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# Gene markers of normal villous maturation and their expression in placentas with maturational pathology

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ABSTRACT

*Introduction:* The placenta demonstrates a recognized sequence of histomorphologic maturation throughout pregnancy, and in some cases, shows abnormally advanced (AVM) or delayed (DVM) villous maturation. While AVM and DVM have important clinical implications, it is unknown whether they truly represent a state of accelerated/delayed normal maturation or a state of pathological maldevelopment. The purpose of our study is, therefore, to address this challenge via a genome-wide search for expression markers of normal villous maturation (NM) and the assessment of these genes in cases of maturational pathology.

*Methods:* A total of 142 placentas, previously evaluated by gene expression microarray, were reviewed histologically and classified as NM, AVM, or DVM. Expression data from healthy NM placentas underwent Pearson correlations with gestational age (GA) and network/pathway analysis to identify candidate gene markers. Candidates were then validated in an independent microarray dataset and used to calculate "molecular GAs" of placentas with maturational pathology.

*Results:* Analysis of NM placentas yielded 17 candidate markers of normal villous maturation, of which 11 were independently validated. Genes with expression increasing across gestation were associated with transcription and metabolism, while those demonstrating decreasing expression were involved in cell cycle and division. Molecular GA was 5.3 weeks older than true GA among AVM placentas ($p < 0.001$), and 1.1 weeks younger among DVM placentas ($p = 0.149$).

*Discussion:* We have found evidence of advanced molecular GA in AVM placentas, while molecular alterations in DVM placentas were merely suggestive of delayed maturation. In the future, these findings will need to be validated with additional techniques such as in situ hybridization or immunohistochemistry.



## 1. Introduction

The placenta undergoes a recognized sequence of anatomical and functional changes over the course of its maturation during pregnancy [1]. These physiologic changes are integral to maintaining appropriate placental function as pregnancy progresses [2] and are reflected by gross and histological changes that are routinely assessed during clinical evaluation of the delivered placenta by pathologists [3,4]. When the placenta does not show appropriate developmental changes for a given gestational age

*Abbreviations:* GA, gestational age; AVM, advanced villous maturation; DVM, delayed villous maturation; NM, normal maturation; PE, preeclampsia; WGCNA, weighted gene correlation network analysis; PVCA, principal variance component analysis.

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(GA), there are significant consequences, including increased risk of fetal and maternal morbidity and mortality [5,6].

For decades, researchers and placental pathologists have employed the concepts of "advanced villous maturation" (AVM) and "delayed villous maturation" (DVM). These describe pathological states of the placenta (Table 1) [7–11] where the maturation of placental villi appears to be either advanced or delayed, respectively, relative to what would be expected in a healthy pregnancy of equivalent gestational age [12,13]. AVM is also known as villous hypermaturity and is closely related to overlapping pathologies such as distal villous hypoplasia, Tenny-Parker changes, and maternal vascular malperfusion. DVM is synonymous with villous immaturity, dysmaturity, or maturation defect. These two diagnoses are essential to the clinical evaluation of placental pathology and show strong correlations to clinical phenotypes in both the mother and the infant (Table 1).

However, although AVM and DVM are routine diagnoses representing the current standard of practice [7], it is an open question as to whether these pathologic states actually exhibit advanced or delayed maturation, respectively [4]. This question is fundamental, and yet, to our knowledge, it remains largely unaddressed. Any progress in this area promises to inform our understanding of these disease processes and whether they represent 1) acceleration or delay of normal maturation, 2) a pathologic adaptation to adverse conditions resembling such changes, or 3) a different process altogether. This understanding will dictate whether we should continue to pursue diagnosis and investigation in this area under the paradigm of alterations in the rate of maturation, with poor outcomes ultimately related to desynchronization of fetal and placental development.

In the present study, we begin to address this problem by 1) developing and validating a gene expression-based molecular signature for normal villous maturation among placentas from healthy pregnancies, and 2) applying this signature to assess the "molecular GA" of placentas with maturational pathologies.

## 2. Methods

### 2.1. Study subjects and microarray analysis

Subjects included in this study were selected from a previously assembled cohort of women with preeclamptic (PE; N = 80) and non-PE pregnancies (N = 77) [14] obtained from the Research Centre for Women's and Infants' Health BioBank (Mount Sinai Hospital, Toronto, Canada). At the time of sample collection and purchase, PE was defined as the onset of systolic pressure ≥140 mmHg and/or diastolic pressure ≥90 mmHg after the 20th week of gestation, accompanied by proteinuria (greater than 300 mg protein/day or greater ≥ 2+ by dipstick) [15]. Patients with diabetes (pre-existing or gestational), sickle cell anemia, and/or morbid obesity (BMI ≥ 40) were excluded, and all samples came from singleton pregnancies. Placental sampling was performed by the BioBank within 30–60 min of delivery, during which quadruplicate tissue biopsies were collected per placenta, pooled, snap-frozen, and crushed into a powder. mRNA was extracted from the snap-frozen placental tissue, gene expression was assessed through microarray (Human Gene 1.0 ST Array Chips, Affymetrix), and the results were deposited in the Gene Expression Omnibus (GEO) under the accession number GSE75010. Additional patient selection and tissue sampling details have been previously published [14].

### 2.2. Histological diagnosis of normal, advanced, and delayed villous maturation

Of the 157 possible samples in our cohort, 142 had placental tissue sections (4 per case) available for histologic assessment. Tissue was prepared for histology as previously described [14]. High-resolution digital images of each section were examined by an experienced perinatal pathologist, blinded to microarray results and clinical data other than gestational age at delivery (determined predominately by ultrasound dating). The presence or absence of maturational histopathology for each placenta was based on established diagnostic criteria (Table 1). Delayed villous maturation was diagnosed at any gestational age when a uniform population of immature villi (villi having substantially more stroma, more centralized vessels, and fewer and less well-formed vasculosyncytial membranes) was identified that occupied at least one-third of the sampled villi within a histologic section. Placentas were then classified as having normal maturation (NM) (N = 50), AVM (N = 73), or DVM (N = 17) (Supplementary Fig. 1). An additional two placentas were diagnosed with mixed maturational pathology (both AVM and DVM). Available corresponding clinical data were compared between the maturational groups using Kruskal-Wallis and Fisher's exact tests, as appropriate.

### 2.3. Linear models of gestational age and gene expression

To identify candidate genes with expression patterns correlating with normal villous maturation, the 35 NM samples that did not have a clinical diagnosis of PE were randomly split into five equally-sized groups, with four groups (28 samples) pooled to comprise the training set and one group (7 samples) withheld for cross-validation. Rotation of these groups for five-way cross-validation generated five linear models. Each model was built as follows (Supplementary Fig. 2). First, data for the training and cross-validation samples were loaded into R 3.1.3 separately from their raw. CEL files, normalized, and converted into log2 values using the *rma* function. Probes with intensity values in the bottom quartile were discarded to minimize issues with background noise, and probes annotated to the same gene were merged to a mean value. Second, using only the training samples, a Pearson correlation

**Table 1**
Placental features and clinical conditions associated with advanced villous maturity (AVM) and delayed villous maturity (DVM).

| | AVM | DVM |
|---|---|---|
| Placental Features | - Small or short villi (relative to gestational age)<br>- Increased syncytial knots<br>- Villi appear hypermature [7] | - Immature villi making up at least one third of the sampled villi, defined as having substantially 1) more stroma, 2) more centralized vessels, and 3) fewer and less well-formed vasculosyncytial membranes<br>- Recapitulates the histology of early pregnancy [7] |
| Fetal Conditions | - Growth restriction<br>- Intrauterine death<br>- Brain injury [8] | - Perinatal death [9,10]<br>- Recurrent stillbirth after 35 weeks gestation [10] |
| Maternal Conditions | - Recurrence in future pregnancies [8]<br>- Hypertension, preeclampsia, diabetes with arteriopathy, autoimmune conditions [11] | - Gestational diabetes [9]<br>- Pre-gestational diabetes [9] |