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Clinical Implications of Primary Cilia in Skin Cancer

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ABSTRACT

The primary cilium is a cell surface organelle that is an important component of cellular biology. While it was once believed to be a vestigial structure without biologic function, it is now known to have essential roles in critical cellular signaling pathways such as Hedgehog (HH) and Wnt. The HH and Wnt pathways are involved in pathogenesis of basal cell carcinoma and melanoma, respectively, and this knowledge is now beginning to inform therapeutic and diagnostic options for patients. The purpose of this review is to familiarize clinicians with primary cilia biology and how this complex cellular organelle has started to translate into clinical care.

Keywords: Basal cell carcinoma; Diagnostic tool; Hedgehog; Melanoma; Primary cilia; Sonidegib; Vismodegib

Key Summary Points

The primary cilium is a cell surface organelle that was discovered in the 1800s, but only in the last 20 years has been recognized for its importance in cellular biology.

The primary cilium has critical roles in signaling pathways such as Hedgehog (HH) and Wnt.

Basal cell carcinoma (BCC) depends on HH pathway activation through the primary cilium, which is the basis for the BCC therapies vismodegib and sonidegib.

Immunofluorescence staining of conventional melanocytic nevi, which retain primary cilia, and melanoma, which shows primary cilia loss, provides the basis for a novel diagnostic tool.
INTRODUCTION

The primary cilium is an antenna-like structure that extends from the surface of nearly all cells in the human body [1]. Acting as the cellular “antenna,” primary cilia help to transmit, regulate, and decode extracellular signals [2]. They have distinct membrane receptors that act as the main signaling depots for common molecular signal pathways in cancer, including the Wnt [3–7], platelet-derived growth factor [5], Hedgehog (HH) signaling [6], among others.

Basal cell carcinoma (BCC) depends on HH pathway activation through the primary cilium. This dependence on the HH pathway is the basis for the BCC therapies vismodegib and sonidegib, which target and inhibit signaling through the primary cilium. Recent studies have shown the ability of BCC to phenotype switch to squamous differentiation in part by reducing dependence on HH signaling through the primary cilium [7].

Melanoma and non-melanocytic skin tumors are the most common cancers worldwide, and are projected to increase in incidence and mortality for the foreseeable future [8]. Given the wide range of clinical and histopathological appearances of melanocytic neoplasms, diagnostic consistency and accuracy can be particularly challenging for both dermatologists and dermatopathologists [9, 10]. Though there are a variety of diagnostic tools available to aid in achieving the most accurate assessment of malignancy [11, 12], diagnostic uncertainty can persist in ambiguous lesions. Multiple studies have now demonstrated that primary cilia are lost in melanoma and retained in nevi [13–17]. These studies suggest the potential for using primary cilia staining as an adjunct diagnostic tool for dermatopathologists.

In this review, we will give a brief history of the primary cilium in modern medicine and cancer, discuss the role of HH signaling in BCC, and explore how primary cilia are giving new insights into melanoma.

METHODS

A literature search using the PubMed database was conducted using the terms ("primary cilia") AND ("history" OR "cancer"); ("basal cell carcinoma") AND ("cilia" OR "treatment" OR "resistance" OR "hedgehog signaling"). For the melanoma component, the search terms included ("primary cilia" OR "primary cilium") AND ("melanoma" OR "nevus"). Only articles focusing on histopathologic diagnosis of cutaneous melanocytic neoplasms were included.

The tissue samples used for generating Figs. 1 and 4 were procured from the archives of the University of California San Francisco (UCSF) Department of Dermatopathology, and all the tissues were collected in accordance with the institutional review board with regard to informed consent. Written patient consent was not required because only existing and de-identified specimens were used. All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1964, as revised in 2013.

RESULTS AND DISCUSSION

Primary Cilia Structure

The primary cilium is a microtubule-based organelle that projects from the surface of most vertebrate cells. Under most circumstances, each cell only has one primary cilium (Fig. 1a, b). Unlike motile cilia, such as those found on respiratory epithelial cells and eukaryotic flagella, primary cilia are not involved in motility. Rather, primary cilia are responsible for transmitting cellular signals, and have a specialized structure that facilitates signal transduction [18]. Vertebrate primary cilium project from the surface of the cell, emerging from a single centrosomal centriole known as the basal body, and are surrounded by the cell membrane. Within the membrane is the axoneme, which consists of a ring of nine microtubule doublets, known as a 9+0 configuration. In contrast, motile cilia additionally have two central
microtubule singlets and dynein arms attached to the outer microtubule doublets, altogether known as the 9+2 configuration (Fig. 1b).

The transition zone at the base of the cilium acts as a gate that controls protein entry and exit [19]. Thus, primary cilia serve as a unique microenvironment where specific proteins and lipids can interact in a dynamic fashion to alter cell signaling, function, and fate. Consequently, dysfunctional primary cilium structure or signaling can lead to cancer and congenital conditions collectively known as “ciliopathies” [20]. Thus, understanding primary cilia is crucial to understanding the pathogenesis and clinical manifestations of many diseases.

**History**

Cilia were initially observed in protozoa by Antony van Leeuwenhoek in 1677, and were defined by their motility as the first cellular organelle [21, 22]. Almost two centuries later, Alexander Kovalevsky perceived that many vertebrate cells had a single, immotile cilium [23]. This discovery was soon corroborated by other scientists, including Karl Wilhelm Zimmernann, who named them “centralfiessel” (central flagella) in mammalian cells and suggested that they may have sensory potential [24, 25]. Despite the short burst of scientific interest in centralfiessel in the late nineteenth century, the organelle was largely neglected and dismissed as vestigial because of lack of any obvious function. In 1968, immotile centralfiessel on mammalian cells were renamed primary cilium because their expression could be identified first during fetal development, before the emergence of motile cilia [26].

Interest in primary cilia smoldered to life in 1993 with the seemingly unrelated discovery of intraflagellar transport (IFT) in the unicellular green alga *Chlamydomonas* [27]. IFT is the method by which cells move cargo, such as proteins, along the microtubules within cilia and flagella. Specifically, scientists discovered that loss of the *IFT88* gene in *Chlamydomonas* resulted in loss of flagella [28]. Interestingly, mice that lack *Tg737* (the murine homolog of *IFT88*) have deformed primary cilia in their kidney cells and develop polycystic kidney disease [28]. This nascent connection between cilia and disease renewed scientific interest in the primary cilium and sparked an explosion of primary cillum investigation that has persisted to the present day. A more detailed timeline and summary of significant advances in the cilia...
literature can be found in Fig. 2 and Table 1, which are based on information from a review article by Bloodgood [29].

Since the connection to polycystic kidney disease, an entire class of disorders, known as “ciliopathies” and spanning almost every organ system, have been linked to dysfunction in the primary cilium [30]. Ciliopathies are inherited genetic disorders that are associated with mutations in genes that are important for the function of primary cilia. Outside of an inherited context, dysfunction of primary cilia has also been implicated in sporadic cancers [31, 32]. The mechanisms underlying both ciliopathies and the primary cilium’s role in cancer have been linked to more recent discoveries implicating the primary cilium as a central hub for many gene expression programs that are crucial for development and adult stem cell homeostasis, such as the HH [6], Wnt [33], and Notch [34] pathways.

**BCC and HH Signaling**

BCC affects approximately two million people per year and is the most common cancer in the USA [35]. BCC typically arises in adult patients as a result of ultraviolet radiation-induced genomic mutations. Indeed, BCCs are the most mutated human cancer, with about 65 mutations per megabase [36]. Although one might expect these numerous mutations to be random, BCCs are unified by misactivation of the HH pathway in both sporadic and syndromic cases. With respect to the latter, individuals with inherited heterozygous mutations in HH pathway inhibitor genes develop nevoid BCC syndrome (also known as Gorlin syndrome), which is characterized by numerous BCCs and other HH-associated cancers such as medulloblastoma and rhabdomyosarcoma [37–41]. Primary cilia play a crucial part in the pathogenesis of BCC because of the primary cilium’s essential role in the HH signaling pathway.

The HH pathway is activated by HH ligands, which are secreted lipoproteins [42]. The best studied HH ligand gene encodes Sonic hedge-hog (SHH), a crucial regulator of embryonic development and adult tissue homeostasis [43, 44]. HH ligands stimulate the HH pathway, often in nearby cells, by binding to their transmembrane receptors, patched 1 (PTCH1) and patched 2 (PTCH2), which are present at the ciliary membrane [45–47] (Fig. 3). HH signaling
| Author(s) (reference) | Year | Discovery/contribution/advancement |
|-----------------------|------|-----------------------------------|
| von Leeuwenhoek [21]  | 1677 | First described most likely ciliate protozoa, “with thin little feet, or little legs, which moved very nimbly…” |
| Muller [94]           | 1786 | Introduced the term “cilium,” meaning hair or eyelash, for cells with multiple cilia |
| Purkinje and Valentin [95] | 1834 | First described ciliary motility in mammalian cells |
| Dujardin [96]         | 1841 | Introduced the term “flagellum,” meaning whip, for cells with a single cilium |
| Ecker [97]            | 1844 | Noted single cilium on the epithelium of the semicircular ear canals in sea lamprey |
| Kolliker [98]         | 1854 | Noted that cilia on epithelium can occur “singly on a cell” |
| Kowalevsky [23]       | 1867 | Noted single cilium on cells during gastrulation of Amphioxus |
| Flemming and van Beneden [99, 100] | 1875/76 | Discovered the centrosome (aka centriole, basal body) |
| Langerhans [24]       | 1876 | Described numerous examples of single cilium on epithelium in Amphioxus and that these cilia were not associated with motility |
| Zimmermann [25]       | 1898 | Discovered that central flagella (primary cilia) are defined, and distinguished from cilia, by a pair of centrioles. In addition, he predicted that primary cilia may act as a “sensory organ” for these cells |
| Henneguy and Lenhossek [101–103] | 1898 | Proposed the Henneguy–Lenhossek hypothesis that centrosomes are basal bodies, which play a key role in the formation of primary cilia |
| Alverdes [104]        | 1927 | Proposed that primary cilium “may represent a cellular receptor, which communicates fluctuations…” |
| Sjostrand [105]       | 1953 | First transmission electron micrograph (TEM) of a mature photoreceptor connecting epithelium (a sensory cilium) |
| de Harven and Bernhard [106] | 1956 | First TEM of a primary cilium |
| de Harven and Bernhard [107] | 1960 | First to describe the role of microtubules in primary cilium |
| Barnes [108]          | 1961 | Proposed requirements for definition of primary cilia: (1) Lack of motility, (2) 9 + 0 microtubule arrangement, and (3) association with pair of centrioles |
| Wilson and McWhorter [109] | 1963 | Proposed that primary cilium on epidermal cell “might conceivably play a part in the initiation of mitosis” |
| Sorokin [26]          | 1968 | Proposed the modern term “primary cilia” and described the difference in formation of primary cilia as distinct from motile cilia |
| Archer and Wheatley [110] | 1971 | First to demonstrate experimentally that primary cilia completely disappear during mitosis |
| Afzelius [111]        | 1976 | Discovered the connection between Kartagener’s syndrome and motile cilium with a defect in the dynein arms |
| Kozminski [27]        | 1993 | Discovered intraflagellar transport in Chlamydomonas |
is transduced by the primary cilium. When HH binds to PTCH proteins, another transmembrane protein, Smoothened (SMO), accumulates at the cilium to activate the downstream pathway [48, 49] (Fig. 3). SMO activates GLI2, the primary activator of the HH transcriptional program, which in turn regulates GLI1, a feedforward amplifier of transcriptional activity [50, 51]. When the HH pathway is inactive, suppressor of fused (SUFU) binds to GLI family members and represses their transcriptional activity [52–54] (Fig. 3).

Primary cilia and the HH pathway play crucial roles in normal skin development and growth, thus their dysregulation in BCC is perhaps not surprising. In the developing skin, SHH secreted by epidermal placodes induces proliferation and hair follicle formation in the underlying dermal condensates [55]. Without SHH, epidermal placodes form but fail to grow down into the dermis [56]. Similarly, in postnatal skin, hair follicle growth also relies on signaling through the HH pathway. For example, SHH is expressed during anagen, the hair follicle phase during which there is downward growth of the follicle into the dermis, and is important for maintaining epidermal stem cells and regulating epidermal growth [57–59].

The most common HH pathway-activating mutations in BCC include biallelic loss-of-function mutations in pathway inhibitors (e.g., PTCH1, PTCH2, and SUFU); amplification of GLI2, the principal HH pathway transcriptional activator; and monoallelic activating mutations in pathway activators, such as SMO [36]. BCC cells possess cilia (Fig. 1a), which transduce HH signals from SMO to GLI2 [60, 61]. In fact, in mouse models of BCC, scientists have shown that both the ciliary gene Intu and HH signaling are necessary for BCC tumorigenesis and progression [61, 62]. Thus, the primary cilium, through HH signaling, acts a crucial nexus in the pathogenesis of BCC.

**BCC Therapy**

BCCs are typically slow-growing and are most often effectively treated with local excision. However, many factors can prevent complete excision, such as number or size of tumors, or proximity to critical structures, including the eye, lip, and nose. In these cases, nonsurgical local treatments, such as topical cytotoxic agents, radiotherapy, photodynamic therapy, and cryotherapy, can be used [63]. In the small subset of patients with locally advanced or metastatic BCC, systemic therapy is indicated. For such cases, HH pathway inhibition with SMO antagonists, such as vismodegib or sonidegib, has been shown to be more effective than chemotherapy [64–66]. Although the proportion of BCC patients who are eligible for molecular therapy is small, the tremendous incidence of BCC cases each year makes the absolute number of patients who may be considered for vismodegib or sonidegib large. Unfortunately, systemic inhibition of the HH pathway can lead to adverse events, such as nausea, muscle cramps, loss of taste, weight loss, and alopecia [67]. Although relatively mild, these symptoms can cause patients to not adhere to treatment regimens, which may lead

| Author(s) (reference) | Year | Discovery/contribution/advancement |
|-----------------------|------|-----------------------------------|
| Pazour [28]           | 2000 | Demonstrated that the ORPK (Oak River polycystic kidney disease) mouse had an underlying primary cilia defect |
| Lehman [112]          | 2009 | Described role of primary cilium in hair follicles |
| Wong [60]             | 2009 | Described role of primary cilium in BCC |
| Ezratty [113]         | 2011 | Described role of primary cilium in skin development |
| Kim [14]              | 2011 | Described role of primary cilium in melanoma |
to BCC recurrence. Thus, the combination of radiotherapy with HH pathway inhibition may be used to achieve durable responses with cessation of systemic therapy for such patients [68].

In addition to recurrence due to lack of adherence, resistance to vismodegib and sonidegib has also been documented, typically via mutations in SMO, the target of both inhibitors [69, 70]. A frequent activating mutation in SMO is W535L, also known as SMOM2, which causes SMO to accumulate in the cilium even in the absence of HH ligands [71, 72]. In medulloblastoma, another HH-driven cancer where HH pathway inhibitors are used, there are examples of resistance that arise from amplification of targets downstream of SMO, such as GLI2 or cyclin D1 [73, 74]. Outside of alternative methods of HH pathway activation, rare examples of BCC resistance have been seen via loss of ciliation, loss of HH signaling, and subsequent activation of alternative signaling pathways, such as the Ras/MAPK pathway [7]. Overcoming resistance to SMO antagonists in BCC is an active area of research, with some efforts focused on targeting downstream

Fig. 3 Hedgehog pathway activation through the primary cilium. When the Hedgehog pathway is off, patched (PTCH1) localizes to the primary cilium and prevents Smoothened (SMO) from entering the cilium. Simultaneously, suppressor of fused homolog (SUFU) binds to and inhibits the activity of glioma-associated oncogene homolog (GLI) transcription factors. Hedgehog ligands (HH) activate the pathway by binding to PTCH1, causing it to leave the cilium, and allowing SMO to enter. SMO in turn activates GLI proteins, allowing for the transcription of Hedgehog target genes.
elements of the HH pathway. HH pathway-independent treatment options, such as cancer immunotherapy, have also been proposed for resistant tumors. Given BCC’s high mutational burden and the correlation between mutational burden and the success of immunotherapy, clinical trials with anti-PD1 therapy have been initiated (NCT03132636, NCT03521830).

Melanoma Pathogenesis

There are diverse genetic changes and transcriptional programs that contribute to melanoma pathogenesis. Prominent activating mutations in key oncogenic driver genes, such as BRAF or NRAS, are present early in melanocytic nevi, but the cellular programs defining proliferative and invasive phenotypes are not defined by particular genetic changes, rather are governed by transcriptional master regulators [75–78]. It is the culmination of these complex changes that translates to biologic function, which is further refined by an individual’s immune system and microenvironment [79]. A detailed review of the genetic and phenotypic changes occurring in melanomagenesis is outside the scope of this review, but can be found elsewhere [75, 76]. Briefly, the initiating mutations result in uncontrolled proliferation of a melanocyte, followed by approximately 5–10 additional pathogenic alterations spread over several signaling pathways to result in melanoma [80]. It is also generally accepted that there are “intermediate” cell states between the “benign” and “malignant” categories of melanocytic neoplasms [79]. Although different combinations of mutations in a variety of genes underlie the diversity of melanocytic neoplasms, they all share the common denominator of activating the mitogen-activated protein kinase (MAPK) pathway. Beyond DNA mutations, deletions and amplifications, DNA methylation [81], microRNAs [82], transcription [83], and translation [84] also play important roles in determining the malignant potential of a particular melanocytic neoplasm [85]. Finally, the tumor’s microenvironment and interaction with an individual’s immune system also contribute to the clinical course of melanoma [86]. Importantly, regardless of the variability of the genetic landscape, there is downstream convergence on a malignant phenotype.

Primary Cilia in Melanocytic Neoplasms

The presence of primary cilia on the cell surface of lesional melanocytes within conventional melanocytic nevi was first reported in 2011 by Kim et al. [14]. This was in contrast to the near-complete loss of primary cilia on in situ, invasive, and metastatic melanoma. At that time, it was also investigated whether the observed loss of primary cilia might be due to ongoing cell cycle progression, as primary cilia reabsorption is a known physiologic response during the interphase portion of the cell cycle [2]. In their study, staining for Ki-67, a protein present during the cell cycle and absent during G0, was performed and found to vastly under-represent the percentage of non-ciliated cells. This was the initial evidence that active cell cycle progression was unlikely to account for the almost complete loss of primary cilia observed in melanoma.

An independent group, Snedecor et al., validated the original findings by evaluating a cohort of melanocytic nevi compared to in situ, invasive, and metastatic melanoma [15]. Interestingly, although still significantly higher than their melanoma cohort, the average percentage of ciliated melanocytes within the nevus cohort was 25%. This is notably lower than that of the Kim et al. study demonstrating an average of 94% ciliated melanocytes in nevi. A direct comparison of these results may be limited by variations in immunofluorescence staining methods used as well as possible variability in the types of melanocytic nevi included. However, Snedecor et al. additionally contributed to the hypothesis that cilia loss in melanoma was unlikely to be solely due to an elevated proliferation rate.

The underlying mechanism of primary cilia loss in melanoma remained a mystery until a recent study examining the role of “enhancer of zeste homolog 2” gene (EZH2) overexpression/amplification in melanoma [87]. Zingg et al. uncovered that many cilia-associated genes
were transcriptionally repressed by EZH2, which functions as the histone methyltransferase unit of polycomb repressive complex 2 (PRC2) [88]. PRC2 is a complex that methylates histone H3 to promote transcriptional silencing. As a global regulator of gene transcription, amplification or gain-of-function of EZH2 has significant downstream effects that have been associated with the transition to a melanoma state [79]. This study also experimentally demonstrated activation of the beta-catenin pathway as a consequence of primary cilia knockdown. These data provide insights into the intricacies of potentially targeting the Wnt/beta-catenin signaling pathway in melanoma therapy. Given the correlation of beta-catenin pathway activation and melanoma immune evasion [89], primary cilia-related proteins may also provide novel candidate targets for patients resistant to immunotherapies.

The function of primary cilia in the context of melanocyte biology and melanomagenesis is still in early phases of investigation; however, it appears to have an active role in suppressing the oncogenic activity of the Wnt/beta-catenin pathway. Importantly, linking the loss of primary cilia to upstream global effectors of melanomagenesis brings us closer to appreciating the potential value of using primary cilia staining as a diagnostic tool in ambiguous melanocytic neoplasms.

**Melanoma Molecular Diagnostic Tools**

The complexity and diversity of biologic checkpoints involved in the development of melanoma reflects the challenges that both clinicians and pathologists can face in making an accurate diagnosis. In particular, the “intermediate” lesions that do not meet histopathologic criteria for melanoma but have atypical features raise the question of their true biologic potential. Many of the most well-established adjunct diagnostic tools available to dermatopathologists for the diagnosis of ambiguous melanocytic neoplasms involve either genetic/genomic analysis or single protein detection by immunohistochemistry [13, 14]. There is now a greater appreciation that a purely genetic analysis may miss critical post-transcriptional and post-translational changes. Techniques such as mass spectrometry [90] and micro-RNA profiling [82] are emerging to address this gap. Each of these tests provides additional information that the pathologist can then use to integrate with the histopathology to reach their best estimate of malignant potential. The decision to initiate additional molecular analysis beyond routine histopathologic assessment largely rests on the ease of performing the test, the availability of sufficient tissue, and expertise for interpretation.

Performing primary cilia staining is no more technically challenging than standard immunohistochemical staining for single proteins, which is currently the mainstay of additional workup for histopathologically challenging melanocytic neoplasms. Immunofluorescence staining for primary cilia requires a single unstained standard thickness tissue section from a paraffin-embedded formalin-fixed tissue block (detailed methods found in Kim et al. [14]), and can be easily incorporated into a routine diagnostic workup. Three primary antibodies are combined in a single cocktail for the identification of melanocytes (SOX-10), the ciliary axoneme (acetylated alpha-tubulin), and centrioles (gamma-tubulin). The corresponding three secondary antibodies are conjugated to fluorophores with distinct excitation and emission wavelengths, which allows for concurrent identification of all three components using an immunofluorescence microscope attached to a camera. An example of this immunofluorescence stain is shown in Fig. 4, which highlights primary cilia staining in a melanocytic nevus and cilia depletion in melanoma.

As this cell surface structure is clearly absent in melanoma when compared to conventional melanocytic nevi, it has the potential for being used in a similar manner as immunohistochemical stains for p16 or PRAME are currently being used [79, 91]. The most common acquired genetic change distinguishing precursor lesions, such as melanocytic nevi or melanoma in situ (MIS), from invasive melanomas is loss of the CDKN2A locus, which is considered an important driver of melanoma [92]. Loss of
Fig. 4 Primary cilia immunofluorescence staining in melanocytic nevus and melanoma. Immunofluorescence staining shows melanocyte nuclei highlighted blue (SOX-10), ciliary axoneme shown in green (acetylated alpha-tubulin), and centrioles in red (gamma-tubulin). For the purpose of quantification for calculation of the ciliation index (% ciliated lesional melanocytes), a cell is considered positive if it contains an elongated ciliary axoneme extending from a centriole and negative if only centrioles are identified. Only melanocytes with identifiable centrioles are counted in at least three high-power fields, with an overall minimum of 150 melanocytes. An example of the immunofluorescence and corresponding hematoxylin and eosin (H&E) stains are shown for a melanocytic nevus (a) and invasive melanoma (b) (scale bar 50 μm).
immunohistochemical staining for the p16 protein can act as a surrogate of the underlying genetic event; however, negative staining for p16 does not always correlate with an underlying mutation being present and conflicting data argues against its use [93]. Conversely, preserved p16 staining does not exclude the possibility of melanoma, and in fact approximately 25% of metastatic melanoma can retain this tumor suppressor gene (TCGA Research Network). With respect to PRAME immunohistochemical staining, there has been rapid adaptation of this stain for clinical use, but as with any single protein, the results must be interpreted with caution in the context of all clinical and histopathological findings. Overall, the cumulative literature results support the need for additional biomarkers, such as primary cilia staining, to help in cases when distinguishing benign from malignant by current immunohistochemical staining practices is insufficient.

CONCLUSIONS

Basic science research in the field of primary cilia biology continues to have implications for translational research and ultimately advances in patient care; therefore, clinicians will need to have a basic understanding of this cell surface organelle. The importance of this organelle is a relatively new discovery, but ongoing research is demonstrating how it relates to cellular function in a context-dependent way.

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Data Availability. Data sharing is not applicable to this article as no datasets were generated or analyzed during the current study.

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