to discuss the implications during the procedure. The MAH considered the observation that no expansion of potentially HA/HCRT cross-reactive CD4 T cells, as detected by DQ0602-tetramers, was seen after vaccination with Pandemrix in healthy subjects, which may reflect immune regulation by regulatory CD4 T cells, or selective expansion of non-cross-reactive T cell clones.

Of note during the tetramer binding studies HCRT-L (N-terminal leader sequence MNLPSTKVSWAAV - Siebold et al., 2004) was chosen as a negative control in the tetramer staining studies based on the assumption the epitope is not presented and that no CD4 T cells against this epitope will exist. The MAH highlight a signal has been detected with the HCRT-L DQB1*0602 tetramer in some samples. Further results from the single cell TCR analysis (see below) suggest the epitope may be presented and recognised by T-cells.

Researchers at Stanford University are also using tetramer-based single cell sorting combined with TCR sequence determination as a complimentary technique to identify novel CD4 T cell responses. This analysis allows the identification of cross-reactive TCRs and if identified enable the determination of their phenotype (e.g. Treg, T17) in terms of expressed transcription factors and cytokines. The original dataset from Prof. Mellins was based on 4 patient samples and provided proof-of-principle for the method: TCR sequences were obtained from tetramer-sorted single CD4 T cells for the HCRT1-13, HCRT87 and HA275 tetramers. Strikingly, TCR sequence identity was observed between CD4 T cells isolated with the HCRT1-13 and HA275 tetramers. A single TCR sequence was identified that was shared between all three tetramers (HA275, HCRT1, HCRT87). This involvement of the HCRT1-13 peptide was surprising since, as discussed above, the HCRT1-13 leader peptide was considered to be a negative control given that no T cell responses to it had so far been described in the literature and also because leader peptides were considered to be not presented to the immune system. The consistent finding that the same TCR was detected when using either the HCRT1-13 or the HA275 DQ0602- tetramer as ‘bait’ suggests that a HCRT1-13-specific CD4 T cell population could exist. In the RSI response, the results from additional samples are reported, such that a total of 12 samples from narcoleptic patients and 12 samples from DQ0602-matched controls have now been analysed by tetramer sorting and TCR sequencing. The major conclusion from the data is that the initial finding of TCR identity between HCRT1-13 and HA275 is reproduced in the additional samples. This TCR match is detected in narcoleptic patients but also in

control subjects. There may be additional cross-reactivity with HCRT87, both with the HCRT1-13 peptide as well as with HA275, consistent with the bulk DQ0602-tetramer expansion and staining experiments.

In order to derive information about phenotype of these cells, a panel of PCR primers was used to amplify transcription factors and cytokines associated with CD4 T cell subsets: Th1, Th2, Th17, T follicular helper cells and T regulatory cells. Overall it is concluded that the initial comparison of the data obtained from single, tetramer-sorted cell from narcoleptic patients and controls does not reveal major differences and suggests that a Th1 phenotype is dominant in both groups.

In summary, H1N1/human hypocretin cross-reactive CD4 T cell epitopes have been identified but they are not unique to patients with narcolepsy and their role in the disease has not been confirmed. It remains possible that, due to limitations in the number of selected proteins from H1N1v (HA/NA) and/or from hypocretin-secreting neurons, the analysis has missed the key epitopes. If molecular mimicry cannot be confirmed as a mechanism, there remains the possibility that a bystander effect may be involved. In this regard one aspect of the plan was to consider the potential for bystander activation of pathogenic cells by the AS03 adjuvant.

Impact of vaccination on CNS/hypothalamus in cotton rats

It has been previously reported as part of the research objectives that neither vaccine nor the AS03 adjuvant alone triggered any detectable changes in the CNS inflammation or changes to blood brain barrier permeability in a cotton rat model (EMEA/H/C/000832/II/0078). AS03-adjuvanted H1N1 vaccine as well as AS03 alone did induce transient increases of neutrophils and monocytes, consistent with its presumed mechanism of action and previously published data. These results do not support a hypothesis that suggests AS03 has a direct inflammatory or toxic effect on the hypothalamus as a potential explanation for the observed association of Pandemrix with the onset of narcolepsy. The absence of a narcolepsy signal associated with administration of AS03 adjuvanted Arepanrix H1N1 vaccine in Quebec provides further evidence against a direct causal role for AS03 in post pandemic narcolepsy.

Comparison of immunological difference between Pandemrix and Arepanrix

Since the adjuvant is the same in Arepanrix and Pandemrix and they are made by somewhat different manufacturing processes, attention has focused on potential immunologic differences between the two vaccines. In terms of exploring immunological differences, the MAH conducted antibody avidity analyses comparing HA immune responses to both vaccines. The results did not reveal significant differences in terms of humoral responses to HA (EMEA/H/C/000832/II/0076). However, the analysis could not exclude minor antigenic differences in the HA proteins of the two vaccines or in other protein constituents of the respective antigen mixes.

In parallel, a study from the laboratory of Prof Mignot using 2-D gel electrophoresis and mass spectroscopy documented a large number of physiochemical/biological differences between the antigen mixtures of Pandemrix and Arepanrix (Jacob et al., 2014). The most significant change noted in HA is a deamidation of asparagine (N) to aspartic acid (D) at residue 146 in Arepanrix. The authors hypothesised this may represent a HLA DQB1*0602 binding epitope although this has not been confirmed. In response the MAH have sequenced the seed banks for Pandemrix and Arepanrix and confirmed the N/D amino acid change for Arepanrix but not for Pandemrix (the data was provided in the MAH HA Sequence Report in Module 5). The significance of this amino acid substitution is currently not clear.

Vaarala and co-workers compared the antigens from the two vaccines and concluded that differences in amount and aggregation state of NP existed – more high-molecular weight forms of NP were observed in the Pandemrix antigen preparation as compared to the Arepanrix antigen preparation (Vaarala et al, 2014). A Company position statement on this publication has been submitted to EMA (EMEA/H/C/000832/MEA/120). It was also found that antibody levels against NP, and in addition against detergent-modified NP, differed when comparing sera from narcoleptic patients and healthy controls. The

MAH concluded that to some extent, this appears to reflect a role for DQB1*0602 in regulating antibody responses to NP. It is not clear whether such antibodies would play any causative role in narcolepsy. Further work is required to better understand the differences between the two vaccines and the putative role that this might have played in the aetiology of narcolepsy.

Recent literature

A recent publication by Ahmed et al. (2015) hypothesized that differences between the reassortant vaccine strains used to produce Focetria (seed virus X-181) and Pandemrix (seed virus X-179A) could explain the increased risk of narcolepsy following vaccination with Pandemrix. They showed that sera from narcoleptic patients and also from healthy control subjects harbour antibodies that bind the human hypocretin receptor 2 (HCRT-R2). Binding of antibodies to HCRT-R2 was outcompeted by peptides from HCRT-R2 but also by potential mimicry peptides from influenza nuclear protein (NP). One important consideration is that the work published by Ahmed and co-workers was based on a purported single amino acid difference at residue 116 of the NP between the MF59-adjuvanted monovalent A/(H1N1)pdm09 vaccine, Focetria, and Pandemrix, with Focetria having a methionine at that position and Pandemrix, supposedly, an isoleucine. However, sequence analysis performed by the MAH of the seed lots of both Pandemrix and Arepanrix has revealed the presence of a methionine at that position. The MAH has recently submitted to the EMA a position paper on this recent publication (EMEA/H/C/000832/MEA/119) which was provided as an annex to the clinical overview addendum.

Recent studies have shown that mice lacking any adaptive immune responses, including autoimmune responses, infected with a H1N1 influenza A virus strain developed sleep–wake changes similar to those seen in models of narcolepsy (Tesoriero et al 2016 PNAS, 113 p368-77). In the brain, the virus was shown to infect orexin/ hypocretin-producing neurons. It is known that narcolepsy has an environmental component and triggering factors including influenza infection have been proposed.

Model of immune-induced narcolepsy

In the RSI response the MAH updated their current thinking on translating the current data and recent publications into a putative mechanism. The MAH working hypothesis is that a subclinical infection of the CNS by H1N1 influenza virus could lead to the death of some HCRT+ neurons, leading to a danger signal able to recruit influenza specific activated CD4 (expanded by influenza vaccination) and CD8 T cells. Such cell death will also release HCRT from dying neurons that will be then available for microglia to present to activated CD4 T cells. CD4 T cell cross reactivity between HA and HCRT has been suggested by the Prof Mellins' work. Therefore, influenza specific CD4 T cells boosted by the vaccination could cross the BBB and be further activated by microglia expressing HCRT (most probably the HCRT-L). Activation of CD4 T cells allows the 'licensing' of pre-existing influenza activated CD8 T cells present in the CNS. Given the specific loss of HCRT+ neurons in narcolepsy, it is likely that these CD8 T cells might also have some cross reactivity with HCRT to be able to specifically target these neurons.

The MAH hypothesis is not consistent with some observations, such as a lack of evidence for H1N1pdm virus infection as a contributing factor in childhood narcolepsy in Finland (Melen et al 2013), and the lack of changes in the CNS of H1N1–infected cotton rats (EMEA/H/C/000832/II/0078 CHMP AR). However, the hypothesis may serve as a framework to shape future research. The MAH stated that, while the model proposed above seems plausible, they would require having additional supportive evidence to pursue new research.

Conclusions

Based on the evidence generated so far, a hypothesis that takes into account the potential role of antigen is more likely to explain the increased risk of narcolepsy observed with Pandemrix than hypotheses that are based on a direct role for the AS03 adjuvant. It is conceivable that that if there is a role for antigenic mimicry or cross-reactivity on the CD4 T cell level, it is likely to reside in the HA protein, as the only 9

amino acid peptide unique to H1N1pdm09 that binds to DQB1*0602 is from the HA head domain. Other research groups have provided evidence that has highlighted the potential role of other antigenic components of the vaccine. The MAH's research has yielded some informative results with regards to the analysis of TCR repertoires in different populations and in identifying CD4 T cell HA/HCRT cross reactivity. However, results remain preliminary and no firm conclusions can be made at this stage. Based on the evidence generated to date, the benefit/risk balance for Pandemrix as defined before the expiry date of the marketing authorisation remains unchanged.

Whilst the post-authorization measures as reflected in Annex II of the SmPC of Pandemrix can be considered completed, it is recommended that the company continue to support further analysis of cross-reactive T cells and data mining of the TCR sequence data. The company should ensure the timely publication of their research work in this area. The company should continue to submit for evaluation any relevant data generated by them or by independent researchers that could help elucidate the association between narcolepsy and Pandemrix.

Scientific Summary for the EPAR

Please refer to the scientific discussion Pandemrix EMEA/H/C/000832/II/0079-AR.

3. Recommendations

Based on the review of the submitted data, this application regarding the following change:

Variation accepted		**Type**	**Annexes affected**
C.I.11.b	C.I.11.b - Introduction of, or change(s) to, the obligations and conditions of a marketing authorisation, including the RMP - Implementation of change(s) which require to be further substantiated by new additional data to be submitted by the MAH where significant assessment is required	Type II	I, II, IIIA and IIIB

Update of annex II of the marketing authorisation regarding the conduct of post-authorisation studies. In addition, the opportunity was taken to correct minor editorial errors in the Product information and to bring the PI in line with the latest QRD template version 9.1.

☒ is recommended for approval.

The variation leads to amendments to the Summary of Product Characteristics, Annex II, Labelling and Package Leaflet and to the Risk Management Plan (RMP)

The following obligation has been fulfilled, and therefore it is recommended that it be deleted from the Annex II to the Opinion:

Conduct non-clinical (including mechanistic) studies in order to elucidate the role of the vaccine and its adjuvant on the association between Pandemrix and narcolepsy:

- *Identify T cell signature from narcoleptic patients by deep sequencing of total CD4 T cells obtained from narcolepsy patients and DQ0602-matched non-vaccinated healthy subjects and, if identified, verify if signature is found in CD4 T cells from healthy subjects after vaccination with Pandemrix or non-adjuvanted H1N1v vaccine.*

- *Verify influenza-specificity of hypocretin-specific CD4 T cells from narcoleptic patients by complementary assays and verify if cross-reactive CD4 T cells are found among influenza-specific CD4 T cells from healthy subjects after vaccination with Pandemrix or non-adjuvanted H1N1v vaccine.*

- *Phenotypic characterization of hypocretin and influenza-specific T cells after stimulation with hypocretin or influenza peptides.*

4. Scientific discussion

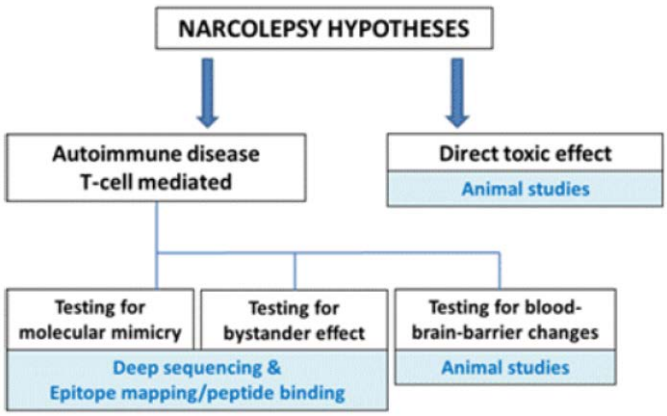
4.1. Introduction

On 27 August 2010, following case reports of narcolepsy after vaccination with Pandemrix originating mainly from Finland and Sweden, a procedure under Article 20 of Regulation (EC) No 726/2004 was initiated to assess these reports and the impact on the product's benefit-risk balance. The MAH has since then been working with the EMA to further investigate the potential association between Pandemrix and the narcolepsy signal.

During the Article 20 procedure, the MAH made the commitment to conduct epidemiological and non-clinical (including mechanistic) studies in order to evaluate the association between Pandemrix and narcolepsy. The MAH submitted a Type II variation (EMEA/H/C/000832/II/0061) containing a non-clinical research plan consisting of a series of *in vitro* and *in vivo* experiments, aiming to assess three hypotheses regarding the potential association between Pandemrix and narcolepsy induction: molecular mimicry, bystander activation and inflammation/damage on the hypothalamus (Figure 1). This research plan was endorsed by the CHMP on 25 July 2013 and included in the Annex II of the Pandemrix SmPC.

In October 2014, the MAH submitted a Type II variation (EMEA/H/C/000832/II/0074) to update the research plan following a data reproducibility issue with the CD4 T cell mimicry work at Stanford University (Palo Alto, USA). This latter variation was approved on 18 December 2014. An overview of the planned non-clinical research activities as per the updated research plan is provided in Table 1. The results from this work package are discussed in the present submission.

Figure 1. Hypotheses to be tested through the proposed non-clinical experiments



4.1. Non-clinical aspects

This submission presents results from the final element of the research plan, which includes the evaluation of the mimicry and bystander activation hypotheses (Step 1 and 2 in Table 1). To this end, the MAH has pursued the following research objectives, in collaboration with Stanford University:

- To study TCR profiles in narcoleptic patients by deep sequencing (step 1A, Table 1) by TCR (TCRA and TCRB) deep sequence analysis of CD4 T cells obtained from narcoleptic patients and DQB1*0602-matched non-vaccinated healthy subjects. If a narcolepsy-specific TCR signature would be identified, then TCR sequence analysis would be conducted to determine whether any TCR (A and B) signatures are induced after vaccination with Pandemrix or non-adjuvanted H1N1v vaccine (step 1C, Table 1).
- To assess the influenza-specificity of hypocretin-specific CD4 T cells identified in narcoleptic patients (step 1B, Table 1) and to assess the presence of any hypocretin-cross reactivity within influenza-specific CD4 T cells in healthy subjects after vaccination with Pandemrix or non-adjuvanted H1N1v vaccine (step 1D, Table 1).
- Evaluate potential H1N1/human cross reactive CD4 T cells epitopes (including HA/NA from H1N1 as well as hypocretin epitopes) and cross-reactive CD4 T cell responses by DQB1*0602 tetramer competition analysis as well as their phenotypic characterization (step 2, Table 1).

Table 2 summarizes the status of post-authorization measures as reflected in Annex II of the summary of product characteristic (SmPC) of Pandemrix. A 'status column' has been added to clearly delineate the activities that are completed as of the August submission of this variation application and the additional ongoing activities that are summarized in Table 1. The MAH anticipated that all commitments would be closed by the end of the procedure, hence the updated SmPC provided in the initial submission did not include this table, as all activities will have been completed by the end (with text indicated by strikeout for deletion in Table 2).

The data are summarized in the following sections and full study reports were provided in Module 5 of the present submission. The MAH re-iterated that it will take into account any emerging data and will update its research plan as needed.

Table 1. Overview of planned research activities to investigate the association between Pandemrix and narcolepsy

Step	Objective	Methods	Status
Evaluation of the mimicry and bystander activation hypotheses			
1A	To study TCR profiles in narcoleptic patients by deep sequencing	Deep sequencing of CD4 TCR (**TCRA and TCRB**) repertoire in narcoleptic patients, with or without influenza vaccination and healthy control subjects. Identification of potential narcolepsy-specific TCR signatures	Completed August 2015; TCRA and TCRB sequencing has been performed in 45/51 patient samples and 33/39 control samples, respectively, thereby exceeding the originiinal plan (n=20) in order to increase power. Results provided in current submission.
1B	To assess the influenza-specificity of any hypocretin-specific CD4 T cells identified in narcoleptic patients	Analysis of DQB1*0602-restricted CD4 T cell responses specific for hcrt1 and hcrt2 using DQB1*0602 tetramer staining following stimulation of PBMC from narcoleptic patients and healthy controls with split influenza or HA epitopes. In addition, DQB1*0602-tetramers for HA275, HRT56 and HCRT87 will be used to single-cell sort tetramer-binding CD4 T cells from which TCR sequence data will be obtained.	Completed August 2015; DQB1*0602 tetramer analysis has been performed on 6 samples from narcoleptic patients and 5 control samples. Results provided in current submission.
1C	To determine whether any TCR (A and B) signatures identified in 1A are induced after vaccination with *Pandemrix* or non adjuvanted H1N1v in healthy subjects	Deep sequencing of CD4 TCR repertoire in vaccinees to assess whether a similar signature (as in 1A) exists after vaccination-Only to be done if a signature is found in 1A.	Ongoing August 2015 - data available by October 2015 ; Because a single Bonferroni significant result was obtained after TCR sequence analysis of the increased number of samples, TCR sequencing of 8 samples from DQB1*0602+ healthy subjects before and after vaccination with *Pandemrix* (16 samples total) has been initiated
1D	To assess the presence of any hypocretin-cross-reactivity within influenza-specific CD4 T cells in healthy subjects after vaccination with *Pandemrix* or non adjuvanted H1N1v vaccine.	DQB1*0602 hcrt tetramer staining of influenza-specific CD4 T cells in vaccinees, after expansion of such cells by in vitro stimulation with split influenza virus antigen (H1N1v) and/or the DQB1*0602-binding HA epitope. In addition, DQB1*0602-tetramers for HA275, HRT56 and HCRT87 will be used to single-cell sort DQB1*0602-tetramer-binding CD4 T cells (if applicable, i.e., if such T cells are detected) from which TCR sequence data will be obtained.	Completed August 2015; DQB1*0602 tetramer staining has been performed on 5 DQB1*0602+ healthy subjects before and after vaccination with Pandemrix (10 samples total). Results provided in current submission.
Step	**Objective**	**Methods**	**Status**
2	To evaluate potential H1N1/human cross reactive CD4 T cells epitopes. This objective evolved into: phenotypic characterization of hypocretin and influenza-specific T cells.	Map DQA1*0102/DQB1*0602 epitopes for HA/NA from H1N1v and for hypocretin as the signature proteins from hypocretin-secreting neurons. Further evaluation of cross-reactive response between HA/NA and hypocretin Test phenotype of specific T cells by flow cytometry and transcriptional profiling.	Completed August 2015: DQB1*0602 binding data have been generated for HA, NA, PB1 and HCRT. Confirmatory HCRT binding data were generated and are provided in the current submission. Completed August 2015: Cross-reactive CD4 T cell responses were evaluated by DQB1*0602 tetramer competition analysis. Results provided in current submission. Ongoing August 2015 - data available by October 2015: Flow cytometry based phenotyping was done by CD25 and CD127 staining but was put on hold due to feasibility issues and prioritization, and replaced by single cell sorting followed by TCR sequencing and PCR-based immune phenotyping: • TCR sequence data are being further analysed (n=3 patients and n+3 controls) or being planned (n=4 patients and n=4 controls) pending samples availability. • PCR phenotyping data are being analysed (n=8 patients and n=8 controls) or being planned (n=4 patients and n=4 contols) pending samples availability.
Evaluation of CNS inflammation/hypothalamus damage and direct toxic effect			
3	To study the impact of vaccination on CNS/ hypothalamus in cotton rats	Vaccinate 4-week old female cotton rats with *Pandemrix* or non-adjuvanted H1N1v vaccine (1/5th of the human dose), in some experiments preceeded by H1N1v infection and analyse impact on CNS: evaluation of hypocretin-secreting neurons, microglia activation, measure of hypocretin in CSF, infiltration of immune cells into CNS, disruption of BBB integrity.	Completed February 2015. CHMP Opinion received on 21 May 2015 (EMEA/H/C/000832/II/0078).
Evaluation of the potential for immunological differences between Pandemrix and Arepanrix H1N1			
4	To evaluate the potential for immunological differences between *Pandemrix* and *Arepanrix* H1N1 using samples from clinical studies in which the two vaccines were compared	Antibody avidity analysis and potentially phage display-assisted epitope mapping (depending on avidity results) from clinical serum samples (Q-Pan H1N1-045) obtained before and at Day 21 after vaccination.	Completed December 2014. CHMP Opinion received on 26 March 2015 (EMEA/H/C/000832/II/0076).

CNS = Central nervous system; CSF = Cerebrospinal fluid; ICS = Intracellular cytokine staining; HLA = Human Leukocyte Antigen; TCRA = T-cell receptor alpha; TCRB = T-cell receptor beta; Th = T helper cell; HA = haemagglutinin; NA = Neuraminidase, BBB = blood brain barrier

Table 2. Overview of planned research activities included in the Annex II of the product information

Description *(Correspondence to Table 1 items)*	Due Date	Status
~~Conduct research (including mechanistic) studies in order to elucidate the role of the vaccine and its adjuvant on the association between Pandemrix and narcolepsy:~~ – ~~Identify T cell signature from narcoleptic patients by deep sequencing of total CD4 T cells obtained from narcolepsy patients and DQ0602-matched non-vaccinated healthy subjects *(1A)* and, if identified, verify if signature is found in CD4 T cells from healthy subjects after vaccination with Pandemrix or non adjuvanted H1N1v vaccine *(1C)*.~~	~~August 2015~~	***1A*** - Done – exceeded number of samples in order to obtain better statistical power ***1C***- Ongoing- Analysis on *Pandemrix* samples
– ~~Verify influenza-specificity of hypocretin-specific CD4 T cells from narcoleptic patients by complementary assays *(1B)* and verify if cross-reactive CD4 T cells are found among influenza-specific CD4 T cells from healthy subjects after vaccination with Pandemrix or non adjuvanted H1N1v vaccine *(1D)*.~~	~~August 2015~~	***1B*** - Done – performed DQB1*0602 tetramer experiments on patient and control samples ***1D*** – Done - performed analysis on Pandemrix samples
– ~~Phenotypic characterization of hypocretin and influenza-specific T cells after stimulation with hypocretin or influenza peptides *(2)*.~~	~~August 2015~~	***2*** - Proof-of-concept for CD25 and CD127 co-staining provided but staining unsatisfactory PCR-based phenotyping based on tetramer-sorted CD4 T cells ongoing

CHMP assessment

The CHMP noted there were ongoing activities that would have been reported at the RSI stage of this procedure. The MAH anticipated that all Annex II commitments will be closed by the end of the procedure; hence the updated SmPC provided in this submission does not include this table.
The CHMP noted that the Company position (EMEA/H/C/000832/MEA/119) on a recent publication, suggesting an immune link between H1N1 Pandemrix vaccination and associated narcolepsy at the antibody level by Ahmed and co-authors, was provided as an annex to the clinical overview addendum.

4.1.1. Evaluation of the mimicry and bystander activation hypotheses

4.1.1.1. TCR deep sequencing (step1A in Table 1)

The objective of the TCR deep sequencing approach was to identify potential TCR sequence biomarkers for narcolepsy and to examine a CD4 T-cell based pathogenesis of the disease.

Methods

Next generation or 'deep' sequencing runs thousands of sequencing reactions in parallel thereby generating large amounts of sequence data in a single run. CD4 T cells from patients and DQB1*0602-matched healthy controls were subjected to RNA extraction, PCR amplification and nucleotide sequencing. The MAH provided a report from Prof Mignot detailing the computational methods used to extract TCRα and β VDJ sequences from Illumina sequencing files. The protocol uses Vx and C primers to amplify all Vx-C TCR α and β segments from RNA extracted from 1 million of cells per sample. For any TCRα and β sequencing project, the end product gives a list of clones with CDR3, V and J IDs and clone frequency.

A total of 59 narcoleptic patients and 47 DQB1*0602-matched controls were studied (Table 3). Final data include Complementarity Determining Region 3 (CDR3) sequences, Variable (V) and Joining (J) IDs and frequencies of individual clones.

Following optimization of the TCR sequencing pipeline and solving error correction issues, several data sets were obtained. For TCRA and TCRB, sequence data were obtained as summarized in Table 3. The changes in TCR repertoire from before to after vaccination with trivalent inactivated influenza vaccine (TIV, Fluzone) were also analysed in 12 cases versus 5 controls.

Table 3. Number of CD4 samples from patients versus matched controls, including samples from before and after TIV vaccination, that were used for TCRA and TCRB deep sequencing

	Narcoleptic patients		DQB1*0602 matched control	
	Total CD4	Memory CD4	Total CD4	Memory CD4
TCRA	14	45	12	33
TCRB	29	51	29	39
TIV (pre/post comparison) (TCRA/TCRB)	-	12*	-	5*

* TCRA and TCRB sequence analysis from CD4 T cells obtained from pre-vaccination results from these subjects was included in the basic cases vs controls TCR sequence data analysis

Effect of vaccination with TIV in narcoleptic patients versus controls

The TCRA and TCRB results addressed two questions:

1. Can analysis of TCR sequence data identify significant repertoire changes resulting from vaccination with TIV? For this analysis, pre- and post-vaccination data from all 17 subjects were analysed with a focus on identifying vaccine-induced changes in TCR repertoires.
2. Is the response to TIV in terms of TCR repertoire different when comparing narcoleptic patients (n=12) and controls (n=5)? For this second analysis, the post vaccination TCR sequence data were compared with the pre-vaccination data per group.

The MAH highlighted that the TCR deep sequencing approach has several limitations:

(1) Since total repertoire is analysed, small changes due to rare CD4 T cell populations are difficult to detect,

(2) Any changes in TCR usage or sequence are not directly linked to antigenic specificity,

(3) Data analysis is limited by statistical power

No statistically significant effects (after Bonferroni correction for multiple comparisons) in terms of TCR repertoire were observed after TIV vaccination of 17 subjects. Usage of one specific TCRB segment, TRBJ2-6*01, was close to reaching statistical significance (P = 0.0066, significance for Bonferroni correction P < 0.003), suggesting that, based on the corrected value, the observed difference is unlikely to be due to chance alone. Table 4 provides an overview of the most important changes in TCR usage after TIV vaccination. Detailed data for each TCR segment with P values were provided in the Mignot report in Module 5.

Table 4. Effect of TIV vaccination on TCRA J and TCRB J and V chain usage

TCR segment	% change after TIV	P value	Bonferroni significance*
TRAJ25*01	+54%	0.013	<0.0008
TRAJ24*02	+12%	0.3	N/A
TRBJ2-6*01	+13%	0.006	<0.003
TRBV04-2*01	+22%	0.06	<0.0009

*P value denoting statistical significance after Bonferroni correction. This P value was calculated by dividing 0.05 by the number of observations and is therefore different for the different analyses.

Further analysis of TCRA and TCRB VJ chain usage revealed that, whereas no single combination reached significance after Bonferroni correction, it was striking that 5 out of 15 combinations that were characterized by (i) P values < 0.05 and (ii) increase over baseline > 5%, contained the TRBJ2-6 segment.

The results from this first analysis indicate that TIV vaccination appears to be associated with changes in TCR repertoire and in particular the segment TRBJ2- 6*01. The small sample size could explain why only marginal statistical significance was observed.

Comparing narcolepsy cases with DQB1*0602 matched controls

Results were presented comparing CD4 T cells from narcoleptic patients with controls that aimed to identify a narcolepsy-specific TCR biomarker. The analysis comprised TCRA and TCRB sequence analysis from both total CD4 T cells and CD45RA-negative non-naive CD4 T cells. For TCR gene segment usage, several differences were nominally significant with some being close to or reaching Bonferroni significance.

The next step of TCR data analysis was to compare results from narcoleptic patients' samples with controls on the individual TCR clone level. This analysis was done for TCRA as well as for TCRB sequences.

On the individual clone level, no Bonferroni significant differences were observed. However several TCRA and TCRB clones were of interest. Detailed results were provided in module 5.

In summary, TCR (TCRA and TCRB) data indicated that differences in TCR repertoire between narcolepsy patients and DQ0602-matched controls exist. From the bioinformatics analysis it was concluded that:

1. Usage of the TRBJ1-3*01 segment was 60% decreased (significant after the Bonferroni correction in narcolepsy patients as compared to controls).

Moreover, marginal significance after Bonferroni correction was reached for two observations:

2. TRBV10-2*01_TRBJ2-2*01 combination was close to Bonferroni significance when comparing narcoleptic patients and controls.
3. Usage of TRBV29-1*01 was 6% increased (P=0.0011, P<0.0009 after Bonferroni correction) in narcolepsy patients as compared control subjects.

4.1.1.2. Evaluation of TCR (A and B) signatures after vaccination with Pandemrix in healthy subjects (Step 1C in Table 1)

It was proposed in the original Research Plan that identification of TCR usage or TCR sequences (defined as "TCR signatures") that could be considered as specific for narcoleptic patients would trigger further analysis of TCR sequences in CD4 T cells pre and post Pandemrix vaccination (Roman et al., 2011). However, because no Bonferroni significant TCR signatures were observed after the data analysis of the first data sets (20 patients and 20 controls) comparing narcoleptic patients and DQB1*0602-matched healthy controls, it was decided to prioritize analyses of additional samples from narcoleptic patients instead of the clinical samples pre/post Pandemrix vaccination. Two important observations were made after the analysis of additional samples (see Table 3 for total sample numbers). First, a single TCR gene segment (TRBJ1-3*01) was found to be significantly different between narcoleptic patients and controls after Bonferroni correction. Second, several TCR sequences appeared to be specific for narcoleptic patients. These observations triggered the decision to also analyse clinical samples obtained before and after Pandemrix vaccination. As a consequence, DQB1*0602- positive PBMC samples were selected from the appropriate clinical study (Roman et al., 2011) and are currently in the sequencing and data analysis

pipeline. Data analysis will focus on differences pre/post vaccination and any emergence of previously identified TCR gene segment and sequences after vaccination with Pandemrix.

CHMP assessment

This aspect of the research plan employed next generation or 'deep' sequencing technology to determine the T-cell receptor repertoire in narcoleptic patients, with or without influenza vaccination and healthy control subjects. The goal of this part of the project was to identify potential TCR sequence biomarkers for narcolepsy to support a CD4-based aetiology of the disease.

It was recognised this aspect of the plan was ambitious, employing state of the art methodology to identify potentially very rare T-cell receptor sequences. The results from these studies were delayed over the course of the programme as significant technical issues had to be resolved before it was considered reliable enough for analyses of potential differences in TCR usage. The researchers have developed the platform to reduce as much as possible the background noise by developing computational methods to remove sequencing errors and increasing the chances of detecting rare signatures by selecting antigen experienced T-cells and including sequencing of both TCR α and β chains. The success of the plan relies upon the robustness and sensitivity of the methodology as the probability of detecting a narcolepsy specific CD4 signature in a small number of patients is low if the frequency and prevalence of corresponding T-cells is low.

The resulting data have been analysed for both TCR V and J chain segment usage and individual clone usage as represented by the CDR3 region sequence. Using this technology no statistically significant effects (after Bonferroni correction for multiple comparisons) in terms of TCR repertoire were observed after TIV vaccination of 17 subjects. However a number of changes in TCR usage approaching statistical significance were detected, in particular the J segment TRBJ2-6*01. A larger sample would be needed to confirm this observation.

Analysis of TCR repertoires between narcolepsy patients and DQ0602-matched controls for TCR gene segment usage identified several differences that were nominally significant with some being close to or reaching Bonferroni significance. On the individual clone level, no Bonferroni significant differences were observed although a number of clones were differentially present in narcolepsy cases and controls.

Note that the Mignot report included in Module 5 does not include detailed results for overall TCRB usage changes in narcolepsy versus controls and only the summary results are included in the overview. The MAH was requested to provide the updated data during the procedure.

The MAH stated that further analysis is ongoing on differences pre/post Pandemrix vaccination. Any emergence of previously identified TCR gene segment and sequences after vaccination with Pandemrix would help confirm if these sequences represent potential markers.

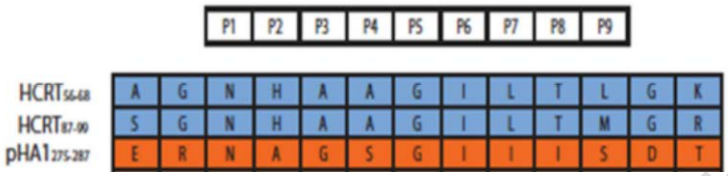
The objective to study TCR profiles in narcoleptic patients by deep sequencing has been formally completed although at this stage only tentative changes in TCR usage have been detected. As discussed due to the potential low prevalence of narcoleptic specific CD4 T-cells, the analysis is difficult and limited by statistical power. Further analysis is ongoing on differences pre/post vaccination and has been reported at the RSI stage of this procedure.

4.1.1.3. Cross-reactive CD4 T cells among influenza-specific CD4 T cells (Step 1B/1D in Table 1)

The original CD4 T cell mimicry hypothesis discussed during the Scientific Advice procedure (EMEA/H/SA/2289/1/2012/III) provided the scientific basis to evaluate the DQB1*0602 peptide binding data (De la Herrán-Arita et al., 2013). HCRT DQB1*0602 peptide binding data for generated by Prof Mignot and his team has been previously reported for HA, NA, PB1 and hypocretin (2013/FUM101).

Among the DQB1*0602-binding peptides, highly overlapping peptides were identified in the HCRT-1 (hypocretin residues 56-68) and HCRT-2 (hypocretin residues 87-99) sequences, differing by a single amino acid (Figure 2). A single DQB1*0602-binding peptide from the HA protein displayed a degree of similarity that was of sufficient interest to pursue further investigation, with the proposed alignment shown in Figure 2. Because the critical residues for DQB1*0602 binding were P1, P3, P4 and P6, this left P2, P5, P7, P8 and P9 as the amino acid residues that potentially interact with the TCR. This peptide sequence alignment was independent of the IFN-γ ELISPOT non-reproducibility issue.

Figure 2. H1N1 HA peptide with strong homology to the DQ*0602-binding HCRT peptides



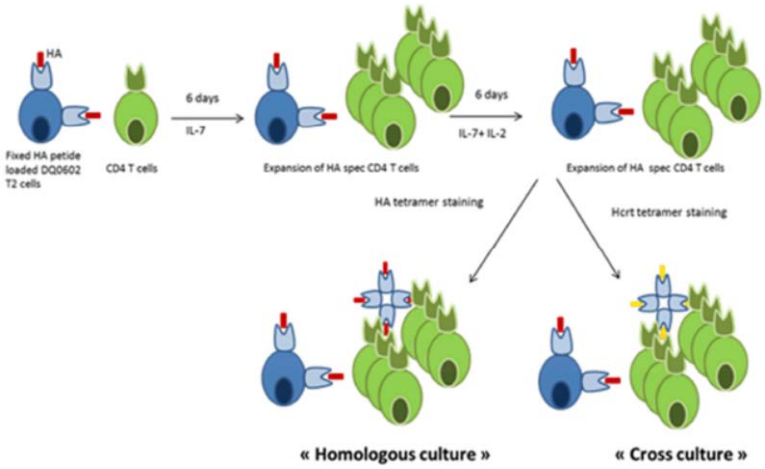
Following the availability of DQB1*0602-binding data, two approaches were originally considered as follow-ups to evaluate potential CD4 T cell cross-reactivity, the IFN-γ ELISPOT assay (De la Herrán-Arita et al., 2014) and direct staining of CD4 T cells with DQB1*0602 tetramers. DQB1*0602 tetramers are tetrameric HLA peptide complexes that contain a fluorescent label and are used to directly visualize antigen-specific T cells by flow cytometry. DQB1*0602 tetramers were constructed for the two HCRT peptides (HCRT-1 and HCRT-2, spanning residues 56-68 and 87-99, respectively), for the HA275-287 peptide (HA-275) (Figure 2), and for the previously identified hypocretin 1-13 peptide (the hypocretin leader peptide, referred to as HCRT-L, sequence: MNLPSTKVSWAAV) (Siebold et al., 2004). It was decided to use the DQB1*0602 tetramer technology to provide independent evidence for the existence of HA and HCRT-specific CD4 T cells. These DQB1*0602 tetramer reagents were constructed by the NIH Tetramer Core Facility for the laboratory of Prof Elizabeth Mellins, also at Stanford University.

At the time when the IFN-γ ELISPOT non-reproducibility issue surfaced, in June 2014 (De la Herrán-Arita et al., 2014), Prof Mellins had brought a preliminary data set to the MAH's attention indicating that DQB1*0602 tetramers carrying the HA275 (HA), HCRT- 1 and HCRT-2 peptides were capable of identifying CD4 T cells in narcoleptic patients (see below). These preliminary data suggested that CD4 T cells specific for these peptides did exist. Frequencies of DQB1*0602 tetramer-positive CD4 T cells were low (much lower than the originally reported IFN-γ ELISPOT data) and CD4 T cells had to undergo *in vitro* expansion to allow visualization of HA and HCRT-specific CD4 T cells. The discrepancy in frequencies between the original IFN-γ ELISPOT data and the DQB1*0602 tetramer staining data suggested that the two approaches were not measuring the same parameters. At the time, the two limitations of Prof Mellins' preliminary data set were (i) the limited number of samples that had been analysed by DQB1*0602 tetramer staining and (ii) the fact that only samples from narcoleptic patients had been analysed, precluding any conclusion on the narcolepsy-specificity of the DQB1*0602 tetramer signal.

Methods and preliminary DQB1*0602 tetramer results

DQB1*0602 tetramers are tetrameric HLA peptide complexes that contain a fluorescent label and that are used to directly visualize antigen-specific T cells by flow cytometry. The preliminary data set provided to the MAH revealed that CD4 T cells binding to the HCRT-1, HCRT-2 and HA-275 DQB1*0602 tetramers were detectable but that detection required in vitro culture of the T cells.

Figure 3. Co-culture and CD4 T cell expansion experiment. Fixed DQB1*0602 T2 cells loaded with HA peptide were co-cultured with purified homologous CD4 T cells in the presence of IL-2 and IL-7. After 12 days of expansion, either homologous HA tetramer ("homologous culture") or heterologous HCRT tetramer staining ("cross culture") was conducted to assess cross reactivity.

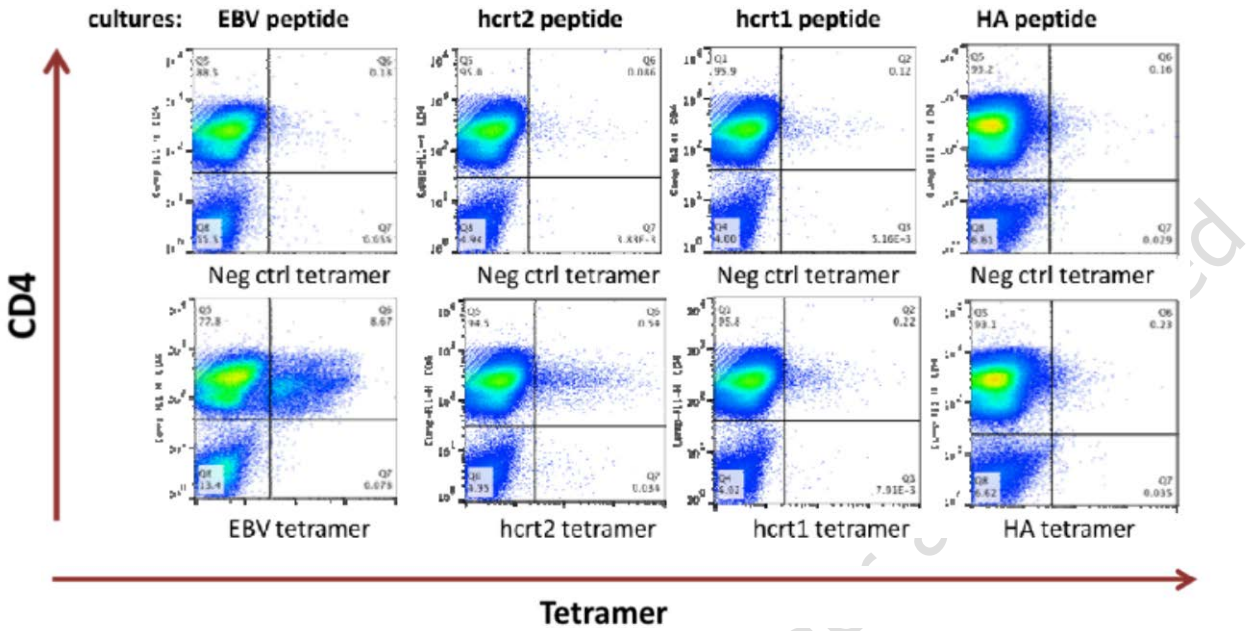


Briefly, purified CD4 T cells were incubated with T2 antigen-presenting cells (Mellins et al., 1991) that express exclusively the DQB1*0602 allele, thereby ensuring that only DQB1*0602-restricted responses are stimulated. T2 cells are human lymphoblastoid cells that are transfected with HLA molecules in order to function as in vitro antigen presenting cells (Mellins et al., 1991). After cell culture for different defined periods of time (12 and 23 days), CD4 T cells are stained with the following DQB1*0602 tetramers: (i) HA275, (ii) HCRT-1, (iii) HCRT-2, (iv) EBV (positive control) and (v) HCRT-L (negative control, sequence MNLPSTKVSWAAV) (Siebold et al., 2004). Of note, the choice of the HCRT-L epitope as a negative control was based on the assumption that no CD4 T cells against this epitope existed. The experiment was performed under homogeneous conditions, i.e. by having the same peptide for stimulation and detection ("homologous culture").

Using PBMC from two narcoleptic patients, these experiments confirmed detection of DQB1*0602 tetramer-positive CD4 T cells, for the HA-275, HCRT-1 and HCRT-2 peptides (De la Herrán-Arita et al., 2013) (Figure 4). These data were interpreted as confirmation that CD4 T cells specific for these epitopes did in fact exist.

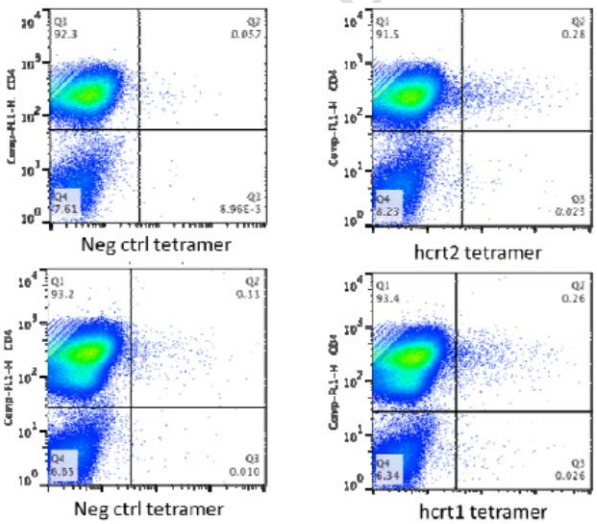
In summary, DQB1*0602-tetramer positive CD4 T cells were observed with the HA-275 epitope, after stimulation with T2/DQB1*0602/HA-275 antigen-presenting cells (Figure 4, far right column). In this assay, CD4 T cells that are binding to the DQB1*0602 tetramer are detected in the upper right quadrant of the FACS plots shown in Figure 4. Furthermore, the HCRT1 and HCRT2 DQB1*0602 tetramers detected CD4 T cells after stimulation with the cognate HCRT1 and HCRT2 epitopes, respectively (Figure 4, middle columns). Culture of CD4 T cells with a DQB1*0602-binding peptide from the EBNA protein of Epstein-Barr virus (EBV) followed by detection with a DQB1*0602/EBV tetramer served as a positive control (Figure 4, left column) (although not all individuals tested harboured CD4 T cells recognizing this epitope). As mentioned, the tetramer of DQB1*0602 with the HCRT-L peptide (Siebold et al., 2014) was used as a presumed negative control, as it was predicted that this epitope might not be presented. Whereas a signal is detected with the HCRT-L DQB1*0602 tetramer in some Q2/Q6 quadrants (Figure 4, upper rows), stronger signals are observed with the homologous tetramers (lower rows). The question that the HCRT-L epitope is a negative control, or not, is currently being tested directly through single cell sorting and sequencing experiments.

Figure 4. CD4 T cells from narcoleptic patient 1 were stimulated with either EBV, hcrt1, hcrt2 or HA peptides loaded DQB1*0602 T2 cells for 23 days (8 days for EBV) and stained with EBV, HCRT-1, HCRT-2, HA-275 (lower rows) or negative control tetramers (upper rows)



As the next step, "cross-culture" conditions were evaluated. In this setting, CD4 T cells purified from PBMC from a narcoleptic patient, were stimulated with T2 cells presenting a different peptide than the peptide in the DQB1*0602 tetramer (Figure 4). In this experimental set-up, CD4 T cells stimulated for 18 days with the HA275 peptide were stained with the HCRT1/2 containing DQB1*0602 tetramers (Figure 5). This cross culture experiment is designed to detect potential cross-reactivity and to directly address the question of whether CD4 T cells that encountered H1N1 influenza epitopes could develop specificity for the HCRT epitopes.

Figure 5. CD4 T cells from narcoleptic patient 2 were stimulated with HA peptide loaded DQB1*0602 T2 cells for 18 days (top graphs) and 23 days (patient 1, bottom graphs) and stained with either HCRT1 or HCRT2 tetramers to assess cross-reactivity



In conclusion CD4 T cells from a narcoleptic patient were stimulated for 12 to 23 days with HA, HCRT1 or HCRT2 and both homologous and cross-culture DQB1*0602 tetramer staining was conducted. Note that

in this set of experiments not every combination was assessed. The results showed the following (Figure 4 and Figure 5):

- HA stimulation yielded CD4 T cells that could be detected with the HA tetramer as well as with the HCRT-1 tetramer.
- HCRT-2 stimulation yielded CD4 T cells that could be detected with the HCRT-1 and HCRT-2 tetramers.
- HCRT-1 stimulation yielded CD4 T cells that could be detected with the HCRT-1 tetramer, but not with the HCRT-L tetramer (presumed negative control).

Overall, this dataset indicated that DQB1*0602 tetramer-positive CD4 T cells could be detected and thus that the CD4 T cell cross-reactivity hypothesis is valid.

DQB1*0602 tetramer results: comparing narcoleptic patients and controls

On the basis of the original dataset, it was decided to continue exploration of CD4 T cell cross-reactivity using DQB1*0602 tetramers as key tools. The following caveats were identified in the preliminary data. First, the initial data set did not comprise DQB1*0602-matched control samples; hence, it was not possible to conclude on the narcolepsy specificity of the observed CD4 T cell populations. Second, the preliminary data comprised only few subjects, implying the need to reproduce the results. Therefore, inclusion of DQB1*0602-matched control samples in the analyses and analysing higher numbers of samples were considered as mandatory to confirm the hypothesis. In addition, PBMC samples from narcoleptic patients and DQB1*0602-matched controls were provided by Prof Mignot's laboratory to Prof Mellins' laboratory in a blinded fashion in order to avoid any bias.

With a preliminary data set that comprised solely data from narcoleptic patients, two scenarios were considered. First, tetramer cross-reactivity might be exclusively linked to narcoleptic patient samples, in which case cross-reactive CD4 T cells could serve as a biomarker and could potentially be directly linked to the etiology of narcolepsy. Second, if cross-reactive tetramer-positive CD4 T cells are detected also in the DQB1*0602- matched control group, then three other hypotheses must be considered: (i) cross-reactive CD4 T cells are phenotypically different between the groups, (ii) the cross-reactive CD4 T cells can occur independent of narcolepsy disease but are required and depend on an additional co-factor, and (iii) cross-reactive CD4 T cells are not associated with narcolepsy.

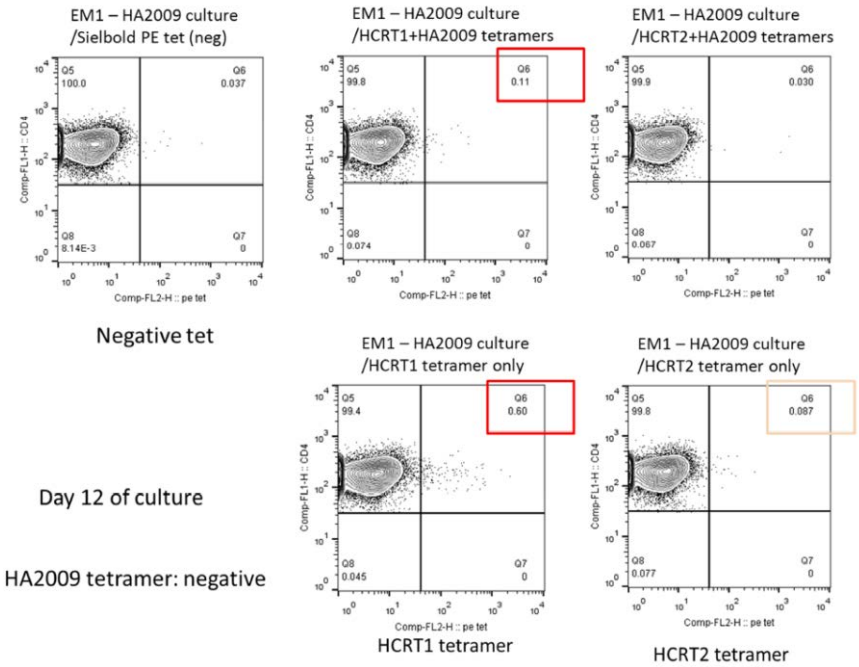
To address these hypotheses, the following studies were performed:

- Ensure that DQB1*0602 tetramer staining data are reproducible in additional PBMC samples;
- Evaluate the narcolepsy specificity of the DQB1*0602/HRCT tetramer-positive CD4 T cells;
- Evaluate potential CD4 T cell cross-reactivity between the HA-275 and HCRT epitopes by DQB1*0602 tetramer competition experiments;
- Evaluate the presence of DQB1*0602/HCRT tetramer-positive CD4 T cells before and after vaccination with Pandemrix;
- Perform single-cell sorting of DQB1*0602 tetramer CD4 T cells followed by TCR sequencing and PCR-based phenotyping;
- Perform phenotypic analysis by FACS of DQB1*0602 tetramer-positive CD4 T cells by co-staining with CD25 and CD127.

In order to evaluate reproducibility and specificity for narcolepsy, a total of 9 PBMC samples were further analysed to date. This included both narcoleptic patient samples and DQB1*0602-matched control

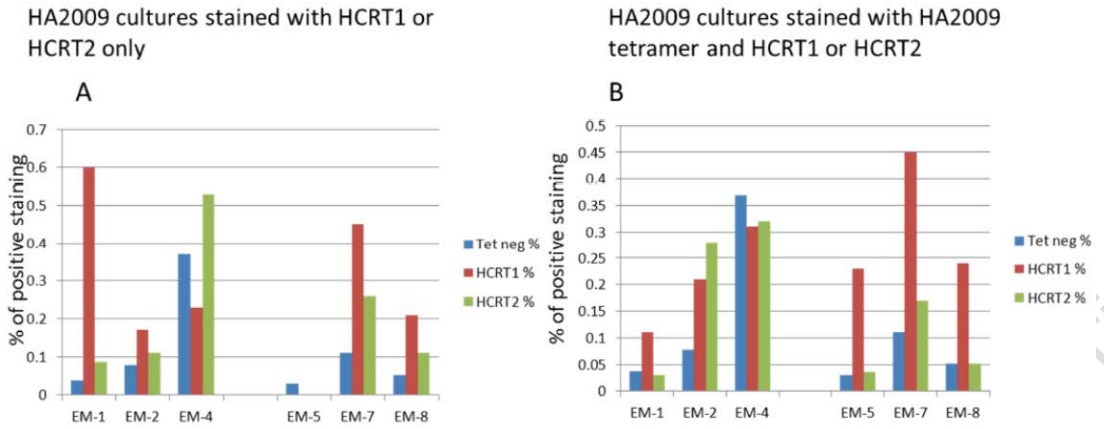
samples, which were analysed in a blinded fashion. For this analysis, CD4 T cells were stimulated with T2/DQB1*0602/HA-275 antigen presenting cells and detected with the DQB1*0602/HCRT-1/2/L tetramers. HCRT-1 DQB1*0602 tetramer-positive signals were detected in samples EM1, EM2, EM7 and EM8 (upper right quadrants in the FACS plots in Figure 6 are indicative of CD4 T cells binding to the DQB1*0602 tetramer). An HCRT-2 DQB1*0602 tetramer-positive signal was detected in samples EM4. The other samples were negative. After unblinding of the samples, it appears that CD4 cross-reactivity can be detected in both narcoleptic patient samples (EM1-4) and in DQB1*0601-matched controls (EM5-9). This implies that cross-reactive CD4 T cells cannot serve as a biomarker of narcolepsy and that their presence in blood is not sufficient to cause disease. Still, these cross-reactive T cells might be required for disease development but might not be sufficient for induction of narcolepsy. An additional co-factor may be required to induce a pathogenic phenotype. Another explanation may be that cross reactive T cells have a different phenotype in narcoleptic patients and controls, with a more regulatory T cell phenotype in the control group. Alternatively, these cells might not be associated with narcolepsy.

Figure 6. CD4 T cells were stimulated with HA peptide loaded DQB1*0602 T2 cells for 12 days and stained with either hcrt1 or hcrt2 tetramers in the presence or not of competing HA tetramer. Example of sample EM1 shown.



The data so far provide evidence for potential cross-reactivity on the basis of T2/DQB1*0602/HA-275 cross-culture. Another way to establish cross-reactivity is DQB1*0602 tetramer double staining. This has been attempted but has proven difficult, possibly due to tetramer competition (data not shown). Another approach to achieve this, is to determine whether the DQB1*0602/HA-275 tetramer could block binding for the DQB1*0602/HCRT-1/2 tetramers (tetramer competition binding analysis). The experimental set-up for this is the T2/DQB1*0602/HA-275 cross-culture, after which CD4 T cells are stained with both the DQB1*0602/HA-275 and DQB1*0602/HCRT-1/2 tetramers, carrying different fluorescent labels. In some of the samples, co-incubation led to reduced DQB1*0602 HCRT-1/2 tetramer staining (most notable EM1 for HCRT-1 – see Figure 6 and EM4 for HCRT-2). No reductions were observed for samples EM5, EM7 and EM8 (Figure 7). At this point, these data need to be interpreted with care because it has not yet been reproduced and it is likely that there is residual variability in this assay.

Figure 7. CD4 T cells from narcoleptic or controls patients were stimulated with HA peptide loaded DQB1*0602 T2 cells for 12 days and stained with either hcrt1 or hcrt2 tetramers in the presence (B) or not (A) of competing HA tetramer. Results are represented as percentage of tetramer-positive CD4 T cells.

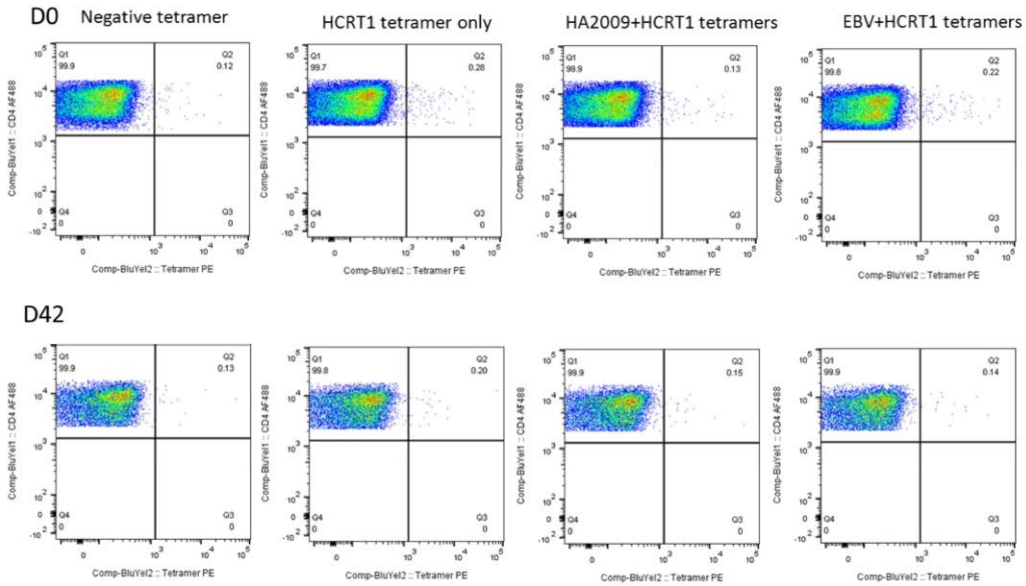


DQB1*0602 tetramer results: comparing responses before and after vaccination with Pandemrix in healthy subjects

If HCRT-specific CD4 T cells are detected after stimulation with the HA epitope, in a DQB1*0602 restricted manner, then it becomes interesting to study the behaviour of such cells after vaccination with Pandemrix. Thus, the results have established that the evidence supporting epitope cross-reactivity is sufficiently convincing, though not yet conclusive, and justify studying with clinical study samples (Roman et al., 2011). To this end, PBMC samples from DQB1*0602+ subjects immunized with Pandemrix were analysed by cross-culture and tetramer staining. Samples were used from a study in which AS03-adjuvanted and non-adjuvanted H1N1v vaccines were compared. PBMC obtained before vaccination and at day 21 post second vaccination with AS03-adjuvanted H1N1 (day 42) (n=5/group, 10 samples total) were subjected to CD4 T cell purification and T2/DQB1*0602/HA stimulation (12 days) after which staining with the different tetramers were performed. Five AS03-adjuvanted H1N1 vaccinated subjects (10 samples total) from the clinical study were analysed for cross reactivity against HCRT1 and HCRT2 after expansion of CD4 T cells with HA peptide. Results demonstrated that 1 out of the 5 analysed subjects exhibits a cross reactive response against HCRT1 (no cross reactivity observed with HCRT2 tetramer) that was already present before vaccination with Pandemrix and that did not expand further after vaccination with Pandemrix (Figure 8).

Interestingly, the signal appeared to be out-competed by the presence of HA tetramer but not with EBV tetramer (Figure 8), highlighting the specificity of the potential HA cross-reactivity. Representative data for a single subject are shown in Figure 8, with full data provided in Module 5 (Report Pr Mellins). The signal deemed positive with the HCRT1 DQB1*0602 tetramer is shown in Figure 8 (upper row, two middle plots, upper right quadrants). Nevertheless, the signals detected with the tetramer reagents are weak, presumably reflecting the low frequencies of such CD4 T cells, and the data can only be interpreted with caution.

Figure 8. CD4 T cells from pre (D0) and post Pandemrix (D42) vaccinated patient cultured with HA275 peptide and T2 cells during 12 days and stained with HCRT1 or HCRT2 tetramer in the presence or not of competing HA275 tetramer.



CHMP assessment

Prof Mignot and colleagues previously reported evidence for molecular mimicry between HA and hypocretin epitopes when using IFN-γ ELISPOT methodology on PBMCs but were unable to repeat these studies and retracted the results prompting a re-evaluation of the research plan. Using a complimentary approach, Prof Mellins' research team, also from Stanford University, used soluble DQ-0602 HLA tetramers and provided preliminary evidence that the key epitopes in HA275-287, HCRT56-68 and HCRT87-99 were recognized by DQB1*0602-restricted CD4 T cells. Unlike the initial IFN-γ ELISPOT data the signals detected with the tetramer reagents are relatively weak, and require a period of T2/DQB1*0602/epitope co-culture and expansion to be detected presumably reflecting the low frequencies of such CD4 T cells. Using cross culture experiments HRCT-specific CD4 T cells were detected after stimulation with the HA epitope suggesting the possibility that CD4 T cells that encountered H1N1 influenza epitopes could develop specificity for the HCRT epitopes.

It is important to determine if the HA/hypocretin cross reactive-specific T cells are uniquely present in narcoleptic patients or are also found in healthy subjects and determine how this is affected by vaccination. A study of a further 9 samples (4 narcoleptic patient samples and 5 controls) has shown HA/HCRT cross-reactive CD4 T cells are also detectable in DQB1*0602+ healthy subjects. This implies that the presence of HA/HCRT cross-reactive CD4 T cells do not cause narcolepsy and that cross-reactive CD4 T cells cannot serve as a biomarker of narcolepsy. It remains possible that HA/HCRT cross-reactive CD4 T cells are required for disease development but do not cause narcolepsy unless another co-factor is present that for example that changes its phenotype or its migration properties into the CNS. It is also possible these cells are not linked at all to narcolepsy.

The sample size in these studies remains relatively small and confirmation of cross-reactivity based on tetramer competition binding analysis has demonstrated cross-reactivity in some samples but not in others and this data needs further confirmation.

PBMC samples from DQB1*0602+ subjects immunized with Pandemrix demonstrated that 1 out of the 5 analysed patients exhibits a cross reactive response against HCRT1 (no cross reactivity observed with HCRT2 tetramer) that was already present before vaccination. The signal was competed out by the presence of the HA tetramer but not with a control EBV tetramer, highlighting again the specificity of the potential HA cross reactivity. However, importantly no expansion of these cross-reactive cells was seen

after vaccination with Pandemrix which might have been expected. The MAH was asked to comment on the implications of this observation during the procedure.
Of note during the tetramer binding studies HCRT-L (N-terminal leader sequence MNLPSTKVSWAAV - Siebold et al., 2004) was chosen as a negative control in the tetramer staining studies based on the assumption the epitope is not be presented and that no CD4 T cells against this epitope will exist. The MAH highlighted that in fact a signal is detected with the HCRT-L DQB1*0602 tetramer in some Q2 quadrants. Also results from the single cell TCR analysis (see results below) suggest perhaps the HLA-DQB1*0602 molecule might present this epitope and that the TCR recognizes it. The MAH stated it is collecting additional data and this question and its potential implications were asked to be addressed by the MAH during the procedure.
One limitation of these studies has been the focus on HA/hypocretin epitopes as the potential cross-reactive targets. Although the results with the HA/hypocretin DQ0602 tetramers have been informative it ignores the potential for other cross-reactive epitopes expressed in hypocretin neurons and the antigenic mix to have role. This aspect was part of the initial plan and going forward it remains important to investigate possibility of other cross reactive epitopes having a role in disease.

4.1.2. Single cell TCR analysis and phenotypic differences evaluation

The observation that cross-reactive tetramer-positive CD4 T cells are detected in both narcoleptic patients and DQB1*0602-matched control subjects now renders the question of potential phenotypic differences relevant. To study phenotypic differences, two approaches were chosen: first, co-staining of tetramer-positive CD4 T cells with two phenotypic markers that could potentially identify regulatory CD4 T cells (CD25, CD127); second, single cell sorting of tetramer-positive CD4 T cells was performed in order to obtain data for TCR sequence analysis and PCR-based immune-phenotyping.

In order to obtain proof-of-principle for the combined T cell expansion, tetramer staining and CD25/CD127 expression analysis, the complete procedure was evaluated using the positive control EBV epitope and DQB1*0602 tetramer. The preliminary data indicate that CD25 staining is compatible with the culture/tetramer staining approach whereas the CD127 staining is weak. Optimization of the staining method is required because it was considered that the culture conditions (which include IL-2 and IL-7; note that CD25 and CD127 are components of the IL-2 and IL-7 receptors, respectively) might affect the CD25 and CD127 staining. No data with HCRT tetramers were generated because the staining with EBV-tetramer-positive CD4 T cells was not deemed satisfactory. At this point the MAH suggested PCR-based phenotyping in DQB1*0602 tetramer sorted CD4 T cells is likely to provide more accurate data.

The additional approach to analysing potential CD4 T cell epitope cross-reactivity and CD4 T cell phenotypes was based on single-cell sorting of DQB1*0602-tetramer-positive CD4 T cells. The underlying hypothesis is that by analysing the TCR sequences from the different DQB1*0602-tetramer-positive CD4 T cells, it is possible to assess cross-reactivity in a direct manner: if the same TCR sequence is identified for HA- and HCRTDQB1*0602 tetramers, then it becomes more plausible that the same CD4 T cell recognizes both epitopes. The technology of single-cell sorting followed by simultaneous TCR sequencing and PCR-based phenotyping has recently been set up. This method has been pioneered in the laboratory from Prof Mark Davis at Stanford University and further modified in Prof Mellins laboratory (Han et al., 2014).

To further evaluate the cross-reactivity hypothesis, Prof Mellins' laboratory performed tetramer staining with DQB1*0602/HA-275, DQB1*0602/HCRT-2, and DQB1*0602/HCRT-L tetramers on CD4 T cells purified ex vivo (without culture) from 5 subjects with narcolepsy and 5 controls. Stained cells were single cell sorted, while preserving the information on the staining level of the sorted cell. TCR sequencing was carried out and analysed. In brief, for each sample, a complete 96-well plate of single DQB1*0602

tetramer-positive CD4 T cells was collected and subjected to PCR for both TCR sequence analysis and immune-phenotyping. TCR data for any single cell were considered valid if at least 100 sequence reads were obtained.

The analysis of the TCR sequences to determine whether potential HA-275/HCRT-1/2 cross-reactive TCRs are present and if so to determine the immunophenotype (expressed transcription factors, cytokines) is ongoing. Based on the data analysis done so far, several preliminary observations can be mentioned.

Among 5 narcoleptic patients from whom ~96 CD4 T cells were Index sorted with each of the 3 tetramers, 4 showed evidence of T cells with candidate cross-reactive TCRs, defined by usage of the same TCRA and TCRB families and identical sequences at both CDR3 loops for more than one tetramer. Sequencing from the 5th patient was less robust as many sequences had fewer reads and there were fewer sequences obtained overall. Among the 4 patients, the numbers of cross-reactive sequences and the apparent cross-reactivity is as summarized in Table 5.

Table 5. Single-cell TCR sequencing results from DQB1*0601 tetramer sorted CD4 T cell

Subject	Tetramer specificity	TRAV	TRAJ	CDR3	TRBV	TRBJ	CDR3
1	HA-275 / HCRT-L	14.D4	44	CAMREGPDTGTASKLTF	12.3	2.3	CASSLSTGGTQYF
1	HA-275 / HCRT-L	10	18	CVVSDRRGSTLGRLYF	25.1	1.1	CASSESQTGSTEAFF
1	HA-275 / HCRT-L	26.2	37		6.1	1.1	CASSEVGGRTEAFF
2	HCRT-2 / HCRT-L	12.2	3	CAVGKYSSASKIIF	19	2.3	CASSIDATSRNTQYF
2	HA-275 /HCRT-L/HCRT-2	6	24	CALTTDSWGKLQF	29.1	2.5	CSVEGDRGRSETQYF
2	HA-275 / HCRT-L	21	43	CAASEGANNDMRF	30	1.2	CAWSSGINYGYTF
3	HA-275 / HCRT-L	12.3	10	CAMSAPGGGNKLTF	20.1	1.4	CSATLDSATNEKLFF
3	HA-275 / HCRT-L	26.1	54	CIVPNKGAQKLVF	28	2.4	CASSFGFFGGPKNIQYF
3	HA-275 / HCRT-L	6	18	CALDLRGSTLGRLYF	5.4	1.1	CASSSPAGGGGNTEAFF
3	HA-275 / HCRT-L	6	43	CALDANNNNDMRF	6.4	1.5	CASSTAGNQPQHF
4	HA-275 / HCRT-L	21	49	CAALTGNQFYF	4.3	1.1	CASSQERTVNTEAFF

Data shown are from 4 narcoleptic patients' samples. TCR segments usage is shown for those cells where identical usage was detected with multiple tetramers, as indicated in the tetramer specificity column. TCRA, TCRB segment usage and CDR3 sequences of those CD4 T cells is indicated in the appropriately labelled columns.

The most striking finding is from patient 2, who had one sequence expressed by 13 cells with the leader peptide tetramer, 3 cells with the HCRT2 tetramer and one with HA tetramer. Of note, this TCR uses J alpha 24, a gene segment, an allele of which has been identified by GWAS as an inherited risk factor for narcolepsy. Notably, the sequencing from the tetramer-sorted CD4 cells from DQ0602 control subjects only yielded one subject with one cross-reactive TCR, shared by 2 HA tetramer-sorted and 2 leader peptide tetramer–sorted cells. This TCR was not sorted from other subjects (control or patient). A different control subject also had 2 sorted cells expressing the first TCR on the above list; surprisingly these cells were sorted only by the HCRT2 tetramer.

Notably, the sequencing from the tetramer-sorted CD4 cells from DQB1*0602 control subjects only yielded one subject out of four with a single cross-reactive TCR, shared by 2 HA-275 tetramer-sorted and 2 HCRT-L tetramer–sorted cells. This TCR was not sorted from other subjects (control or patient).

As the next step, it is important to determine the phenotypic characteristics of these specific and potentially cross-reactive CD4 T cells using PCR primers to CD4 subtype-specific transcription factors and cytokines. These PCR and sequencing experiments are in progress.

Index sorting from 3 more pairs of narcoleptic subjects and controls has been carried out and TCR and phenotype marker sequencing are in process. Analysis of sorted CD4 T cells from an additional 4 pairs is in planning phase. Whereas the single-cell based TCR sequencing data are of obvious interest, it is important to emphasize that the further data analysis of additional subjects is critically important for interpretation.

Prof Mellin's conclusions

1. A single, shared TCR sequence was identified from narcoleptic patient 2 that was expressed by 13 cells with the DQB1*0602/HCRT-L tetramer, 3 cells with the DQB1*0602/HCRT-2 tetramer and one with DQB1*0602/HA-275 tetramer.
2. The TCBJ1.1 segment was found four times.
3. TCR sequence identity in all 4 narcoleptic subjects is observed between CD4 T cells isolated with the DQB1*0602/HA-275 tetramer and the DQB1*0602/HCRT-L tetramer. The HCRT-L (residues 1-13 from HCRT) peptide was previously identified as a strong binder to DQB1*0602 (Siebold et al., 2004) and a crystal structure of DQB1*0601 carrying the HCRT1-13 peptide has been described (Siebold et al., 2004).
4. Shared use of TCR V and J segments is also reflected in the actual CDR3 sequences presented in Table 5, which appear to reveal common sequence elements.

CHMP assessment

Tetramer-based single cell sorting combined with TCR sequence determination is a powerful new technique that potentially could identify novel CD4 T cell responses. If the same TCR sequence is identified for HA- and HCRT DQB1*0602 tetramers, then it becomes plausible that the same CD4 T cell recognizes both epitopes. The reported results are preliminary and need to be treated caution with but are encouraging. For example from one narcoleptic patient, a TRAJ24 and TRBV29.1 containing sequence was detected using all three DQB1*0602 tetramers. TRAJ24 is of interest because it was identified as a SNP linked to risk for narcolepsy whereas TRBV29-1 was highlighted in the current research by comparing sequence data from narcoleptic patients with healthy controls.

As discussed earlier, it is of interest that, from the single cell analysis, tetramers recognising the previously identified hypocretin 1-13 peptide (the hypocretin leader peptide, referred to as HCRT-L) were recognised in the majority of the narcoleptic patients. This peptide was originally selected as a control in the tetramer binding experiments as it was not thought to be presented. The MAH stated it is collecting additional data and this question and its potential implications were asked to be addressed by the MAH during the procedure.

These early results are of significant interest but it is clear that they require confirmation by obtaining TCR sequence data from more patients and controls. This was ongoing and was reported and discussed at the RSI stage. If cross-reactive T-cells are confirmed then determining the phenotypic characteristics of these cells using PCR primers to CD4 subtype-specific transcription factors and cytokines will be essential in understanding their potential pathogenic roles. This additional analysis was reported at the RSI stage.

4.1.3. Discussion

The MAH's in vitro experiments to investigate the association between Pandemrix vaccination and narcolepsy are summarized in Figure 1. The approach was based on two research pathways: i) first on the basis of the strong HLA association and TCRA genetic polymorphism data, that narcolepsy can be studied as a CD4 T cell mediated auto-immune disorder; ii) second, that any direct (toxic and/or inflammatory) effects of the vaccine, including the AS03 adjuvant, can only be studied in an animal model, while acknowledging that there is no available animal model for immune mediated narcolepsy induction. Three non-mutually exclusive hypotheses were considered, molecular mimicry, potential bystander activation by the AS03 adjuvant and a direct toxic effect or inflammation of the hypothalamus.

To study the role of potential cross-reactive CD4 T cell responses ('mimicry'), two parallel approaches were pursued. One approach determining the TCR repertoire using 'deep' sequencing technology to assess whether specific TCR clones or segments might be associated with narcolepsy. However, TCR sequences or clones do not provide information on antigenic specificity or the role of influenza antigens. To address this point, the parallel approach focused on DQB1*0602 CD4 T cell epitope mapping, focussing on the HA, NA and PB1 proteins from H1N1 and hypocretin. PB1, NA and HA are the only proteins from H1N1v that are present in the reassortant viruses that are used to generate the split influenza vaccine preparations. Hypocretin is the signature protein produced by the hypocretin neurons in the hypothalamus that regulate wakefulness, low levels of which are associated with narcolepsy. The original plan (step 2) proposed also to study cross-reactive responses to a set of proteins that are enriched in hypocretin hypocretin-secreting neurons including hypocretin. However, following early encouraging results from the peptide analysis the research has focused on cross-reactivity to hypocretin only, which although a strong candidate for potential auto-reactive epitopes is also a limitation.

The TCR sequence analysis produced multiple differential results when comparing narcolepsy cases with healthy DQB1*0602-matched controls, but in most cases statistical significance was lost following Bonferroni correction for multiple testing. Because this was interpreted as resulting from lack of power, the number of samples sequenced was increased and a single Bonferroni significant result was obtained: a decrease in TRBJ1-3*01 usage. A second result, an increase in TRBV29-1*01 usage, was close to being Bonferroni-significant. Thus, differential usage of at least a single TCR segment was observed and this may indicate that there are indeed differences in the TCR repertoires from patients compared with controls. Interestingly, further analysis to the level of individual sequences, yielded a small number of sequences that were uniquely present or absent in narcoleptic patients. Whilst acknowledging the lack of statistical power the MAH speculate these could represent part of the auto-immune repertoire. From the preliminary results, the MAH concluded that actual differences in TCR repertoire between narcoleptic patients and DQB1*0602-matched controls may exist. This would support the hypothesis of narcolepsy being a CD4 T cell mediated autoimmune disorder and is consistent with a role of DQB1*0602 in presenting antigen to CD4 T cells.

The first step in DQB1*0602 CD4 T cell epitope mapping was to measure binding of overlapping peptides spanning the HA, NA, PB1 and HCRT proteins to DQB1*0602. The analysis revealed multiple binding peptides in each protein. To evaluate the immunological significance of DQB1*0602 binding, CD4 T cell responses to each peptide were measured, initially by IFN-γ ELISPOT. This approach seemed to produce promising results (De la Herrán-Arita et al., 2013) but was not reproducible. This led to retraction of the publication by Mignot and coworkers (De la Herrán-Arita et al., 2014) and the need to re-define the research objectives. The emerging DQB1*0602 tetramer data were considered and it was decided to pursue the epitope mapping work on the basis of the preliminary tetramer data.

Importantly, subsequent analysis of additional samples confirmed that the epitopes HA275-287, HCRT56-68 and HCRT87-99 identified in the initial analysis were indeed recognized by CD4 T cells. This is important because it demonstrates that CD4 T cells that recognize DQB1*0602-restricted epitopes from

hypocretin do exist. In addition, the data show that expansion of CD4 T cells by stimulation with the HA peptide, presented by DQB1*0602, yields CD4 T cells that are recognized by the HCRT-1 and HCRT-2 DQB1*0602 tetramers. The importance of this finding is that it supports the notion of CD4 T cell cross-reactivity.

The preliminary data set was based on only a few narcoleptic patients and it was important to assess the reproducibility and specificity of hypocretin-cross reactivity for narcolepsy. After analysis of an additional samples from both narcoleptic patients and controls in a blinded fashion it appears that HA/HCRT cross-reactive CD4 T cells are also detectable in DQB1*0602+ healthy subjects. This implies that HA/HCRT cross-reactive CD4 T cells per se do not cause narcolepsy. From this, there are two possible interpretations. First, HA/HCRT cross-reactive CD4 T cells exist but do not cause disease unless another co-factor is present that changes, for example, T cell phenotype or migration properties. This possibility is supported by the fact that GWAS analysis identified a number of SNPs related to T cell activation as being associated with narcolepsy. One such scenario could be that cross-reactive CD4 T cells are being kept under tight control, for instance by regulatory T cells, in healthy subjects but can, under certain conditions, acquire a pathogenic phenotype. Alternatively, HA/HCRT cross-reactive CD4 T cells exist but are not linked at all to narcolepsy. Recent work from Prof Mark Davis, also at Stanford University, revealed that CD4 T cell cross-reactivity may be a common feature of the human immune system (Su and Davis, 2013). The current observations are consistent with their studies.

Considering that cross-reactive tetramer-positive CD4 T cells are detected in both narcoleptic patients and DQB1*0602-matched control subjects it is important to determine if potential phenotypic differences are relevant. The researchers are currently using PCR based approaches to phenotype DQB1*0602 tetramer sorted CD4 cells.

They have also used the alternative novel technique of tetramer-based single cell sorting of CD4 cells combined with TCR sequence determination to analyse potential CD4 T cell epitope cross-reactivity and CD4 T cell phenotypes. The preliminary results have provided some noteworthy observations. Shared TCR sequences for narcoleptic subjects are found between CD4 T cells isolated with the DQB1*0602/HA-275 tetramer and the DQB1*0602/HCRT-L tetramer. The HCRT-L peptide was previously identified as a strong binder to DQB1*0602 and a crystal structure of DQB1*0601 carrying the HCRT1-13 peptide has been described (Siebold et al., 2004). Whereas shared TCR sequences are detected in 4/5 narcolepsy patients, it is interesting that the sequences themselves are unique, although some common sequences may be apparent. These early results require confirmation by obtaining TCR sequence data from more patients and this was ongoing during the procedure.

Whereas the TCR and epitope-mapping research activities are providing insight on the potential roles of CD4 T cells and epitope cross-reactivity, they do not address the potential co-factor of CNS-access of immune cells and the impact of the AS03 adjuvant thereon. Since no animal model of immune-induced narcolepsy exists, it was proposed to use immunization and/or influenza infection in cotton rats as a model for immune cell CNS access, rather than a disease model. Effects on the CNS and the blood-brain barrier of different combinations of H1N1 influenza virus, AS03-adjuvanted H1N1 vaccine and non-adjuvanted H1N1 vaccine were evaluated. These results were previously reported and assessed as part EMEA/H/C/000832/II/0078. It was found that neither vaccine nor the AS03 adjuvant alone triggered any detectable changes in the brain. AS03-adjuvanted H1N1 vaccine as well as AS03 alone did induce transient increases of neutrophils and monocytes, consistent with previously published data (Morel et al., 2011; Segal et al, 2015). Thus, the AS03 adjuvant did not induce any CNS inflammation or changes to blood brain barrier permeability. These results do not support a hypothesis that suggests AS03 has a direct inflammatory or toxic effect on the hypothalamus as a potential explanation for the observed association of Pandemrix with the onset of narcolepsy. The absence of a narcolepsy signal associated with administration of AS03 adjuvanted Arepanrix H1N1 vaccine in Quebec (Montplaisir et al., 2014) provides further evidence against a direct causal role for AS03 in post pandemic narcolepsy.

Instead, these data focused the attention on potential immunologic differences between the two vaccines. Since the AS03 adjuvant is the same in the two vaccines, attention was further focused on differences between the antigens. In terms of exploring immunological differences, the MAH conducted antibody avidity analyses comparing immune responses to both vaccines. Serum samples from a head-to-head comparative paediatric study, in which immunological equivalence based on HAI titres had previously been demonstrated, were used for BIAcore-based anti-haemagglutinin antibody avidity analysis. The results did not reveal significant differences in avidity, indicating that any immunological differences between the two haemagglutins were not revealed by avidity analysis (EMEA/H/C/000832/II/0076). Therefore, antibody avidity analysis did not reveal any meaningful immunogenicity differences between Arepanrix and Pandemrix in terms of humoral responses to HA. However, the analysis could exclude minor antigenic differences in the HA proteins of the two vaccines.

In parallel, a study from the laboratory of Prof Mignot using 2-D gel electrophoresis and mass spectroscopy documented a large number of physiochemical/biological differences between the antigen mixtures of Pandemrix and Arepanrix (Jacob et al., 2014). The most significant change noted in HA being a deamidation of asparagine to aspartic acid at residue 146 in Arepanrix. The authors hypothesised this may represent a HLA DQB1*06:02 binding epitope although this has not been confirmed. In response the MAH have sequenced the seed banks for Pandemrix and Arepanrix and confirmed the N/D amino acid change for Arepanrix but not for Pandemrix (the data was provided in the GSK HA Sequence Report in Module 5). The significance of this amino acid substitution is currently not clear.

Vaarala and coworkers compared the antigens from the two vaccines and concluded that differences in amount and aggregation state of NP existed – more high-molecular weight forms of NP were observed in the Pandemrix antigen preparation as compared to the Arepanrix antigen preparation (Vaarala et al, 2014). It was also found that antibody levels against NP, and in addition against detergent-modified NP, differed when comparing sera from narcoleptic patients and healthy controls. To some extent, this appears to reflect a role for DQB1*0602 in regulating antibody responses to NP. It is not clear whether such antibodies would play any causative role in narcolepsy. Although antigenic differences are not unexpected, due to differences in the manufacture of Pandemrix and Arepanrix vaccine antigens further work is required to better understand the differences between the two vaccines and the putative role that this might have played in the aetiology of narcolepsy.

A recent publication by Ahmed et al. (2015) showed that sera from narcoleptic patients and also from healthy control subjects harbour antibodies that bind the human hypocretin receptor 2 (HCRT-R2). Binding of antibodies to HCRT-R2 was outcompeted by peptides from HCRT-R2 but also by potential mimicry peptides from NP. One important consideration is that the work published by Ahmed and co-workers was based on a purported single amino acid difference at residue 116 of the NP between the MF59-adjuvanted monovalent A/(H1N1)pdm09 vaccine, Focetria, and Pandemrix, with Focetria having a methionine at that position and Pandemrix, supposedly, an isoleucine. However, MAH sequence analysis of the seed lots of both Pandemrix and Arepanrix has revealed the presence of a methionine at that position (see Genbank entry KJ942731). The sequence results generated by the Company are included in the MAH NP Sequence Report in Module 5.

The current status of the research into the potential mechanistic links between the Pandemrix H1N1 vaccine and narcolepsy is summarised as follows:

- TCR sequence analysis identifies narcolepsy-specific TCR usage and TCR sequences uniquely present in narcoleptic patients, supporting the potential role of CD4 T cells, consistent with the importance of DQB1*0602.
- Peptides binding to DQB1*0602 were identified for HA, NA, PB1 and hypocretin underscoring the potential for CD4 T cells to recognize these proteins in a DQB1*0602-restricted manner.

- Bioinformatic analysis identified a 9 amino acid sequence from HA as being present in H1N1pdm09 but absent in H1N1 virus isolates from earlier years (HA275).
- DQB1*0602 tetramers indicate that CD4 T cells specific for epitopes in HA (HA275-287) and hypocretin (HCRT56-68, HCRT87-99) exist and that CD4 T cell cross-reactivity in fact exists. The TCR sequence link with the HCRT1-13 sequence is intriguing and requires further study, which was ongoing during the procedure.
- CD4 T cell responses to HCRT may not by themselves be a biomarker for narcolepsy. They may be bystanders or may require a co-factor before developing pathogenic potential.
- Studies in cotton rats do not support the hypothesis that the AS03-adjuvanted vaccine, nor the antigen alone or the AS03 adjuvant alone, trigger any inflammatory responses in the CNS or change the function of the blood brain barrier, indicating that adjuvant-induced inflammatory responses as a generic mechanism do not explain induction of narcolepsy.
- Retrospective studies of the risk of narcolepsy following exposure to Arepanrix in Quebec and to Pandemrix in several Europeans countries generated markedly different attributable risk estimates; it is not known whether the differences reflect differences in the vaccine antigens (they shared the same adjuvant) or in the populations and their environments.
- Immunological differences could not be discerned by studying antibody avidity; however, structural and biochemical analyses identified a single amino acid substitution in HA and different amounts of structurally altered NP when comparing the two antigen preparations. The relevance of these differences for risk of narcolepsy is unknown.

In conclusion from the work done so far, a hypothesis that takes into account the potential role of antigen is more likely to explain the increased risk of narcolepsy observed with Pandemrix than hypotheses that are based on a direct role for the AS03 adjuvant. It is conceivable that if there is a role for antigenic mimicry or cross-reactivity on the CD4 T cell level, it is likely to reside in the HA protein, as the only 9 amino acid peptide unique to H1N1pdm09 that binds to DQB1*0602 is from the HA head domain. Other research groups have provided evidence that has highlighted the potential role of other antigenic components of the vaccine. The MAH's research has started to yield informative results with regards to the analysis of TCR repertoires in different populations and in identifying CD4 T cell HA/HCRT cross reactivity. However, results remain preliminary and no firm conclusions can be made at this stage. Further analysis of the CD4 TCR repertoire after vaccination with Pandemrix and further characterisation and immune phenotyping of cross reactive CD4 T cells was ongoing during the procedure and results have been reported at the RSI stage. The MAH has re-iterated their commitment to continue to take into account any emerging data and to update its research plan as needed.

4.2. Risk management plan

The Company submitted an updated RMP (RMP version 20) with this application. It includes an update of the activities from the narcolepsy research plan, removal of solid organ transplant rejection as potential risk, and the addition of the EPIFLU- H1N1-014 post-authorisation safety study to investigate signal for multiple sclerosis and neuritis.

Summary of changes for RMP version 20

1. Activities from the research plan have been updated in Table 2, SII.3 non-clinical studies to evaluate a signal for narcolepsy. The Pharmacovigilance plan (Part III) has been updated with results currently available from the narcolepsy research activities as well as remaining

outstanding activities, Part VI; summary of activities in the risk management plan by product has been updated accordingly as well.

2. Detailed Description of online signal management tool (OSM) has been deleted in section III.2.3 as it is no longer in use by the MAH.

3. Section SVII.3.4 has been updated with a more recent summary of spontaneous cases reporting Narcolepsy.

4. Final results of study EPI-FLU H1N1-009 have been added (section SVII.3.3 Post authorization studies) and the status of the study adapted to completed in Part III and VI.

5. Solid organ transplant rejection (SOTR) has been removed as potential risk from all relevant tables in the RMP, including Part III-Pharmacovigilance plan, and all information on SOTR has been removed from Part II: Module SVII-Identified and potential risks. Part VI; summary of activities in the risk management plan by product have been updated accordingly as well.

In April 2014, the European Medicines Agency (EMA) recommended the MAH to close the commitment to conduct observational studies on solid organ transplant (SOT) rejection and D-Pan H1N1 (Pandemrix). This decision was based on results of the EPI-FLU-H1N1-012 study together with all other available evidence submitted by the MAH to EMA. This included the results of the feasibility assessment of an epidemiological field-based study to be conducted in Brazil and in the UK, the murine study of the mechanism of action of AS03, the accumulated published scientific literature and the Company's post-marketing experience including an internal observed-to-expected analysis (submitted in 2010). Based on this information and based on the CHMP assessment report received in December 2014 (EMEA/H/C/000832/II/0074), the MAH removed SOT rejection as a potential risk from the current Risk Management Plan.

6. Study PASS EPI-FLU-H1N1-014 has been added to the Part II: Module SVII-Identified and potential risks, SVII.3.3: Post-authorisation studies; and all relevant tables in Part III-Pharmacovigilance plan and in Part VI: Summary of activities in the risk management plan by product. The objective of this study is to assess whether administration of Arepanrix (Q-Pan H1N1) during the 2009/2010 H1N1 influenza pandemic was associated with an increased risk of incident multiple sclerosis and other demyelinating conditions (including neuritis) not ultimately leading to a multiple sclerosis diagnosis in Manitoba, Canada. The results have been submitted to the EMA in December 2015.

Overall conclusion on the RMP

☒ The changes to the RMP are acceptable.

4.3. Changes to the Product Information

The MAH proposed to remove the following from Annex II section D, Additional risk minimisation measures of the product information.

- **Obligation to conduct post-authorisation measures**

The MAH shall complete, within the stated timeframe, the below measures:

Description	Due Date
Conduct non-clinical (including mechanistic) studies in order to elucidate the role of the vaccine and its adjuvant on the association between Pandemrix and narcolepsy:	
Identify T-cell signature from narcoleptic patients by deep sequencing of total CD4 T-cells obtained from narcolepsy patients and DQ0602-matched non-vaccinated healthy subjects and, if identified, verify if signature is found in CD4 T-cells from healthy subjects after vaccination with Pandemrix or non-adjuvanted H1N1v vaccine.	August 2015
Verify influenza-specificity of hypocretin-specific CD4 T-cells from narcoleptic patients by complementary assays and verify if cross-reactive CD4 T-cells are found among influenza-specific CD4 T-cells from healthy subjects after vaccination with Pandemrix or non-adjuvanted H1N1v vaccine.	August 2015
Phenotypic characterization of hypocretin and influenza-specific T cells after stimulation with hypocretin or influenza peptides.	August 2015

5. Request for supplementary information

5.1. Major objections

None

5.2. Other concerns

Non-clinical aspects

The MAH was requested to report the results from the additional ongoing research activities including the outstanding data and updated discussion and conclusion for:

- TCR sequence analysis of CD45RO- CD4 T cells from 9 DQB1*0602+ subjects immunized with Pandemrix, before/after vaccination;
- Single cell TCR sequencing from DQB1*0602 tetramer sorted cells from 7 pairs of samples (narcoleptic patients and controls);
- PCR-based immunophenotyping from DQB1*0602 tetramer sorted cells from 11 pairs of samples (narcoleptic patients and controls);

In the discussion the MAH was requested to comment on studies that suggest HCRT-L (N-terminal leader sequence MNLPSTKVSWAAV) may not be a negative control and may be presented by CD-4 cells and the implications for the current results and future research.

The MAH was also asked to discuss the implications of the observation that no expansion of potentially cross reactive cells was seen after vaccination with Pandemrix.

Since the Mignot report included in Module 5 does not include detailed results for overall TCRB usage changes in narcolepsy versus controls but only the summary results are included in the overview, the MAH was requested to provide the updated data.

6. Assessment of the responses to the request for supplementary information

Non-clinical aspects

6.1. Question 1

The MAH is requested to report the results from the additional ongoing research activities including the outstanding data for:

- TCR sequence analysis of CD45RO- CD4 T cells from 9 DQB1*0602+ subjects immunized with Pandemrix, before/after vaccination

Summary of the MAH's response

Samples of CD4 T cells from 8 DQ0602+ healthy subjects pre- versus post-Pandemrix (Day 0 and Day 42) were sequenced. To address whether significant changes could be detected, two different analyses were performed:

- First, the 8 pre/post Pandemrix samples were subjected to statistical analysis.
- Second, a combined dataset was analysed. This combined dataset consisted of the data from the 8 pre/post Pandemrix samples and data from samples obtained pre/post TIV vaccination (5 narcolepsy patients and 12 controls) to increase robustness of the data.

No change in TCR segment usage in the CD4 T cell population after influenza vaccination was found to be significant after Bonferroni correction, even when all post-influenza vaccination samples (TIV and Pandemrix) were pooled. This means that the putative narcolepsy-specific "TCR signature" (TRBJ1-3*01 defined as the single Bonferroni-significant difference) originally found after the comparison of cases versus controls was not observed after influenza vaccination. These results should, however, be interpreted with caution as the immune response is probably too clonally heterogeneous among individuals to allow detection of significant effects with such a small number of samples, even in a DQ0602 positive background. Thus, CD4 T cell clones with potential specificity for HCRT-secreting neurons are probably rare and not detectable in a bulk analysis. This issue is addressed, in part, by DQ0602-tetramer single cell sorting and TCR sequencing, as reported in response to question 2 and the commitments for TCR sequencing on total or memory CD4 T cell populations are considered to be met.

CHMP assessment of the MAH's response

This aspect of the plan was ambitious, employing state of the art methodology to identify potentially very rare T-cell receptor sequences. Analysis of TCR repertoires between narcolepsy patients and DQ0602-matched control revealed a single TCR gene segment (TRBJ1-3*01) to be significantly different between narcoleptic patients and controls after Bonferroni correction. In addition several TCR sequences appeared to be specific for narcoleptic patients. The analysis of additional samples obtained before and after Pandemrix vaccination did not reveal changes in TCR segment usage.

The objective to study TCR profiles in narcoleptic patients by deep sequencing has been formally completed although at this stage only tentative changes in TCR usage have been detected. As discussed

due to the potential low prevalence of narcoleptic specific CD4 T-cells analysis is difficult and limited by statistical power.

6.2. Question 2

The MAH is requested to report the results from the additional ongoing research activities including the outstanding data for:

- Single cell TCR sequencing from DQB1*0602 tetramer sorted cells from 7 pairs of samples (narcoleptic patients and controls)

Summary of the MAH's response

Analysis of CD4 T cell cross reactivity was done initially based on single-cell sorting of DQB1*0602-tetramer-positive CD4 T cells from 5 cases and controls. To increase the power of the initial sequencing data, 7 pairs of cases and controls were further analysed to make 12 pairs in total.

A full report entitled 'Analysis of CD4T cells with hypocretin and hemagglutinin peptide/DQ0602 tetramers' presenting data generated by the laboratory of Professor Mellins was provided in the annex to this question. The main conclusions that can be drawn from the work done in collaboration with Prof Mellins' laboratory can be summarized as follows:

(1) Cross reactivity between HA-275 and HCRT was observed in both narcolepsy cases and in controls, with most of these cross-reactive CD4 T cell clones being specific for both HA-275 and HCRT-L. This provided the proof of concept for the single cell TCR sequencing methods used in the context of different autoimmune disorders to study the T cell repertoire.

(2) CD4 T cell cross-reactivity is not an exclusive biomarker for disease since it is also observed in control subjects. This suggests that cross reactivity may be necessary but not sufficient to induce disease.

(3) All control subjects harbouring cross-reactive CD4 T cell clones had been vaccinated with influenza vaccines.

(4) As a potential indicator of autoimmune potential, expansion of cross-reactive CD4 T cell clones was compared leading to the conclusion that expansion of such cross-reactive clones in cases and controls was similar.

(5) Sharing of gene segment usage between different individuals is more often observed in autoimmune-related T cells. The observation that a TCR comprising the TRAJ24 and TRBV29-1 segments was found in 5 cross-reactive CD4 T cell clones, across 2 cases and 1 control, may be consistent with this.

CHMP assessment of the MAH's response

The data from the DQ0602 tetramer staining data reported initially and the single cell sequencing data support cross-reactivity between HA and three different epitopes in HCRT. The key observation is the confirmation that cross-reactive T cells can be detected in both cases and controls. Thus cross reactivity is not sufficient to induce disease. It is of interest the controls harbouring these cells have been vaccinated with either Pandemrix or TIV. More data would be needed to confirm the significance of this finding.

Clones reactive with the DQ0602/HCRT-L tetramer were observed in every donor suggesting that the HCRT leader peptides can be presented by MHC molecules.

6.3. Question 3

The MAH is requested to report the results from the additional ongoing research activities including the outstanding data for:

- PCR-based immunophenotyping from DQB1*0602 tetramer sorted cells from 11 pairs of samples (narcoleptic patients and controls)

Summary of the MAH's response

Immunophenotyping has been conducted using the RNA isolated from tetramer-sorted CD4 T cells for PCR-based analysis of a set of immune-related phenotypic markers.

Only IFN-γ transcription is detected in CD4 T cells from patients and controls. The reason for not detecting cytokine mRNA expression can be explained by

(i) assuming that IFN-γ is the dominant cytokine in CD4 T cells from cases and controls
(ii) assuming that TCR-tetramer interaction was not sufficient to stimulate the sorted CD4 T cells to begin expressing cytokine genes
(iii) that the timing between tetramer-staining (and thus TCR stimulation) and mRNA isolation was not optimal to detect cytokine gene expression

The narcolepsy cases and the controls share transcription of TBET.TBX21, which is a Th1-type transcription factor. Combined with IFN-γ expression, this suggests that a Th1 phenotype is dominant among CD4 T cells from cases and controls.

No consistent patterns for FOXP3 (identifying regulatory CD4 T cells) or RORC (identifying Th17 cells) were identified. In some cases, no PCR signals were identified and there may have been a PCR technical issue due to the occurrence of primer-dimers.

Overall it is concluded that the initial comparison of the data obtained from single, tetramer-sorted cell from narcoleptic patients and controls does not reveal major differences and suggests that a Th1 phenotype is dominant in both groups. Hence, this objective is considered completed.

CHMP assessment of the MAH's response

Considering that cross-reactive tetramer-positive CD4 T cells are detected in both narcoleptic patients and DQB1*0602-matched control subjects it was important to determine if potential phenotypic differences are relevant. There were no obvious differences noted between cases and controls when all clones were analysed or when only the cross-reactive clones were analysed. There remain some questions around of the robustness of the PCR analysis with concerns that these data are influenced by the efficiency of the different primer sets.

6.4. Question 4

The MAH is requested to report the results from the additional ongoing research activities including an updated discussion and conclusions.

In the discussion the MAH is requested to comment on studies that suggest HCRT-L (N-terminal leader sequence MNLPSTKVSWAAV) may not be a negative control and may be presented by CD-4 cells and the implications for the current results and future research.

The MAH should also discuss the implications of the observation that no expansion of potentially cross reactive cells was seen after vaccination with Pandemrix.

Summary of the MAH's response

The MAH provided an updated discussion and conclusions taking into account the additional data discussed above and several recent publications.

Novel data from Prof. Mellins' laboratory and rationale for T cell responses to leader peptide

To complement the TCR deep sequencing approach, it was decided to also study CD4 T cells using DQB1*0602 tetramers.

Overall the completed DQ0602 tetramer staining data indicate that

(i) CD4 T cells specific for HCRT56 and HCRT87 are detected, after stimulation with their cognate peptides or with the HA275 peptide (all presented to the T cells by DQ0602)
(ii) such CD4 T cell responses are detected both in narcolepsy patients as well as in DQ0602-matched control subjects
(iii) the frequencies of HCRT specific CD4 T cells are low, as evidenced by the need for in vitro expansion in order to visualize these T cells.

The fact that HCRT56/87-specific CD4 T cells are detected in narcolepsy patients as well as in healthy control subjects suggests that the presence of these cells does not per se predict or explain narcolepsy. Neither does the fact that these CD4 T cells seem to display cross-reactivity with the HA275 epitope. Indeed, it has been shown that CD4 T cell cross-reactivity is relatively ubiquitous in the immune system.

Whereas these data indicate that CD4 T cell cross-reactivity can exist at the polyclonal level, they do not address the question whether it is the same CD4 T cells responding to HCRT and HA, because this would be masked in bulk analysis of cell populations. Therefore, it was concluded that single cell TCR sequence analysis would be needed to address this question, since the TCR sequence is the single unique identifier of a T cell. It was further concluded that the most convincing method to identify potential epitope mimicry was to perform single cell TCR sequencing on DQ0602-tetramer-sorted CD4 T cells from narcoleptic patients and DQ0602-matched controls.

The original dataset from Prof. Mellins, based on 4 patient samples and discussed in the preliminary report, provided proof-of-principle for the method: TCR sequences were obtained from tetramer-sorted single CD4 T cells for the HCRT1-13, HCRT87 and HA275 tetramers. Strikingly, TCR sequence identity was observed between CD4 T cells isolated with the HCRT1-13 and HA275 tetramers. A single TCR sequence was identified that was shared between all three tetramers (HA275, HCRT1, HCRT87). This involvement of the HCRT1-13 peptide was surprising since, the HCRT1-13 leader peptide was considered to be a negative control given that no T cell responses to it had so far been described and also because leader peptides were considered to be not presented to the immune system. The consistent finding that the same TCR was detected when using either the HCRT1-13 or the HA275 DQ0602- tetramer as 'bait', suggested that a HCRT1-13-specific CD4 T cell population could actually exist. The alternative hypothesis, i.e., that the CD4 T cells identified with the HCRT1-13 tetramer are non-specific, would be expected to produce more random TCR sequences after cell sorting. In the updated report, the results from additional samples are provided, such that a total of 12 samples from narcoleptic patients and 12 samples from DQ0602-matched controls have now been analysed by tetramer sorting and TCR sequencing.

From the collective results, it is concluded that

(i) the finding of TCR sequence identity (i.e., for both TCRA and TCRB chains and including both CDR3 regions) between HCRT1-13 and HA275 is detected in 9/10 patient samples
(ii) TCR identity (defined as above) is observed in 6/10 control subjects.

The major conclusion from the data is that the initial finding of TCR identity between HCRT1-13 and HA275 is reproduced in the additional samples. This TCR match is detected in narcoleptic patients but

also in control subjects. There may be additional cross-reactivity with HCRT87, both with the HCRT1-13 peptide as well as with HA275, consistent with the bulk DQ0602-tetramer expansion and staining experiments. Of interest, the TRAJ24 segment was identified 5 times, in different subjects, and always with the same TCRA and TCRB structure but with slightly different CDR3 sequences. This finding is of interest because TRAJ24 was previously identified as being associated with narcolepsy, and the finding is therefore consistent with the hypothesis that the cross-reactive CD4 T cells are involved in narcolepsy.

It was initially surprising to detect a TCR match using the DQB1*0602 tetramer carrying the HCRT1-13 leader peptide because this tetramer was initially thought to be the negative control. The current data indicate that that assumption was most likely incorrect and that the HCRT1-13 carrying DQ0602 tetramer was able to detect specific CD4 T cells. The lack of prior such reports may be due to the very low frequencies of these T cells and the very high sensitivity of the direct ex vivo sorting method that Prof. Mellins has used. Another finding that is of interest is that the TCR sequence identity between HA and HCRT1-13 was detected in control samples from subjects with a history of influenza vaccination. From this, it could be hypothesized that cross-reactive CD4 T cell responses are indeed relatively ubiquitous and do not by themselves lead to narcolepsy symptoms.

In conclusion, the collective dataset (i.e. both the DQ0602 tetramer staining data and the single cell sequencing data) support cross-reactivity between HA and three different epitopes in HCRT. It was remarkable though that the HA/HCRT-1-13 cross-reactivity seemed to be the most obvious in the TCR sequencing studies. One potential explanation considered for this and for the surprising finding of multiple mimicry epitopes within the HCRT sequence, was that the HA peptide itself might bind to DQ0602 in different binding frames (“registers”): the HA275 peptide may contain different DQ0602 anchoring residues that allows the HA peptide to bind the DQ0602 in different binding frames, referred to as registers. In order to test this hypothesis, computer modelling was used to visualize the HA275 peptide in DQ0602 different binding frames. This modelling was then compared to modelling of the HCRT56 (HCRT-1) and HCRT87 (HCRT-2) peptides in DQ0602. The modelling revealed a 3D similarity between the HA275 peptide and a HCRT peptide, but in this case with the HA peptide in a different binding frame.

The MAH states the strong indications that HA/HCRT cross-reactivity does exist implies that the company commitments have been met.

Implications of the observation that no expansion of potentially cross reactive cells was seen after vaccination with Pandemrix

The MAH comment that the observation that no expansion of potentially HA/HCRT cross-reactive CD4 T cells, as detected by DQ0602-tetramers, was seen after vaccination with Pandemrix in healthy subjects may reflect immune regulation.

First, cross-reactive CD4 T cell responses are subject to immune regulation mediated by regulatory CD4 T cells.

Second, the CD4 TCR repertoire for the HA275 epitope appears to be quite broad. Prof Mellins TCR sequencing data revealed that the repertoire is in fact dominated by non-cross-reactive TCR sequences, i.e., only a minority of CD4 T cell clones are cross-reactive. It is possible that there are competitive differences between T cells with cross-reactive and non-cross-reactive TCRs after vaccination with Pandemrix. This could result in the selective expansion of non-cross-reactive T cell clones.

Third, the expansion assay itself may introduce biases. The observation that CD4 T cells specific for HA275 and HCRT are only detected by the DQ0602 tetramers after long in vitro expansion implies that the ex vivo frequencies are very low. Long expansion may obscure small differences in CD4 T cell frequencies.

Translating novel data into a putative mechanism

The MAH conclude by drawing the data together to propose a potential mechanism.

Although the current data support that CD4 T cell cross-reactivity between HCRT and HA can exist, they do not provide final evidence for causality in the development of narcolepsy. Demonstration of CD4 T cell cross-reactivity leaves two key questions unanswered. First, how do these CD4 T cells enter the CNS given that their activation occurs in the lymph node draining the intramuscular injection site? Second, if any such CD4 T cells would enter the CNS, how would they induce disappearance of HCRT secreting neurons, given that these neurons are HLA II negative, yet HLA I positive?

The previously reported studies in the cotton rat model indicate that neither the vaccine nor the AS03 adjuvant alone induce inflammatory changes in the CNS or changes in blood brain barrier (BBB) permeability (EMEA/H/C/000832/II/0078 CHMP AR).

The fact that HCRT-secreting neurons are HLA II negative suggests that cross-reactive CD4 T cells, even if they would enter the CNS, are not likely to directly interact with HCRT neurons. CD8 T cells might play a role because they can recognize HLA I. The MAH highlight two recent publications that point towards a putative role for CD8 T cell responses by revealing increased risk of narcolepsy associated with several HLA I alleles, i.e., B35, B51.

The potential role for CD8 T cells is of interest. In the context of AS03-adjuvanted influenza vaccines, it has been shown that the AS03 adjuvant leads to increased CD4 T cell responses but not CD8 T cell responses. Therefore, any putative role of CD8 T cell responses is not easily explained by the immune responses induced by the AS03- adjuvanted vaccine only.

Indications for a putative role of H1N1 viral infection

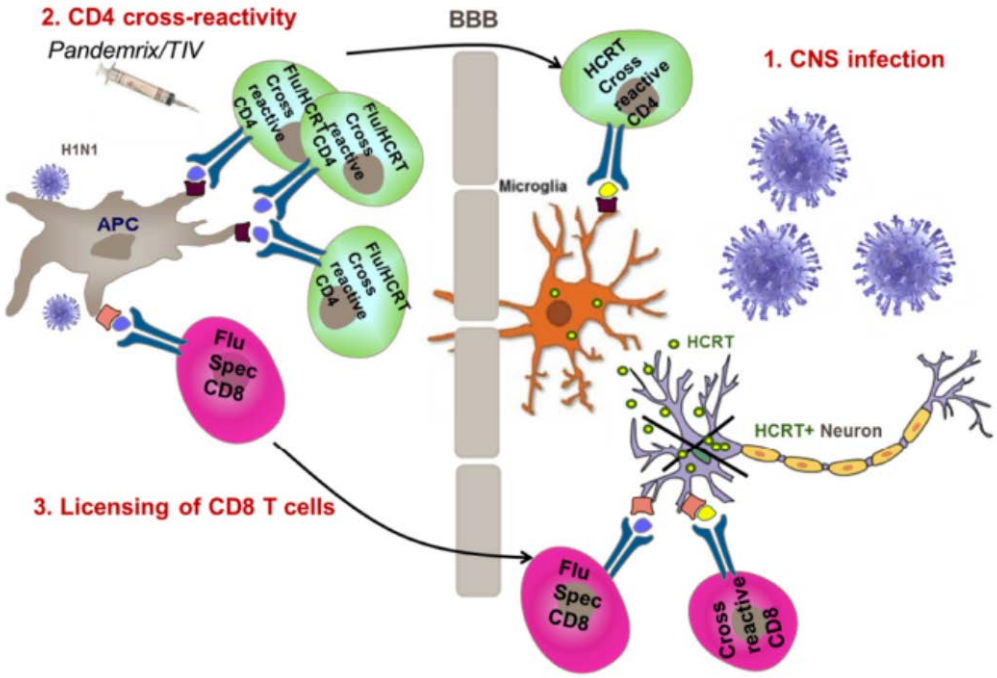
The MAH highlight a recent publication by Tesoriero et al (2016) who used an immune-deficient mouse model to study the effects of H1N1 influenza infection on the CNS and the hypothalamus in particular. RAG-1 knock-out mice (mice deficient for Recombinant Activating Gene -1 resulting in total lack of B and T cells) were intranasally infected with a neuro-adapted strain of H1N1 influenza virus. Viral replication was shown in the lateral hypothalamus and correlated inversely with the presence of HCRT-secreting neurons. In several cases, viral replication was observed in HCRT-secreting neurons. The infected mice developed narcolepsy-like symptoms that closely resembled those of hypocretin- or hypocretin receptor deficient mice. It seems likely that the loss of HCRT-secreting neurons was associated with viral replication and triggered the appearance of narcolepsy-like symptoms. This publication established migration of the neuro-adapted H1N1 influenza virus to neurons with involvement of the olfactory bulb and resulting in narcolepsy-like symptoms. However, when interpreting these results, it is important to consider that the neuro-adapted H1N1 strain that was used (WSN/33) differs from the H1N1/pdm09 influenza virus in HA glycosylation patterns. Another issue to consider is the immune-deficient nature of the RAG-1 knock-out mouse model. On one hand, the lack of immune control may have facilitated detection of viral neurotropism because of more extensive viral replication. On the other hand, normal immune responses, e.g., in infected people, are expected to control viral replication and prevent wide-spread infection in the CNS, consistent with the lack of changes in the CNS of H1N1–infected cotton rats (EMEA/H/C/000832/II/0078 CHMP AR). Indeed, influenza virus-induced encephalopathy is a rare but serious complication of influenza virus infection that may be post-infectious or linked to viral invasion of the brain.

Towards a model of immune-induced narcolepsy

Given the indications for immune involvement in narcolepsy as well as putative H1N1 viral tropism for HCRT neurons, a plausible model involves both. In this hypothetical model, H1N1 infection would display

tropism for HCRT neurons in infected subjects, possibly explaining the Chinese epidemiological data on narcolepsy seasonality. Infection of the hypothalamus in immune-competent people may result in some local cell death, i.e., among hcrt-secreting neurons, and release of pre-pro-hypocretin in an immune-stimulatory context, i.e. associated with viral replication. This local cell death could then serve as a trigger to alert the immune system and any potential CD4 T cell cross-reactivity, such as between HA and the HCRT leader, may be sufficient to break local immune tolerance.

Figure 9. Multi hit model as a way to explain immune-induced narcolepsy



(1) CNS infection: Influenza virus infection has been associated with encephalopathy and subclinical brain injury. In addition a recent paper suggested that in the absence of a competent adaptive immune system, H1N1 virus was able to migrate and destroyed HCRT+ neurons in mice leading to narcolepsy like syndrome. Based on these papers, the MAH think that subclinical infection of the CNS by H1N1 could lead to the death of some HCRT+ neurons leading to a danger signal able to recruit Influenza specific activated CD4 (expanded by influenza vaccination) and CD8 T cells. That cell death will also release HCRT from dying neurons that will be then available for microglia to present to activated CD4 T cells.

(2) CD4 cross-reactivity: CD4 T cell cross reactivity between HA and HCRT has been suggested by the E. Mellins's work. Therefore, Influenza specific CD4 T cells boosted by the vaccination could cross the BBB and be further activated by microglia expressing HCRT (most probably the HCRT-L).

(3) CD8 T cell licensing: Activation of CD4 T cells will allow the licensing of pre-existing influenza activated CD8 present in the CNS. Given the specific loss of HCRT+ neurons in narcolepsy, it is likely that these CD8 T cells might also have some cross reactivity with HCRT to be able to specifically target these neurons.

In summary, the MAH suggest this model is consistent with the following observations:

- Chinese epidemiological data suggesting narcolepsy seasonality and increased incidence in 2010
- German data suggesting that narcolepsy incidence increased already in the spring/summer of 2009, before the vaccination campaign began

- GSK modelling results, based on Norwegian infection and vaccination data, suggesting that a high proportion of subjects immunized with Pandemrix had been exposed to H1N1 influenza virus prior to vaccination (manuscript submitted)
- Associations with HLA I alleles, suggesting a role for CD8 T cells.
- Tropism of H1N1 influenza for HCRT secreting neurons
- Lack of evidence for AS03-impact on the CNS (EMEA/H/C/000832/II/0078)

The company highlight that this hypothesis may serve as a framework to shape future research. If this mechanistic model is correct, the critical role of CD4 T cells suggests that further stimulation of cross-reactive CD4 T cells, e.g., by immunization with an adjuvanted vaccine, could potentially lead to increased risk. This may not be completely black and white, though: enhanced CD4 T cell responses could also decrease risk by ensuring an appropriate immune defence of the CNS. Timing of immunization may play a key role: immunization before infection or when the infection is resolved would not be expected to have any impact in the CNS. An additional variable may be age. As the influenza-specific CD4 T cell repertoire evolves over time with increased exposure to different antigenic variants, the MAH hypothesize that the cross-reactive repertoire, i.e. the repertoire revealed by Prof Mellins, may be less competitive as compared to other CD4 T cell clones.

- Immediate perspectives of the current data are further analysis and data mining of the single-cell TCR sequence data.

Overall, while the model proposed above seems plausible, the Company would require having additional supportive evidence to pursue new research.

CHMP assessment of the MAH's response

In the RSI response the MAH update their current thinking on translating the current data and recent publications into a putative mechanism. The MAH working hypothesis is that a subclinical infection of the CNS by H1N1 could lead to the death of some HCRT+ neurons leading to a danger signal able to recruit influenza specific activated CD4 (expanded by influenza vaccination) and CD8 T cells. That cell death will also release HCRT from dying neurons that will be then available for microglia to present to activated CD4 T cells. CD4 T cell cross reactivity between HA and HCRT has been suggested by the Prof Mellins's work. Therefore, influenza specific CD4 T cells boosted by the vaccination could cross the BBB and be further activated by microglia expressing HCRT. Activation of CD4 T cells allows the 'licensing' of pre-existing influenza activated CD8 T cells present in the CNS. Given the specific loss of HCRT+ neurons in narcolepsy, it is likely that these CD8 T cells might also have some cross reactivity with HCRT to be able to specifically target these neurons.

The MAH hypothesis is not consistent with some observations, such as a lack of evidence for H1N1pdm8H1N1pdm09 virus infection as a contributing factor in childhood narcolepsy in Finland (Melen et al, 2013) and the lack of changes in the CNS of H1N1–infected cotton rats (EMEA/H/C/000832/II/0078 CHMP AR). However, it is agreed the hypothesis may serve as a framework to shape future research. The MAH states while the model proposed above seems plausible they would require having additional supportive evidence to pursue new research.

The post-authorization measures as reflected in Annex II of the SmPC of Pandemrix can be considered completed. However, it is recommended the company continue to support further analysis of cross-reactive T cells and data mining of the TCR sequence data. The company should ensure the timely publication of their research work in this area.

6.5. Question 5

Note that the Mignot report included in Module 5 does not include detailed results for overall TCRB usage changes in narcolepsy versus controls and only the summary results are included in the overview. The MAH are requested to provide the updated data.

Summary of MAH response

The detailed results for overall TCRB usage changes in narcolepsy versus controls have been included as an Appendix to the report. The revised report is provided as annex to question 1.

CHMP assessment of the MAH's response

Data noted.

6.6. Conclusion

The EMA, taking into account the public health impact of narcolepsy and potential implications for the future use of similar vaccines, has finalised the assessment of this application after the expiry of the Marketing Authorisation on 13 August 2015. The MAH confirmed that it did not apply for a renewal of the authorisation due to lack of demand for the vaccine.

The outstanding post-authorization measures as reflected in Annex II of the MA of Pandemrix can now be considered completed.

☒ Overall conclusion and impact on benefit-risk balance has/have been updated accordingly (section 2)

☐ No need to update overall conclusion and impact on benefit-risk balance