NIMBL

PROJECT PERIODIC REPORT

Grant Agreement number:	**241779**
Project acronym:	**NIMBL**
Project title:	**Nuclease Immune Mediated Brain and Lupus-like conditions (NIMBL): natural history, pathophysiology, diagnostic and therapeutic modalities with application to other disorders of autoimmunity**
Funding Scheme:	**Collaborative Project FP7-HEALTH-2009-2.4.4-1: Rare neurological diseases**
Period covered:	from: **01 June 2010** to: **30 November 2013**

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i. Selected abbreviations used in this report

AGS	Aicardi-Goutières syndrome
CADASIL	Cerebral Autosomal Dominant Arteriopathy with Subcortical infarcts and Leukoencephalopathy
CT	Computed tomography
CSD	Cortical spreading depression
CSF	Cerebrospinal fluid
dNTP	Deoxy-nucleotide tri-phosphate
DKO	Double knock-out
DNA	Deoxyribonucleic acid
ELA	European Leukodystrophy Association
EMQN	European Molecular Genetics Quality Network
EQA / PT	External Quality Assessment and Proficiency Testing
FCL	Familial chilblain lupus
IAGSA	International Aicardi-Goutières syndrome Association
IFN	Interferon
ihNSC	Immortalized human neural stem cells
IL	Interleukin (a family of small proteins involved with the immune system)
ISD	Interferon stimulated DNA
ISG	Interferon stimulated genes
KD	Knock-down
KO	Knock-out
LOVD	Leiden Open Variation Database
MLPA	Multiplex ligation-dependent probe amplification
MRI	Magnetic resonance imaging
NIMBL	Nuclease Immune Mediated Brain and Lupus-like conditions
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
RTIs	Reverse transcriptase inhibitors
RVCL	Retinal vasculopathy with cerebral leukodystrophy
SLE	Systemic lupus erythematosus
SNP	Single nucleotide polymorphism
SPENCD	Spondyloenchondrodysplasia
TLR	Toll-Like Receptor (proteins that are involved in nucleic acid sensing)
TNF	Tumour Necrosis Factor (a proinflammatory cytokine)
WP	Work package
WT	Wild-type

1. Executive summary

Nuclease Immune Mediated Brain and Lupus-like (NIMBL) conditions, comprising Aicardi-Goutières syndrome (AGS), retinal vasculopathy with cerebral leukodystrophy (RVCL) and some cases of systemic lupus erythematosus (SLE), are genetic disorders associated with significant morbidity, premature death, and high risks of recurrence. NIMBL conditions are rare, but under diagnosed. No effective treatments or cures exist at present.

To enable optimum patient care, a better understanding of the natural course of these disorders and of their underlying pathological basis, was, and remains, essential. In this project, European and U.S. clinical and basic research scientists united to develop a translational approach to these problems. The Principle Investigators involved were:

1. Prof Yanick Crow, (UNIMAN), Manchester, UK [B1]
2. Prof Arn van den Maagdenberg (LUMC), Leiden, the Netherlands [B2]
3. Dr Simona Orcesi (FMPV), Pavia, Italy [B3]
4. Prof David Bonthron, (LEEDS), Leeds, UK [B4]
5. Prof Taco Kuijpers (AMC), Amsterdam, the Netherlands [B5]
6. Prof Antonio Celada (IRB) and (UB) Barcelona, Spain [B6] and [B9]
7. Prof Dan Stetson (UW), Seattle, USA [B7]
8. Prof Adeline Vanderver (CNMC), Washington DC, USA [B8]

The NIMBL project started in June 2010 and built on very recent discoveries (at that time) of the cell-intrinsic initiation of autoimmunity, having major implications for our understanding of the discrimination of self from non-self. This particular biological paradigm involves intracellular sensors that detect self-derived DNA and RNA, in turn inducing the body to mount an immune response against its own cells. Thus, it was, and is still, considered that the investigation of NIMBL diseases would not only improve the health and well-being of NIMBL patients and their families, it might also lead to better treatments of certain, more common, autoimmune disorders – most particularly SLE.

By the end of NIMBL (November 2013), data had been recorded from 346 affected individuals belonging to 277 families with a confirmed molecular diagnosis of AGS, and from a total of 78 RVCL *TREX1* mutation-positive patients from 11 unrelated families worldwide - representing the largest sets of clinical, radiological and molecular information on these rare conditions ever collected.

The results from the NIMBL project have already had, and will continue to have, major impacts – which can be broadly outlined as: (i) an immediate benefit for affected individuals and their families by allowing for comprehensive diagnostic and carrier testing, (ii) a significant improvement in clinical knowledge of NIMBL-associated conditions, providing guidance on the diagnosis and prognosis of these disorders to physicians and families, and (iii) an increase in scientific knowledge of the underlying basis of the NIMBL-related diseases, with obvious implications for the development of future therapies. As proof of principle, our patient studies, combined with our newly-derived scientific information, have helped to suggest a rational approach to AGS treatment using reverse transcriptase inhibitors, so that the first ever clinical trial will begin in 2014 – a remarkable NIMBL-driven achievement.

The NIMBL project has met the overall project objectives initially stated, and all of the Deliverables and Milestones have been achieved. By the end of the project, 30 papers had been published in peer-reviewed journals. Further manuscripts are being prepared for submission in the coming months, and we are confident that the collaborations established between centres will endure beyond NIMBL into the long-term.

2. A summary description of project context and objectives

Project Context – prior to the start of NIMBL

Aicardi-Goutières syndrome (AGS), which is completely distinct from the similarly named Aicardi syndrome, is a rare inflammatory disorder, most typically affecting the brain and the skin, with onset usually in early childhood. Following an original description of eight cases in 1984, the condition was first referred to as 'Aicardi-Goutières syndrome' in 1992. AGS is a severe disease causing significant physical and mental disability and, frequently, death in childhood. It had been previously recognised that some characteristics of AGS overlap with congenital infection, and with the autoimmune disease systemic lupus erythematosus (SLE) – of note, all three states are recognised to be associated with increased levels of the anti-viral cytokines referred to as type I interferons.

In contrast to AGS, patients with retinal vasculopathy with cerebral leukodystrophy (RVCL) present much later in life, generally aged 30-50 years. RVCL results in visual loss, stroke and dementia - believed to be caused by a systemic condition involving small blood vessels in multiple organs, most particularly the brain, eye and kidney, and death 5 - 10 years later.

When the NIMBL project was proposed, AGS was known to result from mutations in any one of five genes encoding: the exonuclease TREX1 (*AGS1*), the three subunits of the RNASEH2 endonuclease protein complex (*AGS2, 3* and *4*), and the then uncharacterised protein SAMHD1 (*AGS5*). AGS is usually inherited as a recessive trait, although heterozygous (dominant) mutations in *TREX1* had been reported to cause AGS in rare cases. RVCL was also known to be inherited as a result of *TREX1* dominant mutations. In addition, heterozygous *TREX1* mutations had been found in about 2% of cases of SLE.

It was already recognised that the presentation of AGS could be broadly divided into two types; i) infants presenting in the neonatal period with disturbed neurology and brain imaging (CT and / or MRI scans can show various abnormalities, including white matter changes and calcification). This clinical picture can mimic that of *in utero* acquired infection, with which AGS can easily be confused diagnostically, and contrasts with; ii) patients presenting at variable times beyond birth (usually around 3 to 6 months of age) after an initial period of normal development, and who then experience the subacute onset of a severe encephalopathy associated with neuroregression. In either scenario, the initial period of disease 'activity' seems to last for several months, after which time, clinically, the condition appears to stabilise ('burn-out'). Obviously, the severity of the disease, and the associated risk of recurrence, have a devastating effect on patients and families, and a significant cost in terms of health-care.

At the start of the NIMBL project, the mechanisms that cause the associated phenotypes were unclear, and therapies were, and still are, based on ameliorating the various symptoms as they arise, rather than treating the underlying pathology. However, since TREX1 and RNASEH2 are nucleases (enzymes that degrade nucleic acid - DNA and RNA), we had hypothesised that these proteins might be involved in removing 'waste' nucleic acid species, and that a failure of this process in AGS could result in activation of the immune system; that is, in the absence of AGS-related enzyme activity, endogenous nucleic acids accumulate and are sensed as 'non-self' / viral, subsequently inducing an interferon mediated immune response. This would explain the phenotypic overlap of AGS with congenital infection and SLE - where interferon is also recognised to be an important factor in disease pathology.

Although RVCL is associated with genetic changes in the *AGS1* gene *TREX1*, the condition behaves differently from AGS, suggesting that the relevant disease process was possibly

distinct from AGS – and apparently most likely related to a primary problem of blood vessel homeostasis.

We considered that advances in our understanding of the mechanisms underlying the devastating NIMBL-associated conditions would lead to earlier diagnosis, and provide molecular targets for the development of novel therapies. NIMBL was thus conceived as a translational project - directly linking clinical (natural history) and basic research (pathophysiology) to the identification of effective preventive (enhanced clinical awareness, prenatal diagnosis), diagnostic (laboratory, radiological and genetic), and therapeutic interventions in order to alleviate the negative impact of these diseases on the quality of life of affected patients and their families.

Against this background, and with these considerations in mind, we outlined four objectives as being of greatest relevance to address in order to move the field forward, always with a view to the development of therapeutic strategies based on knowledge of disease biology.

Objective 1. Gain insight into the natural history of AGS and RVCL

The rarity of the NIMBL disorders represents a challenge to a proper understanding of their natural history. For this reason, it was considered imperative to derive disease-specific registries, in order to define the true clinical nature of these conditions – in terms of their presenting features, their progression and the final outcome of being affected. Only in this way could we provide families and clinicians with accurate information about the diseases. Additionally, we were interested to determine if there was any relationship between underlying genetic subtype of AGS and clinical presentation and outcome, by linking clinical, radiological and laboratory data (including serial assessments over time) with genetic diagnosis – since this might provide us with diagnostic clues and insights into disease pathology. Of particular importance, we recognised that information on natural history would be crucial when the time came to assess the effects of potential new therapies in future clinical trials.

Objective 2. Acquire knowledge for the development of diagnostic and therapeutic modalities

Although the genetic basis of a majority of patients with clinical features of AGS was known at the start of the NIMBL project, we were aware of some patients who did not have any changes in the already-defined AGS-associated genes. This meant that the diagnosis in these children remained open, and left their parents and wider family in a state of uncertainty with regards to the risk of recurrence. Moreover, in some cases, the interpretation of sequence changes in the known genes was unclear. We therefore wished to define new genetic causes of AGS, and to differentiate diseases showing overlap features with AGS and RVCL. Furthermore, we considered it of the utmost priority to develop gene-specific databases, available in an open-access format, in order to catalogue changes in these genes – and thus aid diagnosis in laboratories worldwide. As an adjunct, in an attempt to ensure uniformity of access to the highest standard of care throughout Europe, and further afield, we recognised a need to encourage quality-control assessments in diagnostic laboratories – informed by outputs of the NIMBL project. Finally, beyond genetic testing, we were cognisant of the need to identify other laboratory markers of disease, so-called biomarkers, which might aid in diagnosis, but, more importantly, could be used as indicators of disease activity in the assessment of outcome of trial therapies.

Objective 3. Develop four new animal models relevant to human NIMBL phenotypes

We appreciated that animal models can help to define the effects, both on the whole body and in specific tissues, of genetic mutations and the subsequent loss of, or change in, the function of an associated protein. Furthermore, we considered that animal models could provide systems in which to initially test potential new forms of treatment. We also recognised the ethical difficulties and responsibilities associated with animal work, and that animal models do not always reflect the situation in the human (where proteins and pathways may not be exactly the same / have identical functions). With these issues in mind, we set out to develop a number of disease-relevant animal (mouse) models of AGS and RVCL.

Objective 4. Explain the pathophysiology of NIMBL phenotypes

At the start of NIMBL, AGS was already considered as an inflammatory disease, associated with an apparently disease-relevant increase in the antiviral cytokines referred to as type I interferons. As discussed above, we had previously derived evidence to suggest that this interferon response was induced by self DNA / RNA, although the precise species of nucleic acid, and the specific mechanisms / signalling pathways involved in sensing their presence to the innate immune system, were undefined. In regards of AGS, it was also clear that the major features of the disease relate to damage to the brain. Again, the manner in which that damage accrued was unknown, and so we were keen to interrogate the interaction of interferon and specific brain cell function. Finally, we wished to consider the pathological basis of RVCL, asking the question as to whether this was common to, or distinct from, AGS.

Summary

The overarching aim of the above objectives was to improve patient care through better diagnosis and management by: (i) allowing for comprehensive diagnostic and carrier testing, (ii) improvement in clinical knowledge of NIMBL-associated conditions, providing guidance on diagnostic criteria and the phenotypic spectrum of these disorders to physicians and patients / families, and (iii) increased scientific knowledge - in terms of genetic, biochemical and cell biological understanding of the pathological basis of the NIMBL-related phenotypes, with obvious implications for the development of directed therapies for these devastating diseases.

Cognisant of the background as set out above, NIMBL was built around eleven work packages (WPs); one (WP1) was concerned with the management of the project as a whole, nine (WP2-10) were focused on research and technology, and a final WP (WP11) was dedicated to the dissemination of NIMBL-related outputs. The results of the NIMBL project in regards of these individual WPs are discussed in the following section.

3. A description of the main results

Work Package 2: *AGS and RVCL phenotypes*

Overall aim: The development of in-depth clinical knowledge of AGS and RVCL in order to inform studies of the molecular and cellular mechanisms of disease, and allow future studies of targeted therapeutics

Involving: B1, B2, B3, B8 (plus B4, B5)

Task 1: Comprehensive characterisation of the phenotype in individuals with mutations in genes known to cause AGS and RVCL.

B1, B3 and B8 have collected extensive clinical data from a cohort of patients with AGS, and other NIMBL-related phenotypes - so-called 'type I interferonopathies', in particular SPENCD patients with mutations in *ACP5* and a monogenic form of lupus due to mutations in *PRKCD*:

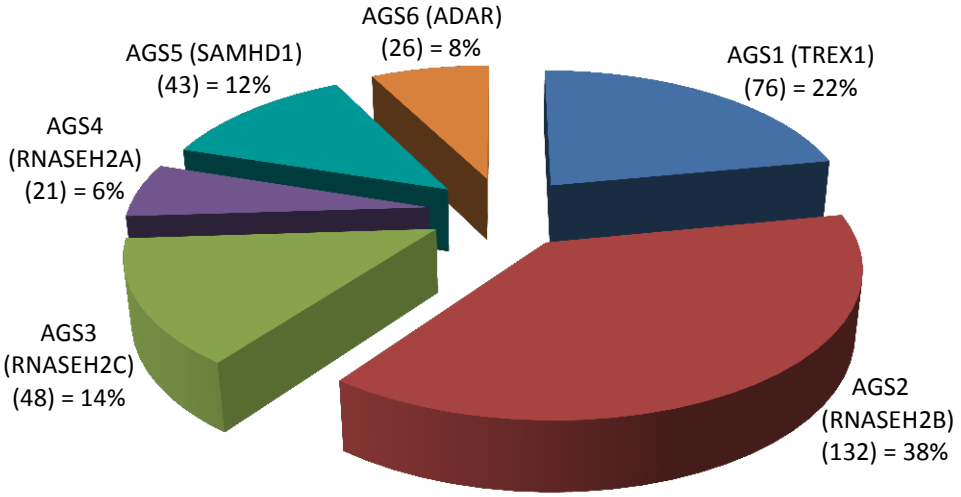
- Briggs et al. Nat Genet 2011;43:127-131 [**B1**]
- Belot et al. Arthritis & Rheum 2013;65:2161-71 [**B1**]

AGS clinics were held in Manchester, Pavia and Washington DC, and these coincided with four family-scientist conferences. At these clinics, each individual patient / family was assessed, data collected (relating to the patient's height, weight, head circumference, clinical history, progression and current status) and, where possible, patient and parental samples were taken (most typically DNA, RNA, serum, plasma and peripheral blood mononuclear cells - PBMCs). The families attending the meetings in Manchester and Pavia were predominantly from the UK and Italy respectively, although families also travelled from the Netherlands, Spain, France and Germany. The US families came from various states including Arizona, California, Colorado, Washington DC, Florida, Illinois, Maryland, Michigan, Missouri, New Jersey, New York, North Carolina, South Carolina, Virginia, Wisconsin, Ohio, and Utah.

In an effort towards uniformitivity of data collection, we established a clinical registry using the online database format REDCap (http://www.project-redcap.org/) for recording information on patients with a molecularly confirmed diagnosis of AGS. This resource has been, and will continue to be, accessible (with relevant identification / access restrictions) to members of the NIMBL project, thus serving as a platform for research on clinical natural history and laboratory associations into the long-term. This database includes information on: molecular diagnosis, MRI and CT findings, available biological samples, plus detailed clinical data (pre-natal and birth history, presentation and diagnosis, developmental history, and specific clinical features such skin lesions, haematological abnormalities, gastrointestinal findings, seizures etc.).

By November 2013, data had been recorded from 346 affected individuals (with a confirmed molecular diagnosis of AGS) from 277 families. Of those for whom recent data are available (n=324, 93.6%), 254 (78%) are alive, with the oldest patient currently 36 years of age; for those who have died, the age at death ranges from 0 to over 27 years. This formidable resource, which represents the largest single collection of clinical and radiological data ever accrued for AGS, is expected to form the basis of many subsequent publications, and serve as a resource for assessing natural history – a function crucial to the interpretation of treatment efficacy in future clinical trials.

Figure 1: Molecularly confirmed cases with AGS1-6 (n=346)



The results have been presented at various scientific meetings, and discrete aspects relating to these data have also been published:

- Vogt et al. Am J Med Genet A. 2013;161A:338-42 [**B1**]
- Olivieri et al. Lupus. 2013;22:1064-9 [**B3**]
- Clifford et al. Blood. 2013 Dec 12 [Epub ahead of print] [**B1**]

B2 completed the systematic collection and evaluation of clinical data of RVCL *TREX1* mutation carriers. In summary, we were able to gather information on a total of 78 RVCL-associated *TREX1* mutation-positive patients from 11 unrelated families worldwide. In addition to DNA, there has been an effort to collect plasma and CSF - which will continue after the completion of the NIMBL project. From our analysis, we conclude that RVCL is a progressive, systemic small blood vessel disease characterized by blindness due to vascular retinopathy, relentless neurological decline caused by cerebral mass and white matter lesions, and premature death. In addition, we found that a large percentage of RVCL patients suffered from comorbid neurological conditions such as Raynaud's phenomenon and migraine. These data represent the largest collection of clinical information yet derived for RVCL, and are currently under review for publication.

Task 2: Establishment of a sample repository

A sample repository was initiated early in the NIMBL project (this is a virtual repository where the samples are recorded as being held by a particular beneficiary), and numerous samples have been added to the collection; for example, B1 has DNA samples on more than 500 AGS patients / family members, and an RNA collection from >150 patients. There have been many transfers between beneficiaries of samples including patients' primary cells and cell lines, antibodies, mouse models, and data analysis results, and with other laboratories (NIMBL has funded over 144 courier shipments from / to B1 alone).

Task 3: Definition of radiologic features seen in AGS and RVCL

Both AGS and RVCL are diseases of the nervous system associated with prominent changes (damage / dysfunction) in the brain. Recognizing these changes on brain scanning is important for diagnostic purposes, and may be important from a therapeutic perspective in

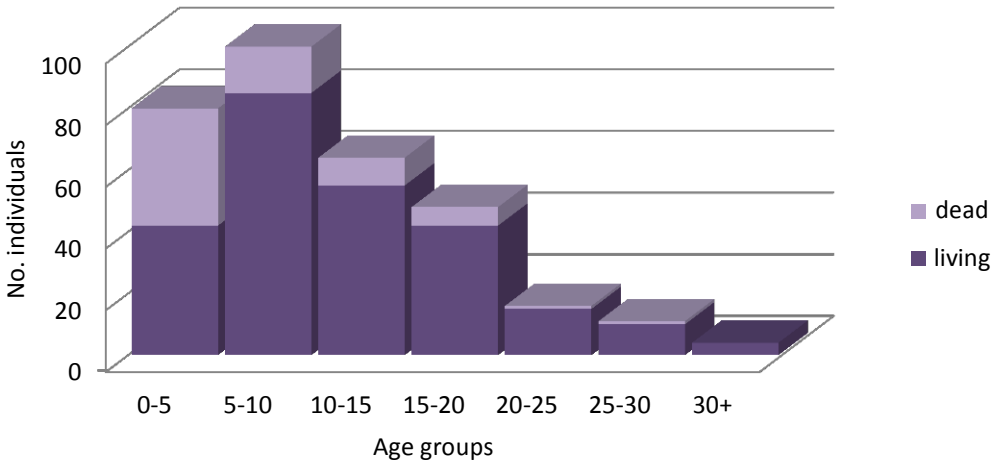
the future (where early diagnosis may be relevant to treatment outcomes). Considering both magnetic resonance imaging (MRI) and computed tomography (CT) scans, a multidisciplinary team of highly experienced neuroradiologists, paediatric neurologists and geneticists (from B1, B2, B3, B4, B8) undertook a detailed and comprehensive characterisation of the radiological phenotype of 94 AGS and 48 RVCL patients in our unique cohorts of molecularly defined cases; where available, serial imaging was used to provide information on progression / stability of radiologic features. The particular purpose of this approach was to define disease diagnostic criteria, and highlight new findings. Some of the derived outputs have already been published (see below), whilst further submissions are expected in the near-term:

- Ramesh et al. Dev Med Child Neurol 2010;52:725-32 [**B4, B1**]
- Livingston et al. Dev Med Child Neurol 2013;55:46-57 [**B4, B1**]
- Livingston et al. J Med Genet. Published on-line 21 November 2013 [**B4, B1**]
- Pelzer et al. J Neurol 2013;260:2188-2190 [**B2**]

Task 4: Development of validated diagnostic criteria for AGS and RVCL

In a significant minority of patients with AGS, problems are recognized at birth, i.e. the disease process begins *in utero*. More frequently, a later-onset presentation of AGS is seen, occurring in some cases after several months of normal development. In either case, over time, severe neurological dysfunction develops - manifesting as progressive microcephaly, spasticity, psychomotor retardation and, in approximately 33% of cases, death in early childhood (by age 8 years).

Figure 2: Known status of molecularly confirmed AGS patients (n=324)



Although the majority of recognized patients conform to the relatively stereotyped 'classical' phenotype just described, our studies have revealed a much broader spectrum of disease presentation, progression and outcome than previously anticipated (a genotype-phenotype submission based on our complete data set is in preparation). These 'non-classical' cases highlight a remarkable paradox relating to the diagnosis of AGS; that is, patients with mutations in the AGS-associated genes are frequently observed to demonstrate the absence of one or more, and even all - in rare cases, of the original diagnostic criteria as outlined by Aicardi and Goutières in their 1984 paper. Thus, neurological dysfunction is not always severe nor, indeed, necessarily present at all; microcephaly is not invariable; onset is not always in the first year of life; intracranial calcification and white matter changes are not inevitable; and a CSF lymphocytosis is often absent. Importantly, disparity in the clinical

phenotype can be seen even within the same family, thus highlighting the role of modifying factors. These issues were discussed in publication form in:

- Crow et al. Clin Exp Immunol. 2014;175:1-8 [**B1, B3, B5, B7**]

B2's research efforts have yielded a set of validated diagnostic criteria for RVCL. Characteristic features of RVCL include, i) progressive visual impairment due to vascular retinopathy; ii) focal and global neuropsychiatric symptoms with relentless neurological decline due to cerebral mass and white matter lesions; and iii) premature death. Other frequently associated features are migraine, Raynaud's phenomenon, and impaired liver and kidney function. The data upon which these criteria have been derived are currently under review for publication.

Task 5: Identification of markers of disease as outcome measures in future therapeutic trials

Despite major advances in molecular diagnosis (in large part brought about through NIMBL), the development of disease biomarkers in the context of AGS remains an important aim for at least two reasons: Firstly, it could be helpful in confirming or excluding a diagnosis in genetically undefined / uncertain cases. Secondly, remarkable progress in the understanding the pathogenesis of AGS, brought about through our work and that of other groups in the field, has led to the near-term possibility of therapeutic interventions. Thus, the identification, and characterisation, of reactive biomarkers which can be used to monitor therapeutic efficacy, has become of the highest priority.

Based on our developing understanding of the pathogenesis of AGS, several different candidate biomarkers have been considered including: 1. Cytokine / chemokine analysis and, 2. Immune phenotyping of CSF and blood lymphocytes. Some of these results have been published / presented internationally:

- Takanohashi et al. Neurology 2013;80:997-1002 [B1, B5, B8]
- Sandza et al. 63rd American Academy of Neurology, Honolulu (April 2011) [**B8**]
- Sandza et al. Society for Neuroscience, Washington DC (November 2011) [**B7, B8**]

Of particular importance has been our characterisation of the upregulation of interferon stimulated genes (ISGs) in the peripheral blood of AGS patients (a so-called interferon signature) – apparently independent of age and of genotype (excepting *AGS2*). We believe that these data represent a major advance for the future management of AGS:

- Rice et al. Lancet Neurology 2013;12:1159–1169 [**B1, B3, B4, B8**]

Overall summary: A major aim of this WP was the development of a comprehensive and uniform phenotype data set relating to NIMBL diseases, most particularly AGS and RVCL. These data, collected across participating clinical centres (Manchester, Pavia, Washington DC, Amsterdam and Leiden), have been incorporated within bespoke databases which will continue to be populated beyond the life-time of the NIMBL project. As such, we now have available information on the largest number of AGS and RVCL patients ever collated – worldwide. Linked to our patient-derived biological sample collection, these data have allowed for relationships to be drawn between clinical, laboratory and radiological information and our experimental results. Highlights of this WP have been a greatly enhanced understanding of the AGS and RVCL clinical and radiological phenotypes – both in terms of 'depth' (numbers of patients) and 'breadth' (phenotypic spectrum), and the identification of biomarkers important for monitoring future therapeutic interventions.

Work Package 3: *Genetic basis of AGS and RVCL*

Overall aim: To develop a comprehensive description of the genetic basis of NIMBL-related disorders, most particularly AGS and RVCL, but also allied type I interferonopathies - recognising that the definition of the mutational spectrum of the NIMBL diseases is crucial to understanding the molecular pathology of these phenotypes, and also allows for uniformitivity of diagnostic laboratory service provision across Europe and elsewhere.

Involving: B1, B2, (plus B3, B5, B8)

Task 1: Establishment of mutation databases

Separate mutation databases for *AGS1* to *AGS6* have been constructed by B2 with the help of B1 within the Leiden Open Variation Database suite (LOVD). These databases are publically available (http://www.lovd.nl/2.0/) and, as such, serve as a source of information for clinicians and molecular geneticists worldwide. In addition, mutation data have been recorded in the REDCap database, with access restricted to members of the NIMBL team.

Task 2: Development of deletion / duplication screen technologies

B1 and B4, in collaboration with MRC-Holland, developed a multiplex ligation-dependent probe amplification (MLPA) kit for the identification of deletions / duplications in *AGS1-5*. A panel of patients with (only) single identifiable mutations in these genes was screened using this kit. This system has now been incorporated into our scanning platform, and into the platforms used in diagnostic laboratories in the UK, mainland Europe and the USA (personal communications).

Our experience indicates that deletions of AGS-related genes, except for *SAMHD1*, are rare. Once further data have been accrued, we plan to publish a detailed description of our experience with deletion / duplication screening across all AGS-relevant genotypes.

Task 3: Mutation scanning in AGS and RVCL

Since the start of the NIMBL project it has become clear that it is not possible to reliably predict AGS genotype from phenotype, and that, although recurrent founder mutations are known, mutations in any of the AGS-related genes are observed across all ethnicities. For these reasons, we now take the approach of Sanger sequencing all genes in a systematic fashion, irrespective of phenotype and ethnicity, combined with MLPA testing in appropriate cases. These observations are captured in the mutation databases that we are curating (see: http://chromium.liacs.nl/lovd2/home.php?action=switch_db) and the quality assessment scheme that we developed (see Task 5). We are currently writing a comprehensive genotype-phenotype paper which will completely capture our AGS-related mutation data. Details of the mutational spectrum in >150 cases were included in the Supplementary Information of the following output:

- Rice et al. Lancet Neurology 2013;12:1159-1169. [**B1, B3, B4, B8**]

Discussion of specific *RNASEH2A* mutations was given in:

- Rice et al. Hum Mutat 34:1066-70 [**B1, B3**]

The identification of eight patients with only a single identifiable heterozygous mutation in *RNASEH2B* led B1 to search a second 'occult' mutation in these patients where we had access to fibroblastoid and / or lymphoblastoid cell lines. An exon 2_5 deletion was identified in a single (A177T heterozygous) patient, whilst retention of the last 11 bases of intron 1_2 was identified as a recurrent mutation in 3 further families (also heterozygous for an A177T mutation). Additionally, a c.136+1del G variant was confirmed to result in a loss of exon 2, and a G146S substitution to cause a deletion of exons 2 to 5, at the cDNA level. These findings are being written up for publication.

B2 continued performing mutation analyses of the *TREX1* gene in patients with RVCL or phenotypes resembling RVCL. *TREX1* DNA mutation scanning is offered in a diagnostic setting in the Leiden clinical genetics department. B2 has now identified five distinct C-terminal *TREX1* frameshift mutations (i.e. V235fs, T236fs, T249fs, R284fs, and L287fs). Six families coming from the Netherlands, North America, or Australia had the same V235fs mutation. T249fs was identified in two families from North America.

Task 4: Identification of the *AGS6* gene

At the start of the NIMBL project, AGS was known to result from mutations in any one of five genes encoding: the exonuclease TREX1 (*AGS1*), the three subunits of the RNASEH2 endonuclease protein complex (*AGS2, 3* and *4*), and the then uncharacterised protein SAMHD1 *(AGS5*). However, approximately 10 - 15% of AGS families did not have identifiable mutations in *AGS1-5*, and B1 had already generated genotype data to show that at least one further AGS-causing gene remained to be identified. The absence of a molecular diagnosis in patients with AGS is of clinical importance, meaning that diagnostic uncertainty remains in such cases, and that carrier and prenatal testing are unavailable to affected families.

Using our existing patient cohort, B1 identified *AGS6* by whole exome sequencing (a technology which only became available during the second half of the NIMBL project) in patients with a clinical diagnosis of AGS, all of who screened negative for mutations in *TREX1*, *RNASEH2A*, *RNASEH2B*, *RNASEH2C* and *SAMHD1.* Visual inspection of the generated data initially identified two patients, each with two non-synonymous coding alterations in *ADAR1*, a gene we had already highlighted as a candidate for AGS in view of its known role as a suppressor of type I interferon signalling in an animal model. Sanger sequencing confirmed the variants in these patients, as well as in two further affected siblings. We went on to sequence the putative *AGS6* gene in other *AGS1-5* negative patients from our cohort. In total, ten affected individuals from seven families demonstrated biallelic *ADAR1* variants which were considered likely pathogenic on the basis of species conservation and the output of pathogenicity prediction packages. In these families, all parents tested were heterozygous for one putative mutation. Two further unrelated patients demonstrated a single heterozygous mutation which was not present in either parent. These findings were published:

- Rice et al. Nat Genet 2012;44:1243-8 [**B1, B3, B4, B8**]

There are still patients conforming to a clinical phenotype of AGS who do not have changes in the *AGS1-6* genes i.e. further genetic heterogeneity likely exists.

Task 5*: Establishment of a pilot External Quality Assessment and Proficiency Testing (EQA / PT) exercise for molecular analysis of *AGS1-5

In order to enhance the delivery of diagnostic testing for AGS, B1 invited clinical genetics centres in Europe and the USA to take part in a pilot scheme, carried out under the auspices of the European Molecular Genetics Quality Network (EMQN; http://www.emqn.org/emqn/Home), aimed at determining the current availability of screening of the genes involved in AGS and related disorders, and assessing the quality and sensitivity of this screening.

A total of seven laboratories across five countries (UK, USA, France, Germany and Italy) took part in the pilot scheme. Depending on the diagnostic testing offered by participating laboratories, up to three anonymised DNA samples were sent to each laboratory, together with mock clinical case information, for genetic testing. The DNA samples were extracted from established cell lines and genotypes validated in the conducting centre (Manchester). AGS testing has now been fully adopted by the Board of EMQN. The final EQA / PT report was authorised by Dr Simon Patton (EMQN Director) on 31 May 2013.

Further aspects of note relating to WP3

1. As B2 is also a major international referral centre for Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy (CADASIL), a small vessel cerebral disorder with some striking similarities to RVCL, 100 patients were identified who were suspected to have CADASIL but who did not carry a *NOTCH3* mutation that defines the disease. Because of the clinical overlap with RVCL, *TREX1* mutation screening was considered, and revealed a heterozygous *TREX1* mutation in two patients. With the identification of the heterozygous *TREX1* mutations p.Tyr305Cys and p.Arg114His in patients with early-onset cerebrovascular disease, these data suggest that (i) ('mild') TREX1 dysfunction may cause disease with adult onset, thus potentially broadening the phenotype of TREX1-associated conditions, and (ii) vascular risk factors may explain part of the phenotypic spectrum of TREX1-related disease. These data have been published:

    - Pelzer et al. J Neurol 2013;260:2188-90 [**B2**]

2. As part of further efforts to define AGS overlap conditions, B1 was involved in the molecular definition and cellular characterisation of the type I interferonopathy spondyloenchondrodysplasia (SPENCD), and a novel monogenic form of lupus due to mutations in *PRKCD*. These data were published:

    - Briggs et al. Nat Genet 2011;43:127-131 [**B1**]
    - Belot et al. Arthritis & Rheum 2013;65:2161-71 [**B1**]

3. In light of the rapid changes in sequencing technologies that have occurred over the last 3 years, B1 is exploring the possibility of high throughput parallel sequencing of the AGS-related genes. The rationale for such a mutation scanning platform is to capture the advantages (specifically, reduced turn-around time and reduced cost) of next-generation sequencing. Thus, considerable time, effort and resources have been dedicated to the development of an analytical pipeline (expected to come into operation in early 2014).

Summary of WP3: NIMBL funding has facilitated the comprehensive description of the genetic basis of AGS and RVCL, the cataloguing of these variants in open-access databases, the harmonisation of genetic testing across diagnostic laboratories internationally, and the identification of new AGS-related / AGS-overlap genes.

Work Package 4: *Development of animal models relevant to NIMBL phenotypes*

Overall aim: To develop a portfolio of mouse models to explore the origin and progression of autoimmune disease in NIMBL-related diseases - these models to be used to interrogate fundamental biological questions relating to NIMBL human phenotypes in allied WPs.

Involving: B2, B4, B6/9

Task 1: Development of mice demonstrating conditional *Samhd1* deficiency

B4 established a re-derived colony of *lox*P-flanked *Samhd1* mice in collaboration with the laboratories of Dr. Caetano Reis e Sousa at the Cancer Research-UK London Research Institute, Lincoln's Inn Fields, London and Dr. Jan Rehwinkel (Oxford). We verified by RNA transcript analysis that the presence of the *lox*P sites did not appear to interfere with gene expression or splicing. This mouse colony was expanded and maintained on a C57Bl/6J background. Extensive genotyping was performed to confirm transmission at expected Mendelian ratios. Initial work in these collaborators' laboratories indicated that *Samhd1*-deficient animals were healthy, with no obvious phenotype.

We pursued plans to generate tissue-specific knockouts of *Samhd1* using the same floxed founder mouse strain referred to above. Specifically, the *Samhd1*^{flox} allele was crossed onto the transgenic SCL-Cre-ER(T) line and doubly heterozygous animals identified by genotyping, and cryopreservation performed (149 embryos). This cross could allow specific ablation of *Samhd1* in vascular endothelial cells - a tissue of interest given the frequent manifestation of vascular pathology in patients with SAMHD1 deficiency. However, the lack of a prominent phenotype in the *Samhd1*-null mouse led us to decide that the production of conditional lines would not represent the best use of available resources.

During the NIMBL project, publications appeared indicating that human SAMHD1 acts as a restriction factor for human immunodeficiency virus (HIV-1), blocking early stage virus replication in dendritic and other myeloid cells. Surprisingly, however, the Oxford / London group found that in the murine system, Samhd1 deficiency did not lead to increased infection by pseudotyped HIV-1 vectors. This was despite the presence of a (mild) type I interferon signature in the relevant cells, and an elevation of dNTP levels in Samhd1-deficient cells. Subsequent work indicated that the manifestation of virus restriction by murine Samhd1 depends on the kinetics of the viral reverse transcriptase; a mutant with an elevated Km for dNTPs was restricted in a Samhd1-dependent fashion, unlike the wild-type HIV-1. This work was published:

- Rehwinkel et al. EMBO J. 2013;32:2454-62 [**B1, B4**]

Task 2: Development of an *Rnaseh2c* knock-in mouse

B4 developed a knock-in *Rnaseh2c* mouse as a model of *AGS3*, by introducing the R69W mutation into the coding sequence; R69W is the predominant human mutation in the *AGS3* gene, and an important cause of AGS in the UK Pakistani community. This strategy was chosen because biallelic null mutations are notably absent at the *AGS2-4* loci (genes encoding the *RNASEH2* complex subunits) and likely to be embryonic lethal. At *AGS3* (*RNASEH2C*), the missense mutations observed in AGS patients are all located at conserved residues, suggesting that they could affect the protein structure or its activity in some way. The R69W mutation, in particular, has been shown to significantly reduce the

enzymatic activity of RNASEH2C *in vitro*. By March 2013, homozygous R69W/R69W offspring showed no apparent ill-health. Tissues were harvested from these homozygous animals for molecular analysis (particularly of DNA ribosubstitution) - results are still in preparation.

Task 3: Development of mice demonstrating conditional *Trex1* deficiency

Trex1 null mice have a shortened life-span due to the development of an immune-driven endocarditis. To obtain further information about AGS, we wanted to explore the genesis of the observed (lethal) endocarditis by considering the different cell types in which Trex1 is expressed. Thus, B6/9 developed a conditional *Trex1* knock-out (KO).

As was expected / hoped, these results show that there was no production of Trex1 in macrophages, whilst normal levels of *Trex1* were observed in other tissues. We are currently testing other cell types (neutrophils, dendritic cells) to determine the specificity of the conditional model. *Trex1* null mice were also crossed with a mouse expressing Socs2-CRE that is expressed in the early stages of development. These results show that Trex1 is lacking in all the tissues tested i.e. that the model represents a true conditional, which could now be used for further studies of the temporal development of the *Trex1* null associated phenotype.

Task 4: Development of mice expressing Trex1 protein with a human pathogenic RVCL mutation

B2 has developed this mouse model. Characterisation of these mice, from a few months of age to almost two years, suggests that they do not show an obvious disease-relevant phenotype (for more details, please see WP9 Task 2). An extended analysis is now being planned.

Further aspects of note relating to WP4

1. In order to compare molecular and neurobiological phenotypes of two clinically overlapping small vessel diseases, i.e. RVCL and CADASIL, B2 had previously generated founders and, towards the end of NIMBL, set out to breed over-expressor CADASIL mutant mice. As migraine is a prominent feature in both RVCL and CADASIL, B2 had the unique opportunity to explore a possible relationship between these conditions. In addition to breeding RVCL knock-in mice in large quantities, CADASIL over-expressor mice, expressing either wild-type human *NOTCH3* or mutant R182C *NOTCH3*, have been successfully bred at B2. As a continuation of this WP, B2 is now backcrossing mutant and wild-type alleles onto a C57/Bl6J background.

Summary of WP4: NIMBL has delivered on three of four mouse models. A strategic decision, based on our derivation and characterisation of the *Samhd1* null mouse, discouraged us from developing a *Samhd1* conditional line (although preparations for this model were made, and floxed / Cre double heterozygote embryos frozen). Outputs relating to our *Rnaseh2c* knock-in, conditional *Trex1* deficient and *RVCL-Trex1* knock-in mice are expected in the future – but the absence of an obvious phenotype relating to the first and third of these models meant that we adapted our experimental efforts / publication priorities during the course of the project.

Work Package 5: *Use of AGS animal models*

Overall aim: To explore the cell-type specific consequences of AGS-related protein dysfunction, and to generate *in vivo* systems to track disease from its earliest initiation to pathology.

Involving: B4, B6/9, B7

Task 1: Transcriptional profiling in conditional mice

The absence of an obvious phenotype in *Samhd1* null mice, and in our *Rnaseh2c* knock-in model, meant that we took a strategic decision not to pursue comprehensive transcriptional profiling in such animals (see WP4 above for justification).

Task 2: Investigations in conditional knock-out mice

Our original plan included the use of a conditional *Samhd1* knock-out (KO) and an *Rnaseh2c* knock-in mouse model. However, our finding that the *Samhd1* null mouse does not demonstrate an overt phenotype, indicated to us that it was no longer appropriate to pursue these experiments as originally envisaged. However, using the full *Trex1* KO mouse model, we made unexpectedly rapid progress in enhancing our understanding of the central role of interferon in AGS pathogenesis. Thus, the knowledge that we hoped would accrue from this Task was, to a large extent, acquired through Tasks 5.3 and 6.1, where we describe the tissue specific relevance of the these AGS-related proteins.

Task 3: Use of novel reporter mice to track the cellular dynamics of cell-intrinsic autoimmune initiation *in vivo*

B7 used an *in vivo* reporter of interferon activity in *Trex1*-deficient mice to explore the cell type-specific consequences of AGS-related protein dysfunction and localize the earliest initiation of disease to non-hematopoietic cells. This groundbreaking work demonstrated that interferons drive T cell-mediated inflammation and a T-cell dependent autoantibody response to abundant, tissue-restricted autoantigens. However, B cells also contribute to mortality, independently of this T cell-mediated tissue inflammation. These findings reveal a stepwise progression of interferon-mediated autoimmune disease in *Trex1*-deficient mice, with implications for the treatment of AGS and related diseases. Accompanied by a three-page Preview article (Pascual V and Banchereau J. Immunity. 2012;36:7-9), these findings were published:

- Gall et al. Immunity 2012;36:120-31 [**B7**]

Task 4: Examination of the contribution of TLRs to autoimmune disease in AGS-related protein deficiency

AGS is a type I interferon -associated autoimmune disease, caused by pathogenic mutations in any one of six published genes, leading to loss of function of the associated proteins. Type I interferons and Toll-like receptors (TLRs), proteins that are involved in nucleic acid sensing, have been shown to play central roles in other severe autoimmune diseases, including SLE and psoriasis. B7 investigated the complex pathway that detects cytosolic DNA within mammalian cells, as this is key to understanding the mechanisms involved in

AGS and NIMBL-relevant pathology. The interferon stimulated DNA (ISD) response, signals activation of the anti-viral response through a specific cascade, involving a protein known as STING (Stimulator of Interferon Genes). The aim of this Task was to understand the involvement of TLRs in the detection of nucleic acids, as relevant to AGS. B7 found that the type I interferon response associated with *Trex1* deficiency is entirely dependent on the STING-dependent ISD pathway and not TLRs. However, our experiments also showed that Myd88 signalling, thus implicating TLRs, is relevant to disease progression / amplification in the *Trex1* null mouse. These findings are being prepared for publication.

Further aspects of note relating to WP5

Following on from our identification of *ADAR1* as the *AGS6* gene, B7 obtained mice with a conditional *Adar1* allele from Prof Stuart Orkin's group at Harvard for analysis of AGS-relevant phenotypes. *Adar1*-deficient mice display embryonic lethality with 100% penetrance by embryonic day 13. This lethality is associated with a massive type I interferon signature in embryonic tissues. To identify the source of the interferon response, and test whether it contributes to early lethality, we generated mice with a complete null allele of *Adar1* and then intercrossed *Adar1*+/- mice on *STING*-/- and *MAVS*-/- backgrounds. Importantly, *STING* deficiency completely rescues *Trex1*-/- from autoimmune inflammation and from lethality. In contrast, we found that *Adar1*-/-;*STING*-/- mice were not rescued from embryonic lethality, and that the interferon response was still present in *Adar1*-/-;*STING*-/- embryos at day E11. These studies are ongoing.

Summary of WP5: Using mouse and cellular models, and informed by human data, the NIMBL consortium has made exceptional progress in defining the cellular basis of AGS related to *Trex1* deficiency in a mouse model. As is inevitably a risk with mouse reverse-genetics (i.e. targeting human-disease related genes in mice without prior knowledge of the animal phenotype), we were disappointed by the lack of obvious disease features related to our *RNaseH2C* and RVCL-*Trex1* knock-ins, and the subtle phenotype associated with our *Samhd1* knock-out. However, further work is warranted in all of these three systems.

Work Package 6: *Cell biology of TREX1 and SAMHD1*

Overall aim: To develop our understanding of the function of TREX1 and SAMHD1, an objective considered crucial to the development of future treatments for NIMBL-related disorders.

Involving: B4, B6/9 (plus B3 and B1)

Task 1: **Definition of the mechanistic function of TREX1**

The cellular mechanisms by which TREX1 deficiency leads to AGS may be multifold. The *Trex1* knock-out (KO) mouse model has a dramatically reduced life expectancy due to the development of an inflammatory myocarditis. We, and others, have demonstrated that an increase of intracellular DNA triggers interferon production that causes a so-called anti-viral state, which, in turn, leads to autoimmunity. In an attempt to better understand AGS-relevant inflammation at a cellular level, we undertook an anatomo-pathological study of Trex1-/- mice, with particular emphasis on the macrophage immune cell type.

Summarising, experiments derived by B6/9 showed that in the absence of *Trex1*, macrophages displayed an overt proinflammatory state. Particularly, following proinflammatory stimulation, *Trex1*-/- macrophages exhibited increased TNF-α and IFN-α production These results reveal a previously unrecognised function of Trex1 as a negative regulator of macrophage inflammatory activation, and suggest that macrophages may play an important role in TREX1-related AGS. These findings have very recently been published:

- Pereira-Lopes et al. J Immunology published online 11 November 2013 [**B6/9**]

B6/9 (has been interested to understand factors important in determining the half-life of *Trex1*. The *Trex1* sequence does not contain premature termination codes, ARE (AU-rich element) sequences or PUF (Pumilio and FBF homology protein) motives. To interrogate the role of miRNAs in relation to *Trex1* metabolism, B6/9 silenced Dicer1, but no modifications were observed on *Trex1* half-life. Pull down experiments with *Trex1* binding proteins were also performed and, through sequencing, several candidates have been identified that might be responsible for regulating the half-life of *Trex1*. Our strategy is to now perform an inhibition of these proteins, with siRNA, in order to investigate this issue further.

B6/9 received mice (generated by B3) with a mutation in the C-terminus of *Trex1* associated with autosomal dominant RVCL. Bone marrow-derived macrophages were obtained from these mice. When activated with interferon gamma, Trex1 was induced – but with a decreased size, as expected, compared to controls. When we treated cells with interferon gamma (IFN-γ) and measured the expression of ifn-β, il-1β, and tnf-α, mutated cells did not show any different activation in relation to controls. Following three days in culture, the percentage of cells that died after IFN-γ or lipopolysaccharide treatment was higher if they had mutated *Trex1*. It was also noted that when treated with IFN-γ or H_2O_2, the number of cells with single DNA breaks was elevated in *Trex1* mutated cells. Experiments to investigate these aspects of TREX1 biology are planned.

Task 2: **Biochemical and structural characterization of SAMHD1**

The *AGS5* disease locus corresponds to the gene *SAMHD1*, which encodes a protein of unknown function at the time of its discovery in 2009. Recognizable domains within the encoded protein include a SAM (sterile alpha motif) and HD (hydrolase) domain, but SAMHD1 is the only protein to have these motifs tandemly arranged. SAM domains serve a wide range of functions within the family of proteins containing them, including protein-protein interaction and nucleic acid binding. The latter is of particular interest given that the products of the *AGS1-4* genes are nucleic acid-degrading enzymes.

B4 obtained biochemical and structural insights into human SAMHD1 using recombinant proteins, and utilised such information to interpret the results of *in vivo* studies in *Samhd1* mutant mice. The later part of this work was informed by the knowledge that in addition to being the mutated gene in *AGS5*, SAMHD1 was shown to act as a restriction factor for HIV1, blocking early virus replication in target dendritic cells. During this work, scientists in another laboratory (in collaboration with B1) determined the crystallographic structure of the catalytic domain of SAMHD1, and demonstrated a GTP-stimulated nucleoside triphosphohydrolase activity. This is postulated to be responsible for the inhibition of HIV replication, via depletion of intracellular dNTP levels. In addition, our *Samhd1* KO mice have provided some evidence for the proposed mechanism of action of SAMHD1 in retrovirus restriction; these animals display elevated dNTP levels, and increased susceptibility to retrovirus replication with characteristics that support the idea that exact levels of dNTP are a critical determinant.

These data have been published:

- Goldstone et al. Nature 2011;480:379-82 [**B1**]
- Rehwinkel et al. EMBO J 2013;32:2454-62 [**B1, B4**]

B1, in collaboration with a research group in Vienna, investigated truncating mutants of *SAMHD1* (found in AGS patients). In this way, a previously unrecognised nucleic-acid-binding activity of SAMHD1 was mapped to residues 164-442, thus overlapping with the HD domain. Furthermore, results showed that although wild-type SAMHD1 displays almost exclusive nuclear localization, 11 of 12 SAMHD1 mutants showed at least partial mislocalization to the cytosol. These data were published:

- Goncalves et al. Human Mutation 2012;33:1116–1122 [**B1**]

B6/9 determined that *Samhd1* is induced in different tissues by pro-inflammatory cytokines (IFN-γ, IFN-α) and lipopolysaccharide, but not by anti-inflammatory cytokines (IL-4 and IL-10). The induction by IFN-γ was STAT1-dependent and required protein synthesis. Because the half-life of mRNA is very long, these data indicate that induction of *Samhd1* mRNA by IFN-γ is mediated at a transcriptional level. B6/9 cloned 1498 bp of the gene promoter for use in transient transfection experiments. It was found that a fragment of 161 bp is critical for the induction of *Samhd1* by IFN-γ. Using gel retardation assays, it was also determined that a protein complex bound to the 161 bp fragment under basal conditions. When cells were treated with IFN-γ, the nuclear extract produced a protein complex with a reduced relative mass. These data suggest that the expression of *Samhd1* may be under negative repression. Further work is actively ongoing relating to these results.

Summary of WP6: Considerable progress has been made through our own NIMBL-funded efforts, and the work of others – particularly relating to the solving of the crystal structure of SAMHD1, and the elucidation of the biochemical function of SAMHD1 – which is now known to be a potent dGTP-stimulated triphosphohydrolase, converting deoxynucleoside triphosphates to the constituent deoxynucleoside and inorganic triphosphate. Because of the importance of both TREX1 and SAMHD1 in the biology of HIV-1, our efforts have synergised with the outputs of multiple groups worldwide – so that knowledge of the function of these molecules has proceeded at a rapid pace.

Work Package 7: *AGS-related nucleic acid substrates*

Overall aim: The aim of this WP was the development of new tools and techniques to define the nucleic acid substrates which accumulate in the NIMBL deficiency states. Since these nucleic acids are postulated to represent the primary trigger of downstream immune system activation, understanding their genesis could be central to the development of rational therapies.

Involving: B7

Task 1: **Generation of tandem affinity purification (TAP)-tagged forms of AGS-related proteins and reconstituted AGS-protein deficient cell lines (with these tagged alleles)**

B7 developed novel tools to characterize Trex1 nucleic acid substrates; specifically, cell lines expressing TAP-tagged forms of Trex1 for purification of Trex1 and its associated DNA substrates have been generated. B7 also performed yeast 2-hybrid analysis of Trex1 in order to gain insight into interacting proteins and determine the biological processes impacted by Trex1. In addition, B7 generated stable cell lines expressing a form of the Rnase H2 complex in which all three subunits are tagged.

B7 also developed a method for direct chemical crosslinking of Trex1 to its DNA substrates in live cells using conventional chromatin immunoprecipitation (ChIP) techniques. ChIP relies on the ability of formaldehyde to reversibly crosslink proteins to closely associated DNA. For this method to be effective, the target protein must contain lysines in close proximity to the bound DNA, because the primary amine of the lysine side chain contributes one essential half of the covalent crosslink. Inspection of the co-crystal structure of Trex1 with single-stranded DNA revealed a complete absence of lysines in proximity to the active site. Moreover, purified, WT Trex1 did not crosslink to DNA in solution when treated with formaldehyde. B7 has therefore engineered a modified form of Trex1 in which lysines have been substituted in place of three non-conserved, solvent-exposed amino acids within flexible loops of the protein in proximity to the DNA but sufficiently distant from the catalytic site. This 3lys-modified Trex1 was constructed within the context of both active Trex1 and a dominant catalytic mutant of Trex1 that can tightly bind to DNA but not excise nucleotides. For stringent purification, B7 has added a tandem N-terminal tag consisting of an *in vivo* biotinylation sequence and a 6x-Histidine tag separated by a cleavage site for tobacco etch virus (TEV) protease. B7 then determined the optimal conditions for recovery of *in vivo*-biotinylated Trex1 from cell extracts, and found that the addition of supplemental d-biotin to culture media facilitates recovery of almost all Trex1 from extracts in a single, rapid step.

Task 2: **Development of *in vivo* cross-linking approaches to capture the nucleic acids that are directly bound to AGS-related proteins in live cells**

Prior to the start of NIMBL, B7 developed a novel method to isolate and purify cytosolic DNA directly from heart tissue of *Trex1*-deficient mice, thus providing initial insights into the nature of Trex1 DNA substrates. However, the exact identity of these nucleic acid substrates remained unclear at that time. During the NIMBL project, B7 has used state-of-the-art methods to define the nucleic acid species which accumulate in this deficiency state, to precisely define the biogenesis and scope of the nucleic acids that likely accumulate in *Trex1* deficiency (and possibly other forms of AGS). The ultimate goal after characterizing the nucleic acid substrates will be to determine the enzymatic activities that generate them. Since these nucleic acids are postulated to represent the primary trigger of downstream immune system activation, understanding their genesis is central to the development of rational therapies.

B7 has extended this analysis to more DNA clones. We noticed that while we were able to map much of this recovered DNA unambiguously to the mouse genome, almost none of the recovered fragments precisely matched the published genome sequence. We then catalogued all of the nucleotide changes in the recovered DNA relative to the genome sequence and found a dramatic bias towards C>T transitions, such that a C encoded in the genome was recovered as a T in the cloned DNA fragment. C>T transitions are indicative of deamination of cytosine to uracil (U), which is then copied as a T during PCR amplification. Such DNA deamination can occur spontaneously by chemical hydrolysis, and is estimated to occur randomly at up to 10,000 cytosines per cell per day. However, when we closely examined the sequence context of the C>T transitions, we found a strong signature of TCT in the genomic sequence recovered as TTT in the cloned DNA fragments. This sequence context argues strongly against spontaneous deamination, but instead suggests the presence of an enzymatic activity that deaminates the accumulated DNA. Interestingly, the TCT signature in our recovered clones is distinct from the known sequence preference of the murine APOBEC3 DNA deaminase, suggesting the existence of a novel deaminase that may be relevant for modifying accumulated Trex1 DNA substrates.

These findings reveal a number of important features of Trex1 DNA substrates. First, the differences between the recovered clones and the genomic sequence means that these DNAs are not simply random genomic fragments that we accidentally recovered in our

isolation protocol. The DNAs were modified by deamination, likely by a specific enzyme, between the time they were generated and the time we harvested the DNA for analysis. Second, we recovered a strong signature of C>T transitions, but did not find evidence for the corresponding G>A transitions that would be present on the DNA strand complementary to the deaminated cytosine. This strand bias is known to occur during deamination of retroviral and retroelement DNA, where the first strand cDNA is targeted but the second strand is not. Thus, our method for isolation, cloning, and sequencing of Trex1 DNA substrates is strand-specific. Third, the TCT sequence context of the deaminated cytosines suggests a novel enzymatic activity, distinct from the known APOBEC3 DNA deaminase, that modifies Trex1 substrates. Interestingly, this signature most closely matches that of APOBEC1, a deaminase that is known to modify specific RNAs *in vivo*, but has not yet been demonstrated to act on DNA. Overall, our analysis of Trex1 DNA substrates further supports our hypothesis that these DNAs arise from reverse transcription of cellular RNA into immunostimulatory DNA.

Our method for identifying Trex1 DNA substrates revealed, for the first time, a potential role for retroelements in AGS, and also demonstrated, again for the first time, a means of innate immune recognition of retroelements and retroviruses based on detection of reverse transcribed DNA. More relevant for AGS, we proposed that the genes mutated in AGS would comprise a system of anti-retroviral defence. This proposal has been validated by the identification of the AGS gene *SAMHD1* as the principal restriction factor that prevents HIV-1 infection of human monocytes and dendritic cells. SAMHD1 was shown to be a dNTP phosphohydrolase that 'starves' the HIV reverse transcriptase of dNTPs, thus preventing reverse transcription. Interestingly, SAMHD1 is targeted for degradation by the Vpx accessory factor of HIV-2 and numerous primate lentiviruses. Moreover, we have also found that RNaseH2 is a potent anti-retroviral enzyme. As such, five of the six known AGS genes have a clear role in the metabolism of retroelement reverse transcription intermediates, thus providing a unifying framework for the study of AGS-related gene / protein function.

To extend this framework, we have also been working on identifying substrates of RNaseH2 – an endonuclease that cleaves the RNA strand of RNA-DNA hybrids. A monoclonal antibody called S9.6 specifically binds to RNA-DNA hybrids in a sequence-independent manner, thus enabling us to enrich for these structures by simple immunoprecipitation of cell lysates. Using S9.6, we have been working to optimize a protocol for immunoprecipitation of RNA-DNA hybrids from AGS patient fibroblasts and control human fibroblasts. Following recovery, we have created a method for ligating unique, barcoded adapters to the DNA strand, copying this strand, amplifying the recovered material, and identifying it using massively parallel next-generation sequencing. We have made substantial progress towards this ambitious goal, on both the practical side of specific and reproducible recovery of RNA-DNA hybrids, as well as the computational strategies required to map these recovered DNAs to the human genome. We have overcome challenges associated with unambiguous identification and absolute quantification of DNA fragments by including a unique molecular identifier (UMI) in the adapter used in the first DNA ligation. We have generated new methods for analysis, including a platform for mapping DNAs to repetitive elements in the human genome. We call this method nascent reverse transcriptase sequencing (NRT-SEQ), and we anticipate that it will be an extremely valuable technique for characterizing the reverse transcription landscape within cells. These data are in preparation for submission.

Summary of WP7: This WP was mainly focused on the development of tools to better define the nature of immunostimulatory nucleic acid species presumed to trigger the proximal innate immune response relevant to AGS. Concentrating initially on Trex1 as a paradigm, these tools have allowed us to extend previous results derived by B7. Of major significance, rapid progress has been made in highlighting a likely key role for the AGS-related proteins in retroelement metabolism - which thus represents a central theme in AGS-causation, and has

immediately suggested a targeted approach to block retrotransposition of such retroelements as a therapy in AGS.

Work Package 8: *Cytokines, autoantibodies and astrocytes in the pathology of AGS*

Overall aim: To address the neuropathological aspects of the human AGS phenotype. Following on from NIMBL-related *Trex1* mouse work, we also considered the role of autoantibodies and cytokines in the context of AGS, with an aim to understand disease pathology (and thus direct therapeutic approaches).

Involving: B5, B8 and B1

Task 1: Investigation of cytokine and autoantibody profiles in AGS patients

B5 analysed plasma from 22 AGS patients and cerebrospinal fluid (CSF) samples from 11 AGS patients using the MILLIPLEX™ MAP Immunobead system. Significant elevations were seen in FMS-related tyrosine kinase 3 ligand (FLT3L), CXCL10, interleukin (IL)-12p40, IL-15, tumour necrosis factor α (TNFα), and soluble IL 2 receptor-alpha (IL2Rα) in both plasma and CSF of AGS patients as compared to healthy age-matched controls. This work, which was performed in collaboration with B8 and B1, resulted in a joint publication:

- Takanohashi et al. Neurology 2013;80:997-1002 [**B1, B5, B8**]

Using the same Luminex platform, B5 also assessed the production and release of cytokines in cultures of astrocytes derived from immortalized human neural stem cells (ihNSC). Those astrocytes were chronically treated with interferon alpha. In these cell cultures B5 described a downregulation of pro-angiogenic factors and other cytokines (vascular endothelial growth factor and IL-1). These findings were also confirmed in brain specimens from AGS donors that had become available during the NIMBL project. The disturbed metabolism of such angiogenic factors in the brains of infants affected with AGS might lead to abnormal vessel formation and proliferation. Indeed, B5 observed an aberrant vasculature with an excessive number of capillary-like blood vessels, especially in cortical areas, in AGS brain tissue specimens. These data have been recently reported:

- Cuadrado et al. Brain 2013;136;245–258 [**B5**]

In parallel to the above, B5 performed a multiplex autoantibody microarray to uncover the presence of autoantibodies in serum samples from 56 genetically confirmed AGS patients. The samples were obtained through exchange and collaboration with B1, B3 and B8. Serum from AGS patients exhibited high levels of IgG against nuclear antigens (gP210, Nup62, PCNA, Ro/SSA, Sm/RNP, SS-A/SS-B), components of the basement membrane (entactin, laminin), the coagulation factor fibrinogen IV, and the wheat protein gliadin. In addition, B5 found that the IgGs were targeting endothelial cells and, more strikingly, astrocytes in brain sections of AGS deceased patients. Furthermore, B8 confirmed, in a proteomics approach, that IgG in serum samples from AGS patients binds to endothelial and astrocytic epitopes present in cerebral white matter. These distinct and novel autoantibody specificities have the potential to provide new insights into the pathogenesis of AGS, and may contribute to the development of therapeutic strategies for the chronic inflammation in AGS. This collaborative work has been submitted for publication.

Finally a large series of patients (n=400) with defined autoimmune diseases (including systemic lupus erythematosus (SLE), neuro-SLE and mixed connective tissue disease) have

been analyzed using a new autoantibody array (that includes 95 IgG and IgM autoantibodies). The measurement of autoantibodies in these patients will help to clarify the role and relevance of autoantibodies in the pathology of AGS compared to SLE and, especially, neuro-SLE. B5 is currently analyzing these data prior to submission.

B8 has studied AGS patient CSF and blood immune phenotypes, using FACS sorting, and demonstrated an increase in circulating B cells in the blood of AGS patients, along with an increase in natural killer (NK) cells in the CSF, relative to patients with multiple sclerosis and genetic immune conditions of the brain (NOMID and CANDLE). The role of these cell populations in AGS is at this point not fully elucidated, but the finding of an increased population of B cells is interesting given the possible role of autoantibodies in AGS pathogenesis. Furthermore, NK cells are known to play a role in the viral response, which may be relevant to AGS pathology based on immune stimulatory nucleic acids. A manuscript relating to these data is in preparation.

B8 has also studied neuronal specific autoantibodies in AGS patient plasma relative to controls. B5 is assisting with validation studies of the proposed antigen candidates, and B7 has helped with experimental design and trouble-shooting. A manuscript is in preparation, and the data have been presented at national scientific meetings.

1. Sandza et al. American Academy of Neurology, Honolulu (April 2011) [**B8**]
2. Sandza et al. Society for Neuroscience, Washington DC (November 2011) [**B7, B8**]

Task 2: Investigation of astrocyte function in AGS

Using the *in vitro* system of ihNSC-derived astrocytes, B5 described that chronic exposure of astrocytes to interferon alpha resulted in an alteration of genes and proteins involved in the stability of white matter (ATF4, eIF2Bα, Cathepsin D, Cystatin F). Interestingly, withdrawal of interferon alpha for seven days barely reversed these cellular alterations, demonstrating that the interferon alpha mediated effects persist over time. These results were confirmed using brain samples from patients with AGS, and indicate a role for interferon alpha as a key factor in the pathogenesis of AGS relating to the observed leukodystrophy and microangiopathy. Because of a sustained interferon alpha effect, even after withdrawal, therapeutic targets for AGS, and other interferon alpha-mediated encephalopathies, may include downstream interferon alpha signaling cascade effectors rather than interferon alpha alone. These data have been recently published:

- Cuadrado et al. Brain 2013;136;245–258 [**B5**]

As a continuation of the above investigations, B5 has examined the effect of silencing the expression of *TREX1*, *SAMHD1*, *RNASEH2A* and *ADAR1* in ihNSC-derived astrocytes and human brain microvascular endothelial cells (hCMEC/D3). For the experiments in ihNSC and other cell types, B5 produced lentiviral particles to effectively knock-down (KD) gene expression and thus mimic the AGS background in experimental cell models. After testing several clones, those shRNA particles capable of knocking down gene expression more than 80% were selected for subsequent experiments.

KD approaches in astrocytes, once differentiated from ihNSC, resulted in a compromise of the viability and proliferation of those differentiated cells, especially in *TREX1* and *ADAR1* KD cells. The amount of cell death of these cells was high, and resulted in a reduction of 70-80% of cells compared to the mock control (non-targeting shRNA). *RNASEH2A* and *SAMHD1* KD cells grew and proliferated normally.

The hCMEC/D3 microvascular endothelial cells were obtained through a collaboration with Pierre-Olivier Couraud (Inserm, Institut Cochin, Paris, France). The reason to study the effect of silencing the AGS-related genes in such endothelial cells is because there is now extensive neuropathological data suggesting that AGS may represent a primary microangiopathy (as supported by our experiments described above). Moreover, we, and others, have published (NIMBL-funded) data showing that AGS patients with *SAMHD1* mutations suffer from cerebral large vessel disease. B5, in collaboration with B3 and B8, is currently preparing a review article highlighting the importance of intracerebral vascular disease in AGS – not limited to the *SAMHD1* genotype.

In contrast to the ihNSC cultures, the same KD strategy did not significantly affect the viability of microvascular endothelial cells. Only *TREX1* KD in the hCMC/D3 cells induced a reproducibly and significant decrease in proliferation associated with a partial cell cycle arrest. Interestingly, and supporting our previous findings relating to proliferation and cell cycle arrest, *TREX1* KD microvascular endothelial cells showed a slower migration capacity to recover an intact monolayer of cells *in-vitro* in a classical, so-called, 'wound healing' assay. The hCMC/D3 endothelial cells did not increase the production of interferon alpha because of intrinsic limitations of this cell line due to the process of immortalization. On the other hand, *TREX1* KD astrocytes demonstrated an increased expression of interferon alpha and of the major histocompatibility complex class I (MHC class I).

Additionally, B5 measured the expression of different interferon-stimulated genes (ISGs) upon KD of the various AGS-related genes. Microvascular brain endothelial cells showed a robust increase in the expression of some of these ISGs, including *IFIT1*, *IFIT2*, *IFIT3*, *IRF9*, *OAS1*, *IFI27* and *RSAD2*, after AGS gene silencing. On the other hand, KD strategies in ihNSC-derived astrocytes demonstrated a notable increase of expression only in *OAS1* and *RSAD2*. In both cells types, and for all the genes tested, *TREX1* KD cells showed persistently higher transcriptional expression levels of these ISGs compared to other KD tissue cells and healthy untransfected control cells. Conversely, *RNASEH2A* KD in these cells resulted in constantly lower levels of ISG expression in all cell types - indicating that silencing of this single factor does not impact the cellular programme in a way that results in the typical 'interferon signature' seen in (predominately other genotypes of) AGS.

Finally, the levels of proinflammatory cytokines were also measured in these different cell cultures – both by quantitative PCR and on a Luminex platform. Results showed that in endothelial cells there was an increase in the expression of CXCL10, especially in *TREX1* KD cells. In astrocytes, there was a general increase of CXCL10 in all KD cells compared to controls, levels being higher on *TREX1* KD. Astrocyte KD cells also demonstrated increased release of pro-inflammatory cytokines (IL6, CXCL10, CCL5 and TNFα). Again, *TREX1* KD resulted in a maximum release of cytokines to the cell medium. In contrast, and again, *RNASEH2A* KD cells produced persistently lower levels of cytokines – corresponding well with the relatively milder phenotype of AGS2 among the various AGS subtypes (*AGS1-6*). The increased expression of ISGs and cytokines in *TREX1* KD cells is congruent with previous observations suggesting that *TREX1* patients suffer from an earlier disease onset and a more severe disease evolution. The *in vitro* data suggest a more robust pro-inflammatory profile on TREX1 KD that might determine a greater degree of neuroinflammation and, as a consequence, a worse prognosis. These data are being prepared for submission to an autoimmune-focused journal.

Further aspects of note relating to WP8

During the second half of the project, B2 was able to collect tissue from three *TREX1*-mutation confirmed RVCL patients living in the Western part of the Netherlands. B2 was able to perform histopathologic examination of the retina at autopsy that consistently showed

scattered micro-infarcts – with retinal arteries having thickened hyalinized walls, and focal areas of disruption to ganglion cells and the inner nuclear layer of the retina. In some areas, the pathologic process had progressed to retinal hemorrhage and neovascularization. With regards to the brain, gross pathology at autopsy demonstrated minimal to marked involvement of the periventricular white matter, particularly of the fronto-parietal lobes. Multiple, often confluent, foci of coagulation necrosis were identified in the white matter with sparing of the grey matter. Larger affected areas had extensive necrosis with focal calcification. On microscopic evaluation a striking vasculopathy affecting the medium and small calibre arteries characterized these necrotic foci and adjacent white matter. Fibrinoid necrosis, adventitial fibrosis, luminal narrowing and mural hyalinization with collagenous material were hallmarks of the vasculopathy. Focal calcifications and reactive astrocytosis were frequent findings. Myelin loss was substantial at autopsy. Neurofilament immunolocalization showed concomitant axon loss and large numbers of swollen axonal spheroids, consistent with an ischemic process. Electron microscopy showed irregular thickening and splitting of the basement membranes in vessel walls, especially in the media, with signs of smooth muscle cell and pericyte degeneration. These data will form part of a planned submission for publication.

Summary of WP8: In this WP, we comprehensively addressed the profile of cytokines and autoantibodies in AGS in relation to the AGS inflammatory-associated brain damage. Furthermore, B5 has specifically defined a central role for astrocytes in AGS pathogenesis. Astrocytes are immune cells responsible for the production of many of the pro-inflammatory cytokines that appear elevated in AGS. Of major significance is the discovery that epitopes expressed by astrocytes are targeted by autoantibodies in AGS patients. As programmed, we have also provided a comprehensive description of the neuropathology of RVCL.

Work Package 9: *Clinical and animal research in RVCL*

Overall aim: To investigate endothelial function in RVCL, both through clinical testing of affected patients, and by using an RVCL *Trex1* knock-in mouse developed in WP4.

Involving: B2

Task 1: Clinical endothelial tests in RVCL patients

As the most prominent phenotype in RVCL patients is a vasculopathy in the retina, the cerebrum, the kidney and other organs, several clinical paradigms to test for vascular / endothelial function (i.e. Pulse Wave Analysis - PWA, Pulse Wave Velocity - PWV, and Flow mediated dilatation - FMD) were performed, in parallel to the clinical characterization of RVCL patients described in WP2. B2 determined the extent of the vasculopathy and assessed whether it is due to impaired endothelium-dependent or -independent (i.e. mediated by direct relaxation of smooth muscle cells) mechanisms. The various vascular tests searched for possible abnormalities in vessel functioning in RVCL patients at different levels of the vascular bed, namely vascular functional changes in arterial stiffness, endothelium-independent vasodilatation of resistance vessels, and endothelial function of conduit arteries. With respect to endothelial profiles in blood, no differences were observed for classical cardiovascular risk profile parameters, such as glucose, total cholesterol, HDL-cholesterol and triglyceride levels. As B2 is also the national referral centre for patients with Cerebral Autosomal Dominant Arteriopathy with Subcortical infarcts and Leukoencephalopathy (CADASIL) - another monogenic cerebral small vessel disease caused by mutations in *NOTCH3* - we compared RVCL and CADASIL patients to determine

any overlap and differences in endothelial profiles. We conclude that in both disorders there seems to be a reduced vascular functionality – but, unlike in CADASIL, in RVCL the systemic vasculopathy does not seem to involve degeneration of vascular smooth muscle cells. These data are almost ready for submission.

Task 2: Testing phenotypes in mice with an RVCL-related *Trex1* knock-in mutation

B2 has begun characterizing the RVCL *Trex1* knock-in mice (+Neo and -Neo), which were generated in WP4, at the molecular and neurobiological level. By analysing tail DNA (and, later, DNA obtained from liver), B2 established that the targeting / selection procedure was exactly as designed when making the targeting construct, and that the desired genomic alteration with the RVCL-associated *Trex1* V235fs mutation was present at the correct position. In addition, no other sequence alterations were identified (e.g. at LoxP sites and restriction sites that were introduced for cloning purposes), with the neighbouring *Atrip* and *Scotin* genes intact. At the RNA level, on northern blots the expected band sizes were seen after probing total RNA of the three genotypes in –Neo mice. Preliminary quantitative PCR of spleen and kidney RNA does not seem to indicate changed expression levels of the neighbouring genes – indicating that the targeting approach had not interfered with sequence elements important for correct expression of these genes. At the protein level, in the homozygous RVCL *Trex1* mutants, a smaller-sized product of around 26 kD was seen compared with 33 kD for wild-type (WT). This result confirms that the correct mutant protein is made in the mutant animals, thereby validating the generated mouse model. However, unlike in *AGS1 Trex1* null mice, RVCL *Trex1* knock-in mice do not show an overt phenotype at first inspection. RVCL knock-in mice seem to breed normally with no deviations from Mendelian distribution in the offspring. A histological analysis with H&E or Nissl staining of several organs (e.g. heart, eye spleen, kidney, liver, skin) does not seem to detect any clear abnormalities. However, at present, it is unclear whether a phenotype in RVCL *Trex1* mutant mice will develop with age. Over the course of the NIMBL project, mice of both genders and various ages (young mice and mice well over one year of age) were tested. Already, B2 has searched extensively for possible structural abnormalities, beyond H&E and Nissl staining, with electron microscopy and on an immunohistochemical analysis platform - to test for abnormalities of potentially relevant marker proteins (e.g. Neu, GFAP, Iba1, CD31/PECAM-1 - for neuronal, glial, microglial, and retinal abnormalities, respectively). Additionally, as all tested commercial TREX1 antibodies, and antibodies provided by B1 and B6, failed at the immunohistochemistry level, B2 – in collaboration with B5 - tested four TREX1 antibodies that were newly generated by Biomatik (these antibodies are required in order to interrogate protein intracellular localisation). Epitopes were chosen from the N- (GSQALPPGPMQT and GSQTLPHGHMQT) and C-terminus (DMEATGLPFSQPK and ATLYGLFLASPGQ) of both human and mouse TREX1, respectively, and peptides were synthesized for immunization in rabbits. The antibodies were optimized for use in western blotting and immunohistochemistry – in order to test a range of post-mortem tissues (including cortex) of RVCL patients and controls, as well as tissues from transgenic RVCL and WT mice. However, despite extensive testing, in close collaboration with B5, of these antibodies under various conditions (e.g. differing titres, testing unpurified serum from rabbits, reducing / non-reducing / denaturing, testing different material i.e. human or mouse cells / tissue), no interpretable banding patterns were seen. More recently, B2 has generated multiple recombinant CMV-promoter containing plasmids (i.e. wild-type and V235fs mutant human and mouse constructs), which might allow detection of recombinant proteins. Attempts by B2 to identify functional pathology in RVCL knock-in mice has included *ex vivo* organ bath measurements of aorta and mesenteric arteries for interrogating differences in endothelial and smooth muscle cell dependent relaxation and constriction. Groups of mutant and wild-type mice of various ages (13, 26 and 52 weeks) are being measured. Finally, given the co-morbidity with migraine, B2 defined an operational platform to undertake neurobiological tests in RVCL *Trex1* knock-in

mice (e.g. using electrophysiological analysis of cortical spreading depression, CSD - an electrophysiological correlate of migraine). We have also generated, in parallel research, CADASIL mutant mice that may be helpful in identifying an RVCL-relevant phenotype (considering the clinical overlap between these two diseases). The search for a functional phenotype in these mice will continue beyond the end of the NIMBL project.

Further aspects of note relating to WP9

Considering the finding of a robust upregulation of interferon stimulated genes (ISGs) in patients with AGS, B1 looked for an interferon signature in patients with RVCL, but did not find evidence of such. Further work is planned to examine the expression of ISGs in RVCL *Trex1* knock-in mice brains, and possibly in human RVCL brain tissue. These data may be important in providing a possible pathological link between AGS and RVCL.

Summary of WP9: In this WP we have comprehensively addressed the clinical features of RVCL. We have also used an RVCL knock-in mouse model to try to better understand the pathology of this devastating adult-onset phenotype. Although initial characterisation of the RVCL mouse has revealed no obvious disease-related features, further work is fully warranted (particularly concentrating on an analysis of older mice).

Work Package 10: *Therapeutics in NIMBL phenotypes*

Overall aim: To consider therapeutic options relevant to NIMBL phenotypes, assess such therapies in the context of NIMBL-derived animal and cellular systems, and determine the possibility of translation into human drug trials.

Involving: All beneficiaries

***Task 1*: Evaluation of current treatments in NIMBL patients**

No effective therapies or cures for any NIMBL-related disorders exist at present. That is, treatment is currently based on ameliorating symptoms as they arise, and is not directed to addressing fundamental pathogenesis. During the first half of the NIMBL project, the clinical partners, notably B3, have collected information from patients relating to their current treatments and any side-effects reported; these data will help in the further evaluation of future drug trials (such as the one due to start in April 2014). Data has been collected on:

- Skin lesions: local treatment in order to protect against low temperature, creams and ointments to promote healing, and vasoprotectives; no significant side effects, but also no important benefits
- Spasticity: baclofen, botulinum toxin; no significant side-effects, but the treatment has shown only a slight improvement in spasticity; in one patient baclofen resulted in a reported significant improved of sleep disturbance
- Seizures: a variety of anti-epileptic medications were considered, although seizure control in AGS is not a major problem in routine clinical practice
- Gastrointestinal problems: omeprazole, gastric feeding tube; benefits: weight gain and also better quality of life, with no significant adverse side effects
- Sleep disorder: antihistamines (niaprazina), benzodiazepines, melatonin: no significant side effects; in two patients sleep disorders apparently improved with niaprazina

B1, B3, B5, and B8, with input from B7, prepared a consensus statement regarding treatments options for patients with AGS. This document has been published:

- Crow et al. Clin Exp Immunol 2014;175:1-8 [**B1, B3, B5, B8**]

***Task 2*: Testing drugs in NIMBL-derived cellular and animal models**

NIMBL associated diseases are severe, so that there is an urgent need to develop effective treatments. The aim of this task was to consider therapeutic options relevant to NIMBL phenotypes, assess such therapies in the context of NIMBL-derived animal and cellular systems, and consider the possibility of translation into human drug trials. Following on from insights derived from other WPs, we have investigated two treatment strategies in our NIMBL-generated disease models, 1. Reverse transcriptase inhibitors (RTIs) in both the *Trex1* null mouse, and in a cellular assay of reverse transcription; and 2. Steroid-mediated immunosuppression in the *Trex1* null mouse. These data are described more fully in Deliverable 10.2, but summarising:

- Following on from previously published work by Beck-Engeser et al. (*Retrovirology* 2011), B7 has confirmed the efficacy of RTIs in the *Trex1* null mouse (using a combination of Emtricitabine, Tenofovir and Nevirapine)
- Furthermore, in preparation for human drug trials, B7 has also assessed the efficacy of RTIs in a cellular assay of LINE-1 (L1) retrotransposition
- Additionally, B6 has demonstrated the positive effect of steroid-mediated immunosuppression in the *Trex1* null mouse

These three sets of data are in preparation for submission for publication.

In regards of RVCL, the lack of an animal or cellular phenotype at this stage has so-far precluded the assessment of treatments in our experimental systems.

Further aspects of note relating to WP10

NIMBL associated diseases are severe, so that there is an urgent need to develop effective treatments. Since RVCL is an adult onset disease, it is possible that early intervention following diagnosis (either at the recognition of clinical features, or through family cascade mutation screening) could, in the future, allow for a cure / amelioration of the clinical phenotype. At this time, our understanding of the biology of RVCL is not mature enough to allow for directed therapeutic approaches, so that this phenotype will not be considered further here. In contrast, led by ourselves and by others, understanding of the biology of AGS-related disease has increased remarkably over the last 4 years. In particular, evidence has accrued to implicate the defective control of retroelements as a central theme in disease pathogenesis (Table 1).

Thus, our proposal is that (certain) RTIs will inhibit the reverse transcription of endogenous retroelements deemed to be responsible for initiating the tissue damage seen in AGS. We believe that the current state of knowledge has advanced to the point where a phase II study is warranted to establish the safety of these RTIs in patients with AGS, and assess their effects on type I interferon outputs (prior to a full efficacy study).

Table 1: Evidence supporting a role for AGS-related proteins in retroelement metabolism

1. TREX1 activity on reverse transcribed DNA
2. Retroelement DNA accumulation in TREX1-deficient cells
3. Rescue of the lethal TREX1-null murine phenotype by treatment with reverse transcriptase inhibitors
4. TREX1 digestion of non-productive HIV reverse transcripts in CD4 T cells and macrophages
5. Role of RNase H2B in 'facilitating' HIV-1 infection (as per TREX1 – point 4 above)
6. SAMHD1 acting as an HIV restriction factor in cells of the myeloid lineage; and silencing of SAMHD1 in non-permissive cell lines being associated with an accumulation of viral DNA
7. SAMHD1 regulation of dNTP availability, a recognised limiting factor in retroviral propagation
8. SAMHD1 regulation of LINE-1 retrotransposition, and defective LINE-1 inhibition associated with *SAMHD1* mutated for pathogenic variants seen in AGS
9. Known role of ADAR1 in editing Alus – retroelements comprising 10% of the human genome

An issue in any drug trial is the assessment of therapeutic efficacy. We have shown that patients with AGS demonstrate an upregulation of interferon stimulated genes (ISGs) in peripheral blood. The features of this AGS-related 'interferon signature' include:

1. An association with mutations in any of the known AGS-related genes
2. Presence of a marked signature beyond the sub-acute encephalopathic stage, and showing no attenuation with patient age
3. No association with patient sex
4. The composite of data from a set of markers, thereby increasing the robustness of the assay and allowing for a quantitative score to be derived
5. Measurement on small volumes of peripheral blood
6. A turn-around time of less than five days

Thus, for the first time, we have a well-characterised AGS biomarker, which we propose to use in the context of a clinical trial. These results have been published:

- Rice et al. Lancet Neurol 2013;12:1159-69 [**B1, B3, B4, B8**]

Founded on the remarkable progress enabled by NIMBL support, we are about to pursue a clinical trial of RTIs in AGS (financed through the European Leukodystrophy Association).

Summary of WP 10: Specifically in regards of AGS, we have collated phenotypic data on 346 affected (molecular confirmed) patients, providing us with a detailed understanding of the natural history of the disease. Additionally, we have liaised with clinical professionals, patients, and pharmaceutical representatives (MedImmune and GSK) to fully assess treatment options in AGS. Following on from this work, we have investigated two treatment strategies in our NIMBL-generated disease models (RTIs in both the *Trex1* null mouse and in a cellular assay of reverse transcription, and steroid-mediated immunosuppression in the *Trex1* null mouse). We have also fully characterised a biomarker (the combinatorial read-out of ISGs) for monitoring treatment efficacy in humans. Building on these research assets, and the remarkable progress made by ourselves and others in understanding the biology of AGS, we have now secured funding for a drug trial (of RTIs) in AGS patients, which will begin in April 2014.

4. The potential impact and main dissemination activities

By combining ideas, skills, resources and data from leading European and North American experts based in eight centres, this project set out to define the natural history and pathophysiology of Aicardi-Goutières Syndrome (AGS) and Retinal Vasculopathy with Cerebral Leukodystrophy (RVCL), prototype immune-mediated neurological diseases due to nuclease deficiencies. The overriding aim of the project was to acquire new knowledge, which would enable the development of diagnostic and therapeutic modalities applicable to AGS, RVCL and related phenotypes. Moreover, by providing fundamental insights into a novel mechanism of autoimmunity caused by inappropriate activation of nucleic acid sensors, it was considered that these data would enhance our understanding of more common autoimmune diseases including systemic lupus erythematosus (SLE).

Scientific Impact

This project has provided seminal insights into NIMBL-related diseases.

- We now have to hand a remarkable collection of phenotypic (clinical, radiological and laboratory) data, which has allowed us / will allow us to better define the natural history of AGS and RVCL, and expand the phenotypic spectrum of NIMBL-associated conditions far beyond the original diagnostic parameters. This information will greatly assist clinical diagnostic practice, and improve the quality of data available to patients and their families.

- Through the identification of new genes which, when mutated, cause AGS, we have extended diagnostic capability worldwide. Under the auspices of the European Molecular Genetics Quality Network (EMQN) we have also established quality controlled, diagnostic testing standards, and AGS testing has now been fully adopted by the Board of EMQN. In this way, we have gone some way towards the adoption / availability of best-molecular-practice in Europe and further afield.

- By providing fundamental insights into a mechanism of autoimmunity involving the inappropriate activation of nucleic acid sensors, we have enhanced knowledge of AGS-related disease processes. In particular, we have further highlighted the importance of type I interferons in the pathology of AGS, and derived data which strongly implicate the defective control of retroelement metabolism as central to AGS biology. These concepts are novel, and are likely to have implications for a broader set of human disease states – most particularly the autoimmune diseases.

Our patient studies, and the use of existing and newly-derived cellular and animal models, have helped to define rationale treatment approaches for AGS – so that the first clinical trial for the disease is now in preparation to begin in 2014 – a remarkable NIMBL-related achievement.

Socioeconomic Impact

AGS, RVCL and other overlap conditions are devastating diseases associated with significant morbidity, premature death and high risks of recurrence. By allowing for early diagnosis through enhanced clinical recognition, improved and extended molecular diagnosis, and increased availability of prenatal and carrier testing, our work has had, and will continue to have, significant socioeconomic impact worldwide. Moreover, although not easily quantifiable, our results and our commitment to research in this field are important in providing hope for the hundreds of families affected by these devastating conditions.

Main dissemination activities

The NIMBL beneficiaries have been involved in a wide range of dissemination activities, most particularly with the following groups: the scientific community including pharmaceutical companies – MedImmune and GSK (though publications and presentations at national and international meetings); key stakeholders (e.g. support groups); patients and their families at clinics (through direct contact and via the website and email); and with the wider community (via the public website). As with many funded projects, many results are disseminated after the end of the study, and this will be true of NIMBL also; research work has continued until the end of the project, and the derived data will continue to be analysed and published / disseminated well beyond the project end-date.

Public website

The public website (at http://www.nimbl.eu/ni/Home), designed by a subcontractor with input from B1, was launched in May 2011. The website, which has been updated regularly, provides basic information (for families, physicians and scientists, and members of the lay public) about the underlying genetics and biochemistry involved in the NIMBL-related conditions, and also describes how research in the NIMBL project helps to expand scientific knowledge. The website also gives email details for clinicians and newly diagnosed individuals and families to contact key NIMBL personnel.

E-newsletters

Four issues of the project e-newsletter (June 2011, April 2012, December 2012, and June 2013) have been circulated to families, with their consent, and to recipient organisations - including the British Paediatric Neurology Association, the European Society of Paediatric Neurologists, the British Society of Human Genetics, the European Society of Human Genetics, the British Society for Investigative Dermatology, the International Aicardi-Goutières syndrome Association (IAGSA), the European Leukodystrophy Association (ELA), and Contact-A-Family. These e-newsletters can be downloaded from the public website.

Family conferences and contact with patients

The clinical partners (B1, B2, B3, B5 and B8) are in regular direct contact with patients and their families, and also with other relevant stakeholders such as patient organisations (most particularly, IAGSA and ELA), to spread awareness of these diseases, discuss wider societal implications, and recruit more patients into the study.

B8, B3 and B1 have held family-scientist conferences (in Washington DC and Manchester, these were held in conjunction with patient clinics) so that families could learn about the project directly from the NIMBL researchers, obtain updates on the current theories of the underlying causes of AGS, and meet other families. These family-scientist conferences give the families – often living far away from any other AGS families – the chance to meet and exchange experiences; they also provide a rare opportunity for the non-clinical scientists to have direct contact with the patients and their families, whose samples form the focus of their research. This can be very inspirational and reinforces the need for these investigations, making the laboratory work even more worthwhile and rewarding.

A total of four such meetings have been held:

1. Washington DC on 30 April 2011
2. Pavia on 3 July 2012 (in combination with IAGSA)
3. Washington DC on 6 October 2012. Patients and families stayed in DC for three days. On the first night there was a family dinner so that families could network and share experiences. During the next two days, each individual family was assessed clinically (see WP2). Speakers also presented on setting up family support groups for rare conditions, and how laboratory-based research might result in future treatments.

Links to the presentations are on the NIMBL website:

- Morning session (duration: 2 hours):
  https://cnmc.webex.com/cnmc/ldr.php?AT=pb&SP=MC&rID=13191317&rKey=8e9c9a39ae098da9
- Afternoon session (duration: 1 hour)
  https://cnmc.webex.com/cnmc/ldr.php?AT=pb&SP=MC&rID=13191327&rKey=6a5dd026d177c34d

4. Manchester on 6 March 2013. Patients and families stayed one or two nights (depending on their travel arrangements) and a family dinner, also attended by the NIMBL PIs, was held the night before the conference. The presentations from the final conference were filmed and are available to view on YouTube:

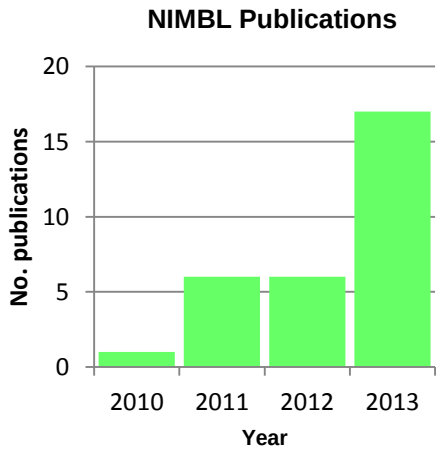
   - NIMBL: an international consortium on AGS: Diana Chase (http://www.youtube.com/watch?v=3gMgXntekxE) [**B1**]
   - Investigation of astrocyte function in AGS: Taco Kuijpers (http://www.youtube.com/watch?v=kfALmBP9_5Q) and Eloy Cuadrado: (http://www.youtube.com/watch?v=ZsGbUAK4lBo) [**B5**]
   - How brain scans (CT and MRI) help in the diagnosis of Aicardi-Goutières syndrome: John Livingston (http://www.youtube.com/watch?v=d-wm8QRVIj4) [**B4**]
   - Investigating the role of RNaseH2 in AGS: Karen McKenzie (Institute of Genetics and Molecular Medicine, Institute of Genetics and Molecular Medicine): (due to the sensitivity of the material presented, this is not on YouTube)
   - Diagnostic mutation testing; 6 years' experience of providing genetic testing to AGS patients and their families: Teresa Lamb (Yorkshire Regional Genetics Service, Leeds, UK): (http://www.youtube.com/watch?v=nsfjdie2GpI) [**B4**]
   - The US based AGS experience: mechanisms and biomarkers: Adeline Vanderver: (http://www.youtube.com/watch?v=G147CX0h1ew) **[B8]**
   - Research Progress and the Future of Treatments in AGS: Yanick Crow: (http://www.youtube.com/watch?v=3HZFFhw-NW0) [**B1**]

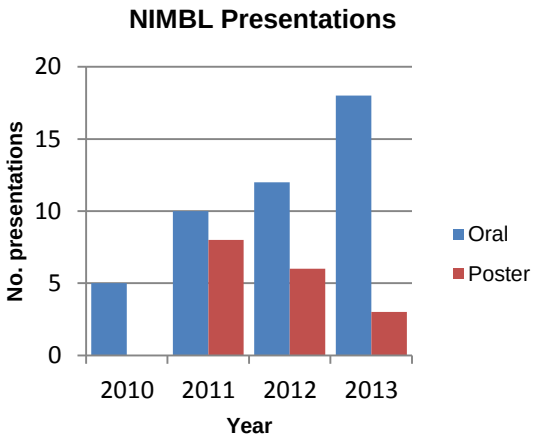
A further family-scientist meeting is planned for October 2014, in Washington DC

Publications in peer-reviewed journals

By the end of the project, the results from this research study have formed the basis of a total of 30 peer-reviewed publications, one on-line review, and a book chapter (for a full list, see Table A1, "Use and dissemination of the foreground"). Several of the publications were in very high-impact journals [Impact Factors quoted from 2012 JCR Science Edition], including *Nature* (Impact Factor 38.597) in 2011 (Goldstone et al. [B1]); *Nature Genetics* (Impact Factor 35.209) in 2011 (Briggs et al. [B1]), and in 2012 (Rice et al. [B1, B3 and B8]); *Immunity* (Impact Factor 19.795) in 2012 (Gall et al. [B7]); and *Lancet Neurology* (Impact Factor 22.917) in 2013 (Rice et al. [B1, B3 and B8]). Of these, the Goldstone et al. 2011, Gall et al. 2011 and Rice et al. 2012 papers have already amassed 107, 48 and 22 citations respectively, which is impressive considering that they were only published in 2011 / 12.

The Goldstone paper attracted a comment in ***ScienceDaily*** (7 Nov 2011) www.sciencedaily.com/releases/2011/11/111107033929.htm, while several other NIMBL publications have attracted editorial comment: Briggs et al. (2011), Gall et al. (2012), Pereira-Lopes et al. (2013) and Rice GI et al. (2013). Indeed, some of the figures from Livingston et al. (e-pub 2013) will be on the front cover of J Med Genet Feb 2014 issue.





More papers are expected to be published in the future, following further analysis of more recently derived data.

Presentations at conferences, seminars and workshops.
During the NIMBL project, a total of 45 oral and 17 poster presentations about the results obtained were given by NIMBL researchers at seminars, workshops, and conferences, both nationally (UK, the Netherlands, Italy, Spain and the USA) and internationally (Turkey, Ireland, Belgium, France, Switzerland, Slovenia, Mexico, and Canada - Toronto and Vancouver). Within the USA, in addition to the 'home' locations of Washington DC and Seattle (University of Washington), presentations were given in many other American states, (California, Texas, Maryland, Georgia, Massachusetts, New York, San Diego, Colorado, Illinois and Hawaii).

Other presentations, based on more recently acquired NIMBL data, are expected to be given in the future.

5. Website address and relevant contact details

The NIMBL project website is at: http://www.nimbl.eu/ni/Home

For further information about the project, please contact Professor Yanick Crow (project coordinator) at yanickcrow@mac.com or Diana Chase (project manager) at Diana.Chase@manchester.ac.uk.

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4	University of Leeds	LEEDS	Leeds UK	Prof David Bonthron d.t.bonthron@leeds.ac.uk
5	Academisch Medisch Centrum bij de Universiteit van Amsterdam	AMC	Amsterdam Netherlands	Prof Taco Kuijpers t.w.kuijpers@amc.uva.nl
6	Fundació Privada Institut De Recerca Biomèdica IRB	IRB	Barcelona Spain	*(Dr Antonio Celada)* ‡
7	University of Washington	UW	Seattle USA	Prof Dan Stetson stetson@uw.edu
8	Children's Research Institute (CRI)	CNMC	Washington DC USA	Prof Adeline Vanderver AVanderv@childrensnational.org
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‡ Antonio Celada relocated from IRB to UB on 01 January 2013

6. Use and Dissemination of the Foreground

Section A

Peer-reviewed scientific publications

By the end of the project, the results from this research study have formed the basis of a total of 30 peer-reviewed publications (for a full list, see Table A1), plus one on-line review, and a book chapter.

*On-line review***:** Aicardi J, **Crow YJ**, Stephenson JBP. Aicardi-Goutières Syndrome. GeneReviews™ [Internet]. Updated 01/03/12. (doi: ncbi.nlm.nih.gov/books/NBK1475) [**B1**]

Book chapter: **Crow YJ**. Aicardi-Goutieres Syndrome. *Handb Clin Neurol* (2013) 113:1629-35 (doi: 10.1016/B978-0-444-59565-2.00031-9) [**B1**]

Presentations at scientific conferences

During the NIMBL project, a total of 45 oral and 17 poster presentations about the results obtained were given by NIMBL researchers at seminars, workshops, and conferences, both nationally (UK, the Netherlands, Italy, Spain and the USA) and internationally (Turkey, Ireland, Belgium, France, Switzerland, Slovenia, Mexico, and Canada - Toronto and Vancouver) (for a full list, see Table A2).

Newsletter

Four issues of the project e-newsletter (June 2011, April 2012, December 2012, and June 2013) have been circulated to families, with their consent, and to recipient organisations. These are also available for download from the NIMBL public website.

Family-scientist conferences

In addition, B8, B3 and B1 have held family-scientist conferences (in Washington DC and Manchester, these were filmed and are on the internet). For more details, please see Section 4 – main dissemination activities.

Table A1: list of scientific (peer reviewed) publications

No.	Title	First author	Periodical	Year	Issue/No	Pages	Identifier (doi)s
1	HIV-1 restriction factor SAMHD1 is a deoxynucleoside triphosphate triphosphohydrolase.	Goldstone DC	Nature	2011	480(7377):	379-82	10.1038/nature10623
2	Mutations in ADAR1 cause Aicardi-Goutières syndrome associated with a type I interferon signature.	Rice GI	Nature Genetics	2012	44(11)	1243-8	10.1038/ng.2414
3	Tartrate resistant acid phosphatase deficiency causes a bone dysplasia with autoimmunity and a type I interferon expression signature.	Briggs TA	Nature Genetics	2011	43(2)	127-31	10.1016/S1474-4422(13)70258-8
4	Assessment of interferon-related biomarkers in Aicardi-Goutières syndrome associated with mutations in TREX1, RNASEH2A, RNASEH2B, RNASEH2C, SAMHD1, and ADAR: a case-control study.	Rice GI	Lancet Neurol	2013	12(12)	1159-69	10.1016/S1474-4422(13)70258-8)
5	Autoimmunity initiates in nonhematopoietic cells and progresses via lymphocytes in an interferon-dependent autoimmune disease	Gall A	Immunity	2012	36(1)	120-31	10.1016/j.immuni.2011.11.018
6	Extensive evolutionary and functional diversity among mammalian AIM2-like receptors.	Brunette RL	J Exp Med	2012	209(11)	1969-83	10.1084/jem.20121960
7	SAMHD1- dependent retroviral control and escape in mice.	Rehwinkel J	The EMBO Journal	2013	32	2454–62	10.1038/emboj.2013.163
8	Chronic exposure of astrocytes to interferon-α reveals molecular changes related to Aicardi-Goutieres syndrome	Cuadrado E	Brain	2013	136(Pt 1)	245-58	10.1093/brain/aws321
9	Cerebral vasculopathy is a common feature in Aicardi-Goutieres syndrome associated with SAMHD1 mutations.	du Moulin M	Proc Natl Acad Sci U S A.	2011	108(26)	E232	10.1073/pnas.1104699108
10	*SAMHD1* is mutated recurrently in chronic lymphocytic leukemia and is involved in response to DNA damage.	Clifford R	Blood	2014	123(7)	1021-31	10.1182/blood-2013-04-490847
11	Endogenous retroelements and autoimmune disease.	Stetson DB	Curr Opin Immunol	2012	24(6)	692-7	10.1016/j.coi.2012.09.007
12	Elevation of proinflammatory cytokines in patients with Aicardi-Goutières syndrome.	Takanohashi A	Neurology	2013	80(11)	997-1002	10.1212/WNL.0b013e3182872694
13	Aicardi-Goutieres syndrome, a rare neurological disease in children: a new autoimmune disorder?	Fazzi E	Autoimmun Rev	2013	12(4)	506-9	10.1016/j.autrev.2012.08.012

No.	Title	First author	Periodical	Year	Issue/No	Pages	Identifier (doi)s
14	Lupus: how much “complexity” is really (just) genetic heterogeneity?	Crow YJ	Arthritis Rheum	2011	63	3661-4	
15	Protein kinase cδ deficiency causes mendelian systemic lupus erythematosus with B cell-defective apoptosis and hyperproliferation	Belot A	Arthritis Rheum	2013	65(8)	2161-71	10.1002/art.38008
16	A type I interferon signature identifies bilateral striatal necrosis due to mutations in ADAR1	Livingston JH	J Med Genet	2014	51(2)	76-82	10.1136/jmedgenet-2013-102038
17	Characterization of*Trex1* Induction by IFN-g in Murine Macrophages.	Serra M	J Immunol	2011	186(4)	2299-308	0.4049/jimmunol.1002364
18	The exonuclease Trex1 restrains macrophage proinflammatory activation	Pereira-Lopes S	J Immunol	2013	191(12)	6128-35	10.4049/jimmunol.1301603
19	Synonymous mutations in RNASEH2A create cryptic splice sites impairing RNase H2 enzyme function in Aicardi- Goutières syndrome	Rice GI	Hum Mutat	2013	34(8)	1066-70	10.1002/humu.22336
20	SAMHD1 is a nucleic-acid binding protein that is mislocalized due to aicardi-goutières syndrome-associated mutations	Goncalves A	Hum Mutat	2012	33(7)	1116-22	10.1002/humu.22087
21	Heterozygous TREX1 mutations in early-onset cerebrovascular disease	Pelzer N	J Neurol	2013	260(8)	2188-90	10.1007/s00415-013-7050-8
22	Therapies in Aicardi-Goutières Syndrome	Crow YJ	Clin Exp Immunol	2014	175(1)	1-8	10.1111/cei.12115
23	Type I interferonopathies: a novel set of inborn errors of immunity. In “The Year in Human and Medical Genetics: Inborn Errors of Immunity I.”	Crow YJ	Ann.N.Y. Acad. Sci	2011	1238	91–98	10.1111/j.1749-6632.2011.06220.x
24	Family history of autoimmune disease in patients with Aicardi-Goutières syndrome	Schmidt JL	Clin Dev Immunol	2012	2012	206730	10.1155/2012/206730
25	Dysregulation of the immune system in Aicardi-Goutieres syndrome: another example in a TREX1-mutated patient	Olivieri I	Lupus	2013	22(10)	1064-9	10.1177/0961203313498800
26	Recognizable phenotypes associated with intracranial calcification.	Livingston JH	Dev Med Child Neurol	2013	55(1)	46-57	10.1111/j.1469-8749.2012.04437.x
27	Inhibition of the de-myelinating properties of Aicardi-Goutières Syndrome lymphocytes by cathepsin D silencing	Pulliero A	Biochem Biophys Res Commun	2013	430(3)	957-62	10.1016/j.bbrc.2012.11.131
28	Bilateral striatal necrosis in two subjects with Aicardi–Goutières syndrome due to mutations in ADAR1 (AGS6)	La Piana R	Am J Med Genet A	2014	164A(3)	815-9	10.1002/ajmg.a.36360

No.	Title	First author	Periodical	Year	Issue/No	Pages	Identifier (doi)s
29	Striking intrafamilial phenotypic variability in Aicardi-Goutières syndrome associated with the recurrent Asian founder mutation in RNASEH2C	Vogt J	Am J Med Genet A	2013	161A(2)	338-42	10.1002/ajmg.a.35712
30	Autosomal dominant inheritance of a heterozygous mutation in SAMHD1 causing familial chilblain lupus	Ravenscroft JC	Am J Med Genet A	2011	155A(1)	235-237	10.1002/ajmg.a.33778

Table A2: list of dissemination activities

No	Type of activity	Main leader	Title	Date	Place	Type of audience	Countries addressed
1	Web sites / Applications	B1	NIMBL public website	01/05/11	www.nimbl.eu	○ Scientific community; ○ Higher education / research ○ Industry ○ Civil society ○ Policy makers ○ Media	International
2	Web sites / Applications	B1	Spotlight on … NIMBL	16/11/2011	www.orpha.net/actor/EuropaNews/2011/111116.html	○ Scientific community; ○ Higher education / research ○ Industry ○ Civil society ○ Policy makers ○ Media	International
3	Web sites / Applications	B1	HIV Study Identifies Key Cellular Defense Mechanism	07/11/2011	www.sciemcedaily.com/releases/2011/11/111107033929.html	○ Scientific community; ○ Higher education / research ○ Industry ○ Civil society ○ Policy makers ○ Media	International
4	Flyers	B1	NIMBL e-newsletter No.1	30/06/2011	Distributed by e-mail; downloadable from the NIMBL public website	○ Scientific community; ○ Higher education / research ○ Civil society ○ Media	International
5	Flyers	B1	NIMBL e-newsletter No.2	27/03/2012	Distributed by e-mail; downloadable from the NIMBL public website	○ Scientific community; ○ Higher education / research ○ Civil society ○ Media	International
6	Flyers	B1	NIMBL e-newsletter No.3	19/12/2012	Distributed by e-mail; downloadable from the NIMBL public website	○ Scientific community; ○ Higher education / research ○ Civil society ○ Media	International

No	Type of activity	Main leader	Title	Date	Place	Type of audience	Countries addressed
7	Flyers	B1	NIMBL e-newsletter No.4	19/06/2013	Distributed by e-mail; downloadable from the NIMBL public website	○ Scientific community; ○ Higher education / research ○ Civil society ○ Media	International
8	Organization of conference	B8	Family conference 1	30/04/2011	Washington DC, USA	○ Civil society	AGS families in USA
9	Organization of conference	B3	Family conference 2	03/07/2012	Pavia, Italy	○ Civil society	AGS families in Europe & USA
10	Organization of conference	B8	Family conference 3	06/10/2012	Washington DC, USA	○ Civil society	AGS families in USA
11	Organization of conference	B1	Family conference 4	06/03/2013	Manchester, UK	○ Civil society	AGS families in Europe & USA
12	Oral presentation at a scientific event	B6	Macrophages and inflammation	26/08/2010	Kansai, Japan	○ Scientific community; ○ Higher education / research	International - Japan
13	Oral presentation at a scientific event	B1	Interferon-opaties: Genetic Disorders of type I Interferon metabolism	6-9/10/2010	Istanbul, Turkey	○ Scientific community; ○ Higher education / research	International
14	Oral presentation at a scientific event	B6	Macrophages and inflammation	26/10/2010	Barcelona, Spain	○ Scientific community; ○ Higher education / research	Spain
15	Oral presentation at a scientific event	B6	Mechanisms of macrophage survival in inflammation	01/11/2010	New York	○ Scientific community; ○ Higher education / research ○ Industry	USA
16	Oral presentation at a scientific event	B1	From childhood encephalopathy to lupus: Mendelian type I interferonopathies	12/11/2010	Newcastle, UK	○ Scientific community; ○ Higher education / research	UK
17	Posters	B7	Origins and progression of a type I interferon mediated disease	22/01/2011	Pacific Grove, USA	○ Scientific community; ○ Higher education / research	USA
18	Oral presentation at a scientific event	B2	Migraine and stroke – genetic evidence	09-11/02/2011	Los Angeles, USA	○ Scientific community; ○ Higher education / research	International - USA
19	Oral presentation at a scientific event	B6	Macrophages and inflammation	18/02/2011	Madrid, Spain	○ Scientific community; ○ Higher education / research	International - Spain
20	Oral presentation at a scientific event	B3	Aicardi-Goutières Syndrome (AGS): from the experience of the First International Association (IAGSA) to the European NIMBL Project	12/03/2011	Liege, Belgium	○ Scientific community; ○ Higher education / research	International - Belgium

No	Type of activity	Main leader	Title	Date	Place	Type of audience	Countries addressed
21	Poster	B8	Family History of Autoimmune Disease in Patients with Aicardi-Goutieres Syndrome	16-20/03/2011	Vancouver, Canada	○ Scientific community; ○ Higher education / research	International - Canada
22	Oral presentation at a scientific event	B7	Connections between antiviral defence and autoimmunity	24/03/2011	Massachusetts, USA	○ Scientific community; ○ Higher education / research	USA
23	Oral presentation at a scientific event	B7	Connections between antiviral defence and autoimmunity	01/04/2011	Seattle, USA	○ Scientific community; ○ Higher education / research	USA
24	Poster	B8	Anti-neuronal auto-antibodies in Aicardi Goutières Syndrome	09/04/2011	Hawaii, USA	○ Scientific community; ○ Higher education / research	International - USA
25	Oral presentation at a scientific event	B1	From childhood encephalopathy to lupus: Mendelian type I interferon-opathies	11/04/2011	Manchester, UK	○ Scientific community; ○ Higher education / research	UK
26	Oral presentation at a scientific event	B3	Aicardi-Goutières Syndrome (AGS): from the experience of the First International Association (IAGSA)	16/04/2011	Paris, France	○ Scientific community; ○ Higher education / research	International - France
27	Oral presentation at a scientific event	B7	Connections between antiviral defence and autoimmunity	26/04/2011	Bethesda, USA	○ Scientific community; ○ Higher education / research	International - USA
28	Poster	B1	Consolidated clinical genetic testing for Aicardi-Goutières syndrome and related conditions: a service package covering *TREX1*, *RNASEH2B*, *RNASEH2C*, *RNASEH2A*, and *SAMHD1*	28/05/2011	Amsterdam, The Netherlands	○ Scientific community; ○ Higher education / research	International - Netherlands
29	Oral presentation at a scientific event	B2	Migraine genetics: new findings and implications for clinicians	2-5/06/2011	Washington, USA	○ Scientific community; ○ Higher education / research	USA
30	Oral presentation at a scientific event	B1	From childhood encephalopathy to lupus: Mendelian type I interferon-opathies	02/09/2011	Dublin, Ireland	○ Scientific community; ○ Higher education / research	Ireland
31	Oral presentation at a scientific event	B1	Lupus: How much 'complexity' is really (just) genetic heterogeneity?"	05-07/09/11	Warwick, UK	○ Scientific community; ○ Higher education / research	UK
32	Oral presentation at a scientific event	B2	Genetics of migraine: the prospects of personalized therapy	10-13/09/11	Budapest, Hungary	○ Scientific community; ○ Higher education / research	Hungary
33	Poster	B1	Phenotypic variation in familial chilblain lupus (FCL) and Aicardi-Goutières syndrome (AGS) associated with *TREX1* mutation in 4 family members	14-18/09/11	Bruges, Belgium	○ Scientific community; ○ Higher education / research	Belgium
34	Oral presentation at a scientific event	B1	SPENCD syndrome and Mendelian variants of lupus	14-18/09/11	Bruges, Belgium	○ Scientific community; ○ Higher education / research	Belgium

No	Type of activity	Main leader	Title	Date	Place	Type of audience	Countries addressed
35	Poster	B8	Recognizable MRI patterns in Aicardi Goutières Syndrome	26-29/10/11	Savannah, USA	○ Scientific community; ○ Higher education / research	USA
36	Oral presentation at a scientific event	B7	Connections between antiviral defence and autoimmunity	05-09/11/11	Chicago, USA	○ Scientific community; ○ Higher education / research	USA
37	Poster	B8	Brain specific targets of autoimmunity in Aicardi Goutières syndrome	12-16/11/11	Washington DC, USA	○ Scientific community; ○ Higher education / research	USA
38	Oral presentation at a scientific event	B1	SAMHD1 dysfunction causes a variable inflammatory phenotype including a cerebral vasculopathy: treatment with anti-retrovirals?	18-20/01/12	London, UK	○ Scientific community; ○ Higher education / research	UK
39	Oral presentation at a scientific event	B1	Aicardi-Goutières syndrome, spondyloenchondrodysplasia (and a little bit of lupus) 'Type I interferonopathies'	09/02/12	Birmingham, UK	○ Scientific community; ○ Higher education / research	UK
40	Oral presentation at a scientific event	B7	Nucleic acid detection in host defence and autoimmunity	12/03/2012	San Francisco, USA	○ Scientific community; ○ Higher education / research	USA
41	Oral presentation at a scientific event	B1	Human Type I Interferonopathies	16/03/2012	Montpellier, France	○ Scientific community; ○ Higher education / research	France
42	Oral presentation at a scientific event	B7	Endogenous retroelements and autoimmune disease	18/03/2012	New York, USA	○ Scientific community; ○ Higher education / research	USA
43	Poster	B3	Aicardi Goutieres syndrome: what about autoimmunity?	19-21/04/12	Santiago de Compostela, Spain	○ Scientific community; ○ Higher education / research	Spain
44	Poster	B8	Neopterin and Tetrahydrobiopterin cerebrospinal fluid elevations in Aicardi Goutieres Syndrome: confirmation of findings in mutation confirmed subjects	22-27/04/12	New Orleans, LA, USA	○ Scientific community; ○ Higher education / research	USA
45	Oral presentation at a scientific event	B7	Nucleic acid detection in host defence and autoimmunity	17/05/2012	New Haven, CT, USA	○ Scientific community; ○ Higher education / research	USA
46	Oral presentation at a scientific event	B1	Trap Deficiency, Autoimmunity and other Mendelian Variants of Lupus	06-09/06/12	Berlin, Germany	○ Scientific community; ○ Higher education / research	Europe
47	Oral presentation at a scientific event	B7	Nucleic acid detection in host defence and autoimmunity	27/06/2012	Snowmass, CO, USA	○ Scientific community; ○ Higher education / research	USA
48	Poster	B5	Aicardi-Goutières syndrome (AGS): The Role of Astrocytes in Interferon alpha-mediated Leukodystrophy and Microangiopathy	14-18/07/12	Barcelona, Spain	○ Scientific community; ○ Higher education / research	Spain

No	Type of activity	Main leader	Title	Date	Place	Type of audience	Countries addressed
49	Oral presentation at a scientific event	B5	Role of astrocytes in Aicardi-Goutières Syndrome	31/08/2012	Amsterdam, Netherlands	○ Scientific community; ○ Higher education / research	The Netherlands
50	Poster	B6	Mechanisms of macrophage survival in inflammation	5-8/09/12	Glasgow, Scotland, UK	○ Scientific community; ○ Higher education / research	UK
51	Oral presentation at a scientific event	B1	RNase H2 in the context of Aicardi-Goutieres syndrome	5-7/09/12	Edinburgh, Scotland, UK	○ Scientific community; ○ Higher education / research	UK
52	Oral presentation at a scientific event	B1	Human Type I Interferonopathies	18-19/09/12	Gaithersburg, USA	○ Scientific community; ○ Higher education / research	USA
53	Oral presentation at a scientific event	B1	Aicardi-Goutieres syndrome: treating an autoimmune disease with antiretrovirals. Surely some mistake?	22-25/10/12	Manchester, UK	○ Scientific community; ○ Higher education / research	UK
54	Oral presentation at a scientific event	B1	Inborn errors of enhanced IFN production and auto-immunity	03-04/11/12	Newport Beach, USA	○ Scientific community; ○ Higher education / research	USA
55	Poster	B8	Elevations of pro-inflammatory cytokines/chemokines in patients with Aicardi-Goutières Syndrome	06-10/11/12	San Francisco, USA	○ Scientific community; ○ Higher education / research	USA
56	Poster	B3	Novel mutation in TREX1 gene associated with Systemic Lupus Erythematosus (SLE)	21-24/11/12	Sorrento, Italy	○ Scientific community; ○ Higher education / research	Italy
57	Poster	B3	NIMBL project: Italian contribution to the genetic analysis of Aicardi-Goutières Syndrome	21-24/11/12	Sorrento, Italy	○ Scientific community; ○ Higher education / research	Italy
58	Oral presentation at a scientific event	B1	ADAR1 mutations in the context of the human type I interferonopathy Aicardi-Goutieres syndrome	06-11/01/13	Galveston, Texas, USA	○ Scientific community; ○ Higher education / research	International - USA
59	Oral presentation at a scientific event	B7	Endogenous retroelements and autoimmune disease	18/02/2013	Minnesota, USA	○ Scientific community; ○ Higher education / research	USA
60	Oral presentation at a scientific event	B5	Chronic exposure of astrocytes to interferon alpha reproduces Aicardi-Goutières syndrome defects and reveals novel molecular changes related to leukodystrophy and microangiopathy	28/02/2013	Amsterdam, Netherlands	○ Scientific community; ○ Higher education / research	The Netherlands
61	Oral presentation at a scientific event	B8	New strategies for the treatment of Aicardi Goutières Syndrome	28/02/2013	Bethesda, MD, USA	○ Scientific community; ○ Higher education / research	USA
62	Poster	B8	CNS reactive autoantibodies in Aicardi Goutières Syndrome: autoimmunity in a genetic disease	16-23/03/13	San Diego, CA, USA	○ Scientific community; ○ Higher education / research	USA

No	Type of activity	Main leader	Title	Date	Place	Type of audience	Countries addressed
63	Oral presentation at a scientific event	B7	Endogenous retroelements and autoimmune disease	28/03/2013	La Jolla, CA, USA	○ Scientific community; ○ Higher education / research	USA
64	Oral presentation at a scientific event	B1	Interferonopathies and Monogenic SLE	17-20/04/13	Rockefeller, NY, USA	○ Scientific community; ○ Higher education / research	International - USA
65	Oral presentation at a scientific event	B5	Chronic exposure of astrocytes to interferon alpha reproduces Aicardi-Goutières syndrome defects and reveals novel molecular changes related to leukodystrophy and microangiopathy	17-20/04/13	Merida, Mexico	○ Scientific community; ○ Higher education / research	International - Mexico
66	Poster	B3	Disregulation of the immune system in Aicardi Goutières Syndrome: another example in TREX1-mutated patient	18-20/04/13	Brescia, Italy	○ Scientific community; ○ Higher education / research	Europe - Italy
67	Oral presentation at a scientific event	B2	New perspectives on migraine genetics	27/04/2013	Porto, Portugal	○ Scientific community; ○ Higher education / research	Portugal
68	Oral presentation at a scientific event	B1	Inborn errors of enhanced IFN production and autoimmunity	14/05/2013	London, UK	○ Scientific community; ○ Higher education / research	UK
69	Oral presentation at a scientific event	B3	Update on Neuroradiological Findings in Aicardi-Goutières Syndrome: Expanding the Phenotype	20-22/05/13	San Diego, CA, USA	○ Scientific community; ○ Higher education / research	International - USA
70	Oral presentation at a scientific event	B9	Macrophages and inflammation	27-28/05/13	Nijmegen, Netherlands	○ Scientific community; ○ Higher education / research	Europe – The Netherlands
71	Oral presentation at a scientific event	B5	AGS and autoimmunity	17/06/2013	Amsterdam, Netherlands	○ Scientific community; ○ Higher education / research	Amsterdam, Netherlands
72	Oral presentation at a scientific event	B2	Genetic Modulation of Spreading depressions	23-26/06/13	Berlin, Germany	○ Scientific community; ○ Higher education / research	International - Germany
73	Poster	B2	Novel transgenic mouse models for monogenic cerebral small vessel diseases related to migraine	23-27/06/13	Boston, MA, USA	○ Scientific community; ○ Higher education / research	International - USA
74	Oral presentation at a scientific event	B7	Negative regulation of cell-intrinsic nucleic acid sensing	29/06/2013	Boston, MA, USA	○ Scientific community; ○ Higher education / research	USA
75	Oral presentation at a scientific event	B1	Screening for a type I interferon signature identifies a frequent cause of 'idiopathic' bilateral striatal necrosis	16-18/09/13	Liverpool, UK	○ Scientific community; ○ Higher education / research	UK
76	Oral presentation at a scientific event	B1	Inborn errors of enhanced IFN production and autoimmunity	19/09/2013	London, UK	○ Scientific community; ○ Higher education / research	UK

No	Type of activity	Main leader	Title	Date	Place	Type of audience	Countries addressed
77	Oral presentation at a scientific event	B1	SLE in monogenic disease	25-29/09/13	Ljubljana, Slovenia	○ Scientific community; ○ Higher education / research	Europe – Slovenia
78	Oral presentation at a scientific event	B1	Interferonopathies: New inborn errors of immunity	04/10/2013	Paris, France	○ Scientific community; ○ Higher education / research	International - France
79	Oral presentation at a scientific event	B2	Migraine – present and future	14-15/10/13	Leiden, The Netherlands	○ Scientific community; ○ Higher education / research	The Netherlands
80	Poster	B1	Aicardi-Gouteire syndrome carrier screening in Ashkenazi Jewish families	22-26/10/13	Boston, MA, USA	○ Scientific community; ○ Higher education / research	International - USA
81	Poster	B8	Accumulation of Endogenous Retroelements in Aicardi Goutieres Syndrome- a potential therapeutic avenue	30/10/2013	Austin, TX, USA	○ Scientific community; ○ Higher education / research	USA

Section B

List of applications for patents, trademarks, registered designs, etc.

No applications were made for patents, trademarks, registered designs, etc.

List of Exploitable Foreground

Purpose:

The purpose of all exploitable foreground (listed in Table B2) is to advance the knowledge of AGS and RVCL.

How the foreground might be exploited, when and by whom:

Over the next one to five years, all the foreground will be exploited through:

- Improved clinical understanding of AGS, RVCL and related disorders, provided to researchers world-wide;
- Improved clinical diagnosis of AGS, provided to clinicians world-wide;
- Improved standardized accredited clinical genetic testing for AGS families
- The development of genetic tests where currently none are available.

IPR exploitable measures taken or intended:

None

Further research necessary, if any:

Further research in this area will be needed for the foreseeable future; there are still many unknowns, including (a) appropriate therapies that could lead to clinical trials and successful treatment; (b) greater understanding of the biological mechanisms of the AGS proteins, and involvement of relevant signalling pathways.

Potential/expected impact (quantify where possible):

The NIMBL project has already benefitted, and the exploitable foreground will continue to benefit:

1. Physicians: by providing guidance on diagnostic criteria;
2. Affected individuals and their families: by allowing for comprehensive diagnostic and carrier testing, and by laying the foundation for future treatments
3. The scientific community: through greater knowledge - in terms of genetic, biochemical and cell biological understanding of the underlying basis of the NIMBL-related phenotypes.

It is impossible to quantify these benefits.

Planned use and dissemination of foreground after the end of the NIMBL project

Personal contact with families, clinicians and other specialist physicians will continue.

Attendance at scientific meetings will also continue; indeed, several researchers attend some specific conferences annually (e.g. Réunion de la Société Européenne de Neurologie Pédiatrique, (SENP), American Academy of Neurology (AAN)) and will continue to do so. The researchers plan to give further oral and/or poster presentations at such future scientific conferences.

In addition, further scientific publications are currently either being drafted, or are at the outline plan stage; there is every expectation that many more NIMBL-related outputs will be published in the next 12 months, in peer-reviewed, high-impact scientific publications, by the NIMBL partners (via either individual or joint publications)

Table B2: Exploitable Foreground

Type of Exploitable Foreground	Description of exploitable foreground	Confidential Click on YES/NO	Foreseen embargo date	Exploitable product(s) or measure(s)	Sector(s) of application	Timetable, commercial or any other use	Patents or other IPR exploitation (licences)	Owner & Other Beneficiary(s) involved
Exploitation of R&D results via standards	Identification of two further genes (*AGS6* and *AGS7*)	NO	n/a	Genetic testing now available where it was not possible before	1. Q86.2.2 - Specialist medical practice activities 2. P85.5 - Other education	New genetic tests expected to be available: 2014-2020.	None	No 'owner'. Involvement of B1
General advancement of knowledge	Generation of the largest sets of clinical, radiological and molecular information on AGS and RVCL ever collected	YES	These datasets will be made available through scientific publication in anonymised / aggregate form	Improved clinical understanding of AGS and RVCL.	1. M72.1 - Research and experimental development on natural sciences and engineering; *2.* Q86.2.2 - Specialist medical practice activities; 3. P85.5 - Other education	Potential use in interpreting the results of future clinical trials: 2015-2030.	None	No 'owner'. Involvement of B1, B2, B3, B8
General advancement of knowledge	Increased knowledge of the pathophysiology of AGS (and potentially other auto-immune disorders);	NO *(after publication)*	n/a	Improved clinical understanding of AGS	1. M72.1 - Research and experimental development on natural sciences and engineering; *2.* Q86.2.2 - Specialist medical practice activities; 3. P85.5 - Other education	Scientific publications: 2014-2020. Education (of physicians): 2014-2020.	None	No 'owner'. Involvement of all beneficiaries
General advancement of knowledge	Increased knowledge of the genetic cause(s).	NO *(after publication)*	n/a	Improved AGS genetic testing	1. M72.1 - Research and experimental development on natural sciences and engineering; *2.* Q86.2.2 - Specialist medical practice activities; 3. P85.5 - Other education	Scientific publications: 2014-2020. Education (of physicians): 2014-2020.	None	No 'owner'. Involvement of all beneficiaries