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A Sub-Type of Familial Pancreatic Cancer: Evidence and Implications of Loss-of-Function Polymorphisms in Indoleamine-2,3-Dioxygenase-2.

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A Sub-Type of Familial Pancreatic Cancer: Evidence and Implications of Loss-of-Function Polymorphisms in Indoleamine-2,3-Dioxygenase-2

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BACKGROUND: Variation in an individual’s genetic status can impact the development of pancreatic ductal adenocarcinoma; however, the majority of familial pancreatic cancers (FPC) cannot yet be attributed to a specific inherited mutation. We present data suggesting a correlation between loss-of-function single nucleotide polymorphisms (SNPs) in an immune regulator gene, indoleamine-2,3-dioxygenase-2 (IDO2), and an increased risk of FPC.

STUDY DESIGN: Germ line DNA from patients who underwent resection for pancreatic ductal adenocarcinoma (n = 79) was sequenced for the IDO2 SNPs R248W and Y359Stop. Genotypes resulting in inactivation of IDO2 (Y325X homozygous, R248W homozygous) were labeled as homozygous, and the other genotypes were grouped as wild-type or heterozygous. Genotype distributions of each SNP were analyzed for Hardy-Weinberg deviation. A genotype frequency set from the 1000 Genomes Project (n = 99) was used as a genetic control for genotype distribution comparisons.

RESULTS: A significant 2-fold increase in the overall prevalence of the Y359Stop homozygous genotype compared with the expected Hardy-Weinberg equilibrium was noted (p < 0.05). Familial pancreatic cancer was noted in 15 cases (19%) and comparison of the FPC cohort set to the genetic control set showed a 3-fold increase in Y359Stop homozygous rates (p = 0.054). Overall in our cohort, the homozygous genotype group was associated with increased risk of FPC (odds ratio 5.4; 95% CI 1.6 to 17.6; p < 0.01). Sex, age at diagnosis, and history of tobacco use were not found to be significantly associated with FPC.

CONCLUSIONS: Our preliminary data suggest a strong association between the IDO2 inactivating Y359Stop SNP and an increased risk of FPC when compared with the control group. Future studies will evaluate the value of IDO2 genotyping as a prognostic, early detection marker for pancreatic ductal adenocarcinoma and a predictive marker for novel immune checkpoint therapies. (J Am Coll Surg 2018;226:596-603. © 2018 The Authors. Published by Elsevier Inc. on behalf of the American College of Surgeons. This is an open access article under the CC BY-NC-ND license [http://creativecommons.org/licenses/by-nc-nd/4.0/])

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Pancreatic ductal adenocarcinoma (PDA) will soon become the second leading cause of cancer-related deaths in the US.1 Although the majority of PDAs are sporadic, roughly 10% are classified as familial pancreatic cancer (FPC).2 Although different definitions of FPC are found in the literature, a common practice is to define the familial form of PDA as instances in which multiple family members (first-degree relatives) are afflicted with PDA.3,4 Thorough genomic and epidemiologic analyses of familial registries have identified FPC cases and have provided the field with important insights,2 yet only a subset of cases can be attributed to inherited mutations; single nucleotide polymorphisms (SNPs); or environmental elements 4,5 (Tables 1 and 2). In fact, only a small portion of FPC cases are related to specific inherited syndromes, such as hereditary breast-ovarian cancer (eg BRCA2), Peutz-Jeghers syndrome, Lynch syndrome (or hereditary nonpolyposis colorectal carcinoma), familial adenomatous polyposis, ataxia-telangiectasia, hereditary pancreatitis, and familial atypical multiple mole melanoma (Table 1).7 The remaining FPC cases have not been attributed to a common driving inherited genetic alteration. It was recently reported that the frequent genetic drivers (ie Kras and TP53) of FPC are virtually identical to the drivers of sporadic PDA.3 Together, these and other studies support the notion that unidentified genetic susceptibility alterations exist, along with interactions with the environment or host (eg the immune system) that cooperate to influence the unusual frequency of PDA found in certain families (ie FPC). Single nucleotide polymorphisms are subtle genetic alterations that are frequently found in the general population (>1%); inherited; and not classified as somatic-tumor mutations. Associations have been made with SNPs and FPC (Table 2); however, these associations cannot completely explain FPC susceptibility found in high-risk individuals, with the best odds ratios not reaching the influence of smoking as a risk factor (eg odds ratio < 2.0).8

We previously carried out expression and genotype analysis of the indoleamine 2,3-dioxygenase-2 (IDO2) gene in sporadic PDA.9 The IDO2 gene is adjacent to and structurally similar to the IDO1 gene on chromosome 8p12.10 Functionally, IDO2 is also similar to its paralog, IDO1, in that it can catabolize tryptophan. Notably, several studies have shown that the IDO system (both the IDO1 and IDO2 genes) functions in restraining the activity of the immune system in its interactions with multiple tumor systems.11 Previous work has postulated that functional IDO2 enzymatic activity represses immune responses in a host, which in turn, facilitates PDA tumorigenesis. In theory, a functional IDO2 enzyme in tumor cells could aid PDA to avoid the immune system. Alternatively, Køllgaard and colleagues12 demonstrated that a functionally intact IDO2 enzyme could be presented to elicit an immune response compared with an inactive IDO2 protein. We previously discovered and described the presence of 2 loss-of-function polymorphisms within the coding region of the IDO2 gene, with a high prevalence in the general population.10 The 2 SNPs are the R248W polymorphism, defined as having a >90% reduction in IDO2 catalytic activity and the Y359STOP polymorphism, generating a premature stop codon, completely inactivating IDO2 activity10 (Fig. 1). Taking into account the importance of chronic inflammation on PDA pathogenesis and based on previous work showing IDO2’s role in immune regulation, we used our vast PDA clinical database (the Jefferson Pancreatic Tumor Registry) and patient population to determine whether the IDO2 genotype had any correlation to FPC susceptibility.

### Table 1. Inherited Syndromes and Associated Lifetime Risk of Pancreatic Ductal Adenocarcinoma

<table>
<thead>
<tr>
<th>Inherited syndrome</th>
<th>Lifetime risk of pancreatic cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hereditary breast and ovarian cancer syndrome, RR5</td>
<td>3.5–5.9</td>
</tr>
<tr>
<td>Peutz-Jeghers syndrome, %</td>
<td>11–36</td>
</tr>
<tr>
<td>Hereditary pancreatitis, %</td>
<td>25–40</td>
</tr>
<tr>
<td>Hereditary nonpolyposis colorectal cancer syndrome (Lynch syndrome), %</td>
<td>3.7</td>
</tr>
<tr>
<td>Familial atypical multiple mole melanoma, %</td>
<td>17</td>
</tr>
<tr>
<td>Familial adenomatous polyposis, %</td>
<td>1.7</td>
</tr>
</tbody>
</table>

*Relative risk (RR) associated with BRCA2 specifically. Data obtained from multiple sources."
MATERIALS AND METHODS

Patient population

The cohort used for this data set included 79 patients (approximately 130 normal and tumor tissue samples) diagnosed with PDA, who underwent primary pancreatic resection at the Thomas Jefferson University Hospital (TJUH) between August 2006 and February 2013. Patients in the study all had available tissue for DNA analysis. Medical history, preoperative laboratory tests, surgical and histologic findings, and oncologic follow-up data were recorded from the patients’ medical records. Cases in which the index patient had at least 1 first-degree relative with a history of PDA were considered FPC, and the rest of the cases were classified as sporadic PDA. We used the Jefferson Pancreatic Tumor Registry as a valuable resource to evaluate whether the indexed patient’s tumor was classified as FPC. The Jefferson Pancreatic Tumor Registry is IRB approved, and participating patients provided appropriate informed consent.

DNA sequencing of IDO2 polymorphisms

Genomic DNA from surgically resected pancreatic tissue specimens (normal and tumor tissues, n = 79 patients) was isolated using the DNAeasy Blood and Tissue Kit (Qiagen Inc). Polymerase chain reactions (PCR) were used to amplify exons containing the IDO2 coding region polymorphisms rs4503083 and rs10109853, based on previously validated primer sets (R248W (rs10109853) Forward Primer 5’-GAA-CATTCTATCCCCGTTGC-3’; R248W (rs10109853) Reverse Primer 5’-TTACCTGAGAGTGGATCCCTAG-CA-3’; Y359Stop (rs4503083) Forward Primer 5’-TCTT-GTGCTCCCTCCAAACA-3’; Y359Stop (rs4503083) Reverse Primer 5’-TGTTTGGCTTCCATGCTT-3’). The PCR reactions were performed in 25 μL reactions using 2 μL DNA, 0.5 U/μL Taq polymerase (USB), 2.5 μL 10× PCR buffer (USB), and 0.5 μL 10 mM dNTP Mix (Invitrogen). Conditions were set for 35 cycles at (95°C for 2 minutes, 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds, followed by an extension of 4 minutes at 72°C). Sequencing reactions

<table>
<thead>
<tr>
<th>Table 2. Inherited Syndromes, Related Genes, and Single Nucleotide Polymorphisms Associated with Familial Pancreatic Cancer</th>
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<tbody>
<tr>
<td>Familial disorder</td>
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<tr>
<td>-------------------</td>
</tr>
<tr>
<td>Hereditary breast and ovarian cancer syndrome</td>
</tr>
<tr>
<td>Peutz-Jeghers syndrome</td>
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<td>Hereditary pancreatitis</td>
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<tr>
<td>Hereditary nonpolyposis colorectal cancer syndrome (Lynch syndrome)</td>
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<tr>
<td>Familial atypical multiple mole melanoma</td>
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<tr>
<td>Familial adenomatous polyposis</td>
</tr>
<tr>
<td>1q32.1 (NR5A2 or LRH-1)</td>
</tr>
<tr>
<td>5p15.33 (CLPTM1/TERT)</td>
</tr>
<tr>
<td>6q25.3 (FOXQ1)</td>
</tr>
<tr>
<td>9p34.2 (ABO)</td>
</tr>
<tr>
<td>12p11 (BICD1)</td>
</tr>
<tr>
<td>13q22.1 (KLF5)</td>
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</tbody>
</table>

APC, adenomatous polyposis coli; ATM, ataxia-telangiectasia; HNPCC, hereditary nonpolyposis colorectal carcinoma. Data obtained from multiple sources.5,6

Figure 1. Representative chromatograms of direct sequencing of patient constitutional genomic DNA showing the 3 possible sequence of R248W polymorphism (left) and Y359STOP (right).
included PCR purified products using DNA purification columns (Qiagen) and the forward primers for each PCR reaction. Each PCR reaction was separated by DNA electrophoretic separation on a 0.75% DNA agarose gel. Sequencing was then performed by capillary electrophoresis in the Sidney Kimmel Cancer Center DNA core facility at Thomas Jefferson University. Genotyping steps were blinded and annotated by number to clinical data and familial-sporadic patient status. The representative sequencing chromatograms were used to identify a wild-type, heterozygous, or homozygous IDO2 genotype (see Fig. 1). Genotypes considered as resulting in inactivation of the IDO2 enzyme (Y325Stop homozygous and R248W homozygous) were categorized as the homozygous group, and the other genotypes were grouped as wild-type or loss-of-function heterozygous. All genotypes provided in this study reflect the germline and were not from microdissected samples enriched with neoplastic cells.

Genetic distribution data from the 1000 Genomes Project. The 1000 Genomes Project is a global effort to map, through sequencing, human genetic variation across the globe (ie a global reference for human genetic variation). In brief, it contains genetic variation data of more than 2,500 subjects from around the world. The data were obtained through a planned sequencing of target populations and, as such, can be divided into specific geographical subsets. The CEU subset (Utah residents with northern and western European ancestry) was selected to serve as a genetic distribution control due to its closeness to the TJUH patient cohort in terms of ethnорacial distribution.

### Statistical analysis

Genotype distributions of each polymorphism were analyzed for Hardy-Weinberg (HW) deviation using chi-square test and Fisher’s exact test. A genotype distribution set of Utah residents with northern and western ancestry available from the 1000 genomes project (CEU, n = 99) was used as a genetic control for distribution comparisons. Distribution comparisons were also performed using chi-square test and Fisher’s exact test. Age, sex, tobacco use, familial history positive for any type of cancer, R248W genotype, Y359Stop genotype, and IDO2 homozygous status were assessed individually for association with FPC. Correlative analysis was performed using Spearman’s test. Factors with p < 0.2 were subsequently included in a multivariate regression model for correlation with FPC. The model was further optimized by sequential exclusion of statistically non-relevant factors (p ≤ 0.2) until achievement of a final optimal model fit (p ≤ 0.05). A p value ≤ 0.05 was considered statistically significant. Statistical analysis was performed using IBM SPSS, version 20 (IBM Corp).

### RESULTS

An increased frequency of the Y359Stop SNP but not the R248W SNP uncovered in FPC patients. Sanger sequencing revealed that 52 cases (66%) of the TJUH cohort had the wild-type Y359/Y359 genotype configuration, 19 (24%) had the Y359/Y359Stop configuration, and 8 (10%) had the completely inactive Y359Stop/Y359Stop configuration (Table 3). The frequency of Y359STOP alleles significantly correlated with increased rates of FPC compared
with sporadic PDAs (Fig 2A, p < 0.05), resulting in increased rates of FPC in Y359Stop heterozygous carriers and even higher rates in Y359Stop homozygous patients (50%). Furthermore, overall comparison of Y359Stop genotype distribution demonstrated a strong trend showing a greater representation of the Y359Stop/Y359Stop
configuration in the FPC subset compared with the CEU normal control group (Fig. 2B, \( p = 0.054 \)).

By way of comparison, Sanger sequencing determined that 25 cases (32%) of the TJUH cohort had a R248/R248 genotype configuration, 39 (49%) had a R248/R248W configuration, and 15 (19%) had the homozygous R248W/R248W configuration. Overall and with stratification to FPC and sporadic PDA, this genotype distribution did not deviate from the HW equilibrium. Allelic distributions among the FPC and CEU control cohorts (33% vs 27% with an R248/R248 genotype configuration, 28% vs 44% with an R248/R248W configuration and 39% vs 29% with a homozygous R248W/R248W configuration (Fig. 2C, Table 3). Fisher’s exact test revealed no significant differences in genotype distribution between the TJUH cohort (or its sub-populations) and the CEU control group. The R248W polymorphism, although having slightly increased FPC rates with the homozygous configuration (39%) compared with the heterozygous and wild-type genotypes (33% and 28%, respectively), did not significantly correlate with FPC (Fig. 2D).

The combined homozygous group (Y359STOP and R248W) was strongly associated with FPC (Fig. 3), with an odds ratio of 5.4 (95% CI 1.6 to 17.6; \( p < 0.01 \)) compared with sporadic PDA cases. Taken together, these data demonstrated that in our cohort the inactive IDO2 genotype (ie the homozygous group) correlated with individuals with FPC compared with the control cohort or patients with sporadic PDA (Fig. 3). A full bivariant distribution table is available (Table 4).

Evaluations of clinical risk factors for FPC were not significant in these cohorts. Age, sex, smoking, and familial cancer history were not associated with FPC. However, due to a large body of work linking smoking to PDA and increased risk for PDA in families with known PDA,15,16 we included smoking in 3 separate regression models (with Y359Stop, R248W, and the combined homozygous grouping). A regression model analyzing the interaction of the combined IDO2 homozygous status with smoking status showed the non-homozygous genotypes combined with non-active smoker status suggested an association with a decreased risk for FPC (nonsmoker/quit \( \geq \)15 years ago: relative risk 0.27; 95% CI 0.6 to 1.1; \( p = 0.07 \); quit <15 years ago: relative risk 0.17; 95% CI 0.2 to 1.5; \( p = 0.11 \)).

DISCUSSION
Pancreatic ductal adenocarcinoma has an overall 5-year survival rate of 9%.17 To date, the molecular drivers (eg BRCA2 and PALB2) of PDA prevalence are only known for a small percentage of high-risk families2 (Tables 1 and 2). In this study, we evaluated the frequency of loss-of-function SNPs in the IDO2 gene in our institutional patient population that contained FPC. Although this was a small patient cohort, we found a high frequency of the inactive, homozygous IDO2 genotype in FPC patients (see Figs. 2 and 3).

These data are compelling, yet this study has a number of limitations. First, due to our limited numbers, we restricted our definition of FPC to cases in which the index patient had at least 1 first-degree relative with a history of PDA. It is possible that our results would be stronger if we included only patients with 2 or more affected family members. Second, increasing our numbers could dilute our positive signal in the FPC cohort. Third, additional molecular correlates from the patients would support our conclusions, including the knowledge of any predisposing genotypes (eg BRCA2 mutations) or immune signatures. Fourth, having more clinical data about the individuals genotyped in this study including their
history of autoimmune disorders or pancreatitis could be informative. Finally, it would be interesting to see if in \textit{IDO2} \((+/-)\) heterozygote germline genotyped patients there was evidence of selection for a loss of the wild-type and/or SNP allele in the tumors of these patients.

In theory, these data appear counterintuitive, as an inactive \textit{IDO2} host genotype has been predicted to produce an overactive immune system that would suppress PDA tumorigenesis.\textsuperscript{9,10} According to this theory, an inactive host \textit{IDO2} system (as indicated by homozygous loss of function SNPs), can enable a heightened, pro-inflammatory host environment that cooperates with \textit{Kras} activation to induce PDA tumorigenesis.\textsuperscript{18} Simply put, an inactive host \textit{IDO2} genotype could contribute to a tumor-promoting, inflammatory environment.\textsuperscript{19} Yet, the data from this study might support an opposing theory of how the host immune (IDO) system can facilitate FPC tumorigenesis. Findings from Kollgaard and colleagues\textsuperscript{12} work led them to postulate that individuals harboring the inactive homozygous Y359STOP host genotype are unable to mount a specific, \textit{IDO2}-directed immune response. The investigators discovered that specific T cells primed against different HLA-A2-restricted peptides derived from the \textit{IDO2} protein were restricted to wild-type or heterozygous \textit{IDO2} genotyped individuals, and no T-cell responses were observed in individuals homozygous for the Y359STOP \textit{IDO2} alleles. Therefore, these data support the notion that to achieve an \textit{IDO2} specific T-cell response, an individual must have a functional \textit{IDO2} enzyme (ie genotyped \textit{IDO2} heterozygous or wild-type). A possible explanation for why the R238W does not elicit a comparable effect is that, although the R238W appears to interfere with substrate accessibility to the active site, the Y359Stop allele eliminates an essential histidine that, from studies of the \textit{IDO1} enzyme, has been shown to be essential for coordinating with the heme iron. Breaking this heme iron-histidine bond results in conformational changes that are thought to be responsible for enhanced proteosomal degradation of \textit{IDO1}. The Y359Stop allele might not only abrogate activity, but could also lead to the elimination of the \textit{IDO2} protein itself, so that any non-enzymatic effects it might have are also eliminated.

In the scenario mentioned, high-risk individuals who are homozygous for the inactive \textit{IDO2} Y359Stop genotype might be unable to mount a proper immune response against PDA cells due to the lack of presentable \textit{IDO2} antigens. The evidence for these 2 opposing hypotheses highlights the dual-edged sword of the \textit{IDO2} system. That is, either dysregulated inflammation and/ or an inactive immune response can facilitate the tumorigenesis process. Ongoing studies in both a mouse model for PDA tumorigenesis\textsuperscript{20,21} and human specimens are being performed to further investigate these countervailing hypotheses.

**CONCLUSIONS**

Other possible implications of this study relate to early detection and predictive biomarker strategies in the PDA field. Future studies will demonstrate whether high-risk individuals in FPC families, with unknown genetic drivers, should be \textit{IDO2} genotyped. In one scenario, these individuals could be identified for immune-suppressing therapies in an effort to modify an overactive host immune system facilitating PDA tumorigenesis. More realistic deliverables of this work are the immediate clinical implications for FPC patients with an inactive \textit{IDO2} genotype. These patients might be refractory to novel \textit{IDO} inhibitor-based therapies, yet they might respond better to other immune checkpoint therapies (eg PD-1/PDL1 inhibitors).\textsuperscript{22} Larger-scale validation studies and future retrospective studies from immunotherapy-based clinical trials will be required to assess the prognostic and predictive value of \textit{IDO2} genotyping.

**Author Contributions**

Study conception and design: Nevler, Muller, Winter, TP Yeo, Lavu, CJ Yeo, Prendergast, Brody Acquisition of data: Nevler, Cozzitorto, Goetz, Brody Analysis and interpretation of data: Nevler, Muller, Winter, TP Yeo, Lavu, CJ Yeo, Prendergast, Brody Drafting of manuscript: Nevler, Muller, Winter, TP Yeo, Lavu, CJ Yeo, Prendergast, Brody, Goetz Critical revision: Nevler, Muller, Winter, TP Yeo, Lavu, CJ Yeo, Prendergast, Brody

**REFERENCES**

Discussion

DR SELWYN VICKERS (Birmingham, AL): I commend Drs Yeo and Brody and your team for creating an outstanding clinical enterprise, and also for continuing to push the boundaries of science and trying to address this difficult disease of pancreatic cancer. As you look at the indoleamine-2,3-dioxygenase-2 (IDO2) molecule, but particularly the Y-359 allele, it is fairly prevalent throughout human species, but it has a unique distribution that is not uniform across the races—approximately 12% in African Americans, and as high as 37% in Asians. Have you taken what the distribution is into account in your familial allele, at least in your familial pancreatic group? How does that compare with your validation cohort, which is from the state of Utah? As you know, this is a very homogeneous population. What impact does that have on the conclusion?

As it relates to a driver, with the prevalence of the allele, is it likely that it is a driver? And if it is, how might you actually prove it even more so in vivo? It may affect not only T cells, but the dendritic cells. And might this also be a selector? In some ways, if you highlight it for precision medicine, might it be a selector for vaccine therapy? Might these patients actually be more qualified for a vaccine trial because the immune system may have a prevalent role in affecting dendritic cells?

As it relates to the broader picture, please give your thoughts on the ability to further stratify patients in the context of either familial disease or, as it relates to our sporadic disease, from using either whole genome or exome sequencing looking for targeting mutations. I think you really are onto something, that the immune system is truly suppressed in major ways with pancreas cancer. Might broader sequencing give you some ideas for targeted or at least actionable targets related to immune checkpoint inhibitors?

DR CHRISTOPHER WOLFGANG (Baltimore, MD): I would like to congratulate Drs Yeo and Brody on a well-done and important study. It is yet another example of high-quality translational science from this surgical group. The details of the study are quite elegant, with sophisticated genetic work and a complex statistical analysis. The authors demonstrated that inactivation of the IDO2 gene is linked to familial pancreatic cancer, and these results suggest that this single nucleotide polymorphism (SNP) may play a mechanistic role through alteration of immune function. The conclusions have the potential to guide patient management in the near future.

I would like to break from the obviously interesting findings of this study to point out the importance of this work in terms of the big picture, something that may be lost in the details of the presentation unless one thinks often about cancer genetics. In past decade, we, as a scientific community, have focused on cancer genomes and the somatic mutations that occur within tumors. For pancreas cancer, this era was kicked off by Dr Bert Vogelstein and the Hopkins group with the publication of the pancreatic cancer genome in the journal Science in 2008. This somatic tumor sequence is important because it serves as the code book for tumor behavior. However, tumor behavior is not realized at the level of the genotype—it is realized at the level of the phenotype. Tumors are a population of cells that initiate and grow under many of the same principles as do an animal species.

In Darwinian evolution, it is the phenotype, not the genotype, which is selected by the environment. The environment in which a species evolves influences the characteristics of the animal—polar bears are big, hairy, and white, all adaptations to their environment. When we study cancer genomes to study the development of cancer, we ignore the environment in which tumor grows. You can only learn so much about the polar bear by studying it in the zoo. Dr Yeo and his