Stanford Center for Sleep Sciences and Medicine
Wake Up Narcolepsy Report 2016

The Stanford Center for Sleep Science and Medicine's narcolepsy group has had an extremely busy year and we are very appreciative of the support from Wake Up Narcolepsy. The support has been particularly important this year as our National Institute of Neurological Disorders and Stroke's (NINDS) Program Project Grant is up for renewal and required a full re-application followed by a revised submission. We expect to find out in February 2017 if the grant will be renewed, but current funding has been suspended since July 2016. The funding from Wake Up Narcolepsy is helping us bridge this gap so we can continue research and retain important research personnel. Below is a summary of some of the lab's recent efforts.

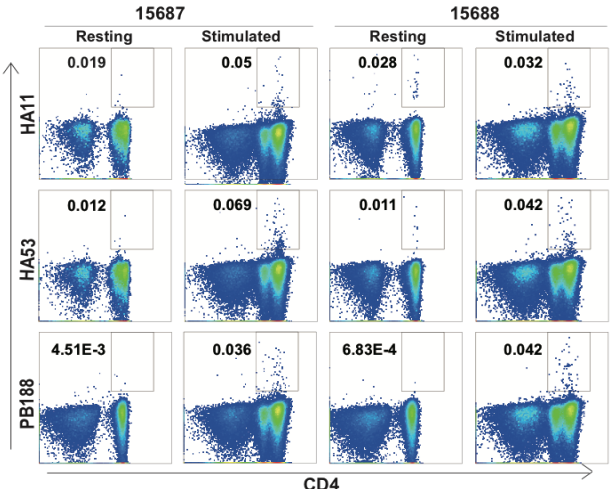
Genetic Research:
We performed a Genome Wide Association Study (GWAS) in Asian, African American and Caucasian samples including 5,000 cases and 30,000 controls which was presented at Sleep2016[1]. Regulatory effects of the top findings were examined using data from the ENCODE and GTEx consortiums. In addition, direct (subsample) genotyping and HLA imputation at high resolution (8-digit) was performed using HIBAG. Case control matching and conditional analysis were performed. In addition to well-known DQB1 effects, HLA-DPA1*01:03~DPB1*04:02 was highly protective, while other alleles at DPB1 and HLA-Class I increased susceptibility. **We confirmed existing risk associations and also found significant genetic associations with eight novel genetic locations that predisposed to narcolepsy.** The majority of these novel loci are either shared with other autoimmune diseases or are known regulators of immune response. The results highlight both the importance of HLA in narcolepsy and the effect of individual risk variants. **The novel genes may explain how hypocretin cells are destroyed and support a T cell mediated autoimmune attack in the cause of narcolepsy, notably involving a sub-group of T cells called CD8 cells. A couple of other genetic variants are also suggesting possible brain pathways that are affected in narcolepsy.**

Immunological Research:
Vaccine composition: We conducted 2D-DIGE and MS biochemical studies of one 2009 Pandemrix® batch and one 2010 Arepanrix® batch, showing that the composition of these vaccines was similar and included multiple instances of protein degradation[2, 3]. Approximately 40% of all mappable peptides were of influenza-A origin, a third mapped to chicken proteins, a few mapped to Bos Taurus (due to the addition of deoxycholate, Gallbladder extracts), and the rest were unknown. Arepanrix® displayed a larger diversity of viral and chicken proteins, with the exception of five chicken proteins (PDCD6IP, TSPAN8, H-FABP, HSP and TUB proteins) that were more abundant in Pandemrix®. A significant finding was an HA mutation N146D present in ~60% of peptides in Arepanrix® but not present in Pandemrix® (3%). This mutation occurs within one of our identified DQ0602 binder (PHDSNKGVT), with the N position predicted to be in position 2 of the 9-position register of the DQ peptide binding groove, therefore contributing to DQ0602 binding. Although 146N is present in the PR8 backbone and some post 1918 H1N1-derived strains, it is located within a very different sequence context unlikely to produce a similar epitope[4]. Since in recent vaccine strains and Focetria® after 12/2009, 146N is mutated to D (X-181 associated with increased production yields), the mutation could possibly explain why Pandemrix® increased susceptibility versus other vaccines[2]. This represents a first interesting epitope to test further for T-cell activation assays in

narcolepsy cases. **The study shows that there are differences in vaccine composition that may explain why some flu vaccines and strains and not others can trigger narcolepsy in susceptible individuals. These differences are next assayed in T cell activation and T cell tetramer studies.**

T-cell activation and tetramer studies: DQ0602 tetramers were constructed as described by Kwok et al. who also reported the successful use of DQ0602 tetramer using a known HSV Type 2 binder[5]. Tetramers are reagents that contain a labeled DQ0602 molecule that is also binding any peptide of interest (for example a peptide derived from a flu vaccine). This labeled complex can then be used to stain and study T cells that recognize this complex and may be different in controls versus narcolepsy (Figure 1).

Figure 1. FACS plots showing the % frequency of 3 different DQ0602 (DQA1*0102/DQB10602) tetramer-peptide restricted CD4 T cells in a discordant twin pair (15687-narcolepsy & 15688- control) before and after enrichment using 100 ng/ml of Pandemrix® for 7 days, followed by 10U/ml of IL-2 for 3 more days. The biotinylated monomer DQ0602 was pre-incubated with peptide for 3 days at 37C, and conjugated with PE labelled streptavidin at a molar ratio of 6:1. For tetramer staining, PBMCs were incubated with DQ0602 tetramer-peptide for 1 hour at 37C and then stained with anti-CD3/CD4/CD8 antibodies on ice, and analyzed with FACS. The cells were gated on $CD3^+$.



We next screened 35 tetramers containing strong binders in influenza HA, NA, and PB1 and NP of X-179A following 10 days Pandemrix® culture in 1 recent onset (<6 month) patient, 1 post-Pandemrix case and in one twin pair discordant for narcolepsy. Of 8 PB1 peptides tested, only 2 gave interesting signals (positive in the post-Pandemrix® or the recent onset sample), suggesting PB1 is not an important antigen, in line with its low abundance. Of 11 NP, 10 HA and 3 NA peptides, 9, 7 and 3 had interesting signals, respectively. Of these candidates, signal has been tested at baseline in the same subjects for 9 NP, 3 HA, 1NA and 1 PB1 peptides and in all cases except for 4 subjects signal was enriched by the culture. None of these first signals were present in all 3 cases but not the controls. These experiments are using state of the art immunological techniques to study differential reactivity of T cells of patients versus controls to various pieces of the H1N1 vaccine. **We hope to find specific T cell tetramers that will differentiate T cells from control versus narcolepsy.**

As mentioned, we just resubmitted our NINDS Program Project Grant application titled Genetic, Neurobiological and Immunological Basis of Type 1 Narcolepsy. It is a multidisciplinary grant with integrated projects in three different disciplines: genetics, immunology and neurobiology. The overarching topic is how genetic and environmental factors converge to create a breakdown of immune tolerance leading to HCRT neuronal loss. We believe our proposal will unravel the pathophysiology of narcolepsy.

Machine Learning Research:
Electroencephalography (EEG) power spectral density (PSD) was computed in 136 type 1 narcolepsy patients (narcolepsy with cataplexy) and 510 sex- and age-matched controls[6]. Features reflecting

differences in Power Spectra Density curves were computed. A regression model was used to find an optimal feature subset, which was validated on 19 type 1 narcolepsy patients and 708 non-narcolepsy patients from a sleep clinic. Reproducible features were analyzed using receiver operating characteristic (ROC) curves. Results showed that narcolepsy patients show (1) increased alpha power in REM sleep, (2) decreased sigma power in wakefulness, and (3) decreased delta power in stage N1 versus wakefulness. Sensitivity of these features ranged from 4% to 10% with specificity around 98%, and it did not vary substantially with and without treatment. Therefore, we believe EEG spectral analysis of REM sleep, wake, and differences between N1 and wakefulness contain diagnostic features of NC. These traits may represent sleepiness and dissociated REM sleep in patients with type 1 narcolepsy.

However, the features are not sufficient for differentiating type 1 narcolepsy from controls. Therefore, we are developing a single algorithm that combines the findings from this work with several other polysomnogram (PSG) biomarkers discovered and validated by our team[6-9]. **We will use a combination of 11 PSG biomarkers to establish a detector that will identify individuals who have narcolepsy with a sensitivity and specificity similar to the MSLT based on data recorded during a single overnight sleep study. This would greatly facilitate the detection of narcolepsy cases as a simple sleep study would be used instead of the more costly MSLT.**

REFERENCES:

1. Ollila, H., Hillary R, Faraco J, Lin L, Hallmayer J, Han F, Mignot E, *Genome-wide Association Study Identifies Novel Genetic Loci in Narcolepsy.* Sleep 2016. **39**(Abstract Suppl.): p. A2.
2. Jacob, L., et al., *Comparison of Pandemrix and Arepanrix, two pH1N1 AS03-adjuvanted vaccines differentially associated with narcolepsy development.* Brain Behav Immun, 2015. **47**: p. 44-57.
3. Jacob L, L.R., Bonvalet M, Luo G, Ling Lin, Chris Adams, Danuta Skowronski, Gaston DeSerres, Emmanuel Mignot *Letter regarding article « Antibodies to influenza nucleoprotein cross-react with human hypocretin receptor 2".* Science Translational Medicine, 2016 In Press.
4. Medina, R.A., et al., *Glycosylations in the globular head of the hemagglutinin protein modulate the virulence and antigenic properties of the H1N1 influenza viruses.* Sci Transl Med, 2013. **5**(187): p. 187ra70.
5. Kwok, W.W., et al., *HLA-DQ tetramers identify epitope-specific T cells in peripheral blood of herpes simplex virus type 2-infected individuals: direct detection of immunodominant antigen-responsive cells.* J Immunol, 2000. **164**(8): p. 4244-9.
6. Christensen, J.A., et al., *The diagnostic value of power spectra analysis of the sleep electroencephalography in narcoleptic patients.* Sleep Med, 2015. **16**(12): p. 1516-27.
7. Andlauer, O., et al., *Nocturnal rapid eye movement sleep latency for identifying patients with narcolepsy/hypocretin deficiency.* JAMA Neurol, 2013. **70**(7): p. 891-902.
8. Christensen, J.A., et al., *Sleep-stage transitions during polysomnographic recordings as diagnostic features of type 1 narcolepsy.* Sleep Med, 2015.
9. Christensen JA, M.E., Carrillo O, Moore HE, Peppard PE, Young T, Sorensen HB, Mignot E, Jennum P, *Diagnostic Value of Polysomnographic EEG in Narcoleptics.* Sleep, 2014. **37**(Abstract Supplement): p. A230.