Noninvasive Screening of a Fecal Biomarker for Human Necrotizing Enterocolitis (NEC)

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SANTA CLARA UNIVERSITY

Department of Bioengineering

I HEREBY RECOMMEND THAT THE THESIS PREPARED
UNDER MY SUPERVISION BY

Daniela Campa, and Kyle Sullivan

ENTITLED

Non-invasive Screening of a Fecal Biomarker for Human
Necrotizing Enterocolitis (NEC)

BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF

BACHELOR OF SCIENCE
IN
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NONINVASIVE SCREENING FOR A FECAL BIOMARKER FOR HUMAN NECROTIZING ENTEROCOLITIS (NEC)

By

Daniela Campa and Kyle Sullivan

SENIOR DESIGN PROJECT REPORT

Submitted to
the Department of Bioengineering

of

SANTA CLARA UNIVERSITY

in Partial Fulfillment of the Requirements
for the degree of
Bachelor of Science in Bioengineering

Santa Clara, California

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ABSTRACT

Necrotizing enterocolitis (NEC) is a disease that predominantly affects preterm infants. Of the 10% of preterm neonates that develop NEC, about one in four cases results in death. The pathophysiology presents as inflammation of intestinal epithelial cell lining and subsequent tissue death, sometimes resulting in intestinal perforations. The underlying mechanisms have yet to be comprehensively identified. Diagnosis—the rate limiting step in reducing critical disease-onset-to-treatment time—is based on relatively nonspecific signs, impeding early and accurate diagnosis. This project, a collaborative effort between Santa Clara and Stanford Universities, aims to improve current diagnostic methods by identifying the first verifiable and useful fecal biomarker for NEC. The primary molecule of interest is referenced as “Candidate-X” to protect intellectual property. Stool samples from healthy and NEC-affected infants at Stanford’s hospital, taken before and after treatment and analyzed at UC Davis, showed a strong correlation between elevated Candidate-X levels and NEC. The effects of Candidate-X and several other molecules on cell morphology were first studied in well plates seeded with either human intestinal epithelial cells (HIEC-6) or human colon cancer cells (HT-29). After HT-29 was verified as a biologically relevant cell line, two bacteria-related factors of interest, butyrate and Candidate-X, were plated with HT-29 for further study. In light of recent publications and unpublished data, Candidate-X was ultimately selected. Key results show that Candidate-X causes a comparatively high level of cell death and its effects are cell-correlated, suggesting a causative relationship between Candidate-X and NEC. Morphological changes with Candidate-X treatment reveal an unidentified cell death pathway, which will be the topic of future exploration. This paper proposes that using Candidate-X as a noninvasive fecal biomarker in combination with current methods and predictive models may improve speed and accuracy of NEC diagnosis, and outlines potential future directions for research.

Keywords: Necrotizing Enterocolitis, NEC, Fecal Biomarker, Intestine, Preterm Infants, Premature Infants, HT-29, HIEC-6, Morphology, Gut Dysbiosis, Fermentation Products
ACKNOWLEDGEMENTS

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Chapter 1: Introduction

1.1 Background

Human necrotizing enterocolitis (NEC) is an intestinal disease that predominantly affects preterm neonates [1, 2, 3], or infants who have had a gestation period of less than 37 weeks [4]. About 10% of infants are born premature; NEC affects approximately one in ten of these infants [1, 2]. Pathophysiology presents with inflammation of intestinal tissue upon feeding-induced bacterial colonization of the gut, which can lead to tissue death, holes in the intestinal wall, and in about one in four cases, fatality [2, 3]. While the precise and comprehensive etiology remains undefined, the working theory for susceptibility is that it stems from a combination of genetic factors and the premature intestinal environment, which may exist in a state of immaturity, hyper-reactivity/sensitivity, and microbial imbalance [2].

Currently, NEC diagnosis in the early stages is based on relatively nonspecific clinical and radiographic signs, some of which mimic sepsis [2, 6]. Infants are unable to communicate symptoms such as pain levels and discomfort, so physicians are missing important information that could help more efficiently diagnose the disease. This also makes studying the disease in humans more difficult, compounding factors such as a premature infant’s fragility and size [6]. Treatment options for NEC are nonspecific as well [2]. The current standard of care includes ceasing feeding; administering intravenous fluids, broad-spectrum antibiotics, and additional oxygen or assisted breathing; and in severe (later-stage) cases, performing surgery [2, 3, 6, 7]. For cases requiring surgery, fatality rates for NEC rise to 50% [2, 7]; if the infant survives the surgery, he/she will experience long-term health impacts [2, 3, 8]. This sobering statistic emphasizes the importance of early diagnosis and treatment to a NEC infant’s survival and future quality of life.

Improving current diagnostic methods and understanding of the disease would have a significant impact on modern medicine’s ability to fight NEC because diagnosis is the rate limiting step in reducing disease-onset-to-treatment time and understanding the disease is vital to discovering better treatments. Through in vitro and in vivo (animal model or noninvasive human) disease studies, researchers may accomplish this in the near future and pave the way for development of more effective, NEC-specific treatment [5].

1.2 Review of the Field/Literature Search

An article by Neu and Walker published in the New England Journal of Medicine addresses that current diagnostic methods need improvement [7]. Neu and Walker estimate NEC costs the
United States healthcare system $500 million to $1 billion annually [7]. In agreement with other sources [2, 3, 6], they state that the most common interventions for NEC are “abdominal decompression, bowel rest, broad-spectrum intravenous antibiotics, and intravenous hyperalimentation,” along with prebiotic and probiotic treatments [7]. Surgery is required for patients who have intestinal perforations or other factors that render the disease stage severe [7]. Surgeries usually involve abdominal drainage, exploratory laparotomy that results in a resection of the diseased bowel, and the creation of a stoma from an enterostomy [7]. Preterm infants with NEC will on average be hospitalized 20 days longer than healthy preterm infants if no surgery is required, and 60 days longer than healthy preterm infants if surgery is required [7]. Because of the difficulties associated with diagnosing NEC, Neu and Walker note that a significant portion of cases require surgery due to prolonged disease progression [7]. Spencer et al. states that one of the most common severe long-term effects of NEC is short-bowel syndrome in pediatric patients, on average costing an estimated $1.5 million over a 5-year period [8]. These sources highlight the necessity of catching the disease early, as well as the significant cost (monetary, physical, and psychological) that current standards of care present patients’ families, hospitals, and insurance companies.

Sylvester et. al. offer an interesting supplement to traditional clinical diagnostics: predictive models to assess risk and provide physicians with additional context for diagnosis [9]. The Sylvester Lab previously conducted a retrospective analysis on 94,110 preterm births over a 3-year period with a validation cohort of 22,992 preterm births over a 1-year period to develop their predictive model [9]. They were able to identify approximately 90% of NEC cases using the model, demonstrating its efficacy [9]. Sylvester et al., and later, Sinclair et. al., both noted an association between prematurity of infants, the development of NEC, and abnormal fatty acid metabolism [5, 9]. Sylvester et. al. hypothesize that metabolic profiling may serve as a way to objectively screen preterm infants for having or being at risk for developing NEC [9].

Furthermore, other sources note that certain acids known to be fermentation products of bacteria can sometimes cause intestinal injury in rodent and human neonates, likely due to abnormal metabolism of these products [5, 10, 11]; this points to imbalanced microbial colonization as a potential factor in NEC.

Niño et. al. reiterate and condense much of the previously listed information, as well as identify some important considerations, developments, and promising biomarkers for NEC [2]. Comparing the preterm and full-term infant gut environments, they state that toll-like receptor-4 (TLR4), a surface protein expressed more strongly in the premature gut and activated by gram-negative bacteria, increases intestinal epithelial cell apoptosis, impairs mucosal healing, and upregulates the release of proinflammatory cytokines [2]. It is proposed that gram-negative bacteria, in activating this receptor, keep the expression levels of TLR4 high and contribute to
the onset of NEC [2]. This could in part explain why premature infants are more susceptible than full-term infants [2]. Reviewing potential biomarker candidates, this paper lists those found in blood (acute-phase reactants like C-reactive protein and proinflammatory cytokines like TNF-alpha, interleukin-6, and interleukin-8) and in urine (intestinal fatty acid-binding protein, liver fatty acid-binding protein, fecal calprotectin, trefoil factor 3, and claudin-3) [2]. Dr. Sylvester and others have been exploring urine biomarkers [2, 13].

1.3 Critique of Current Technologies and Literature

Though predictive models may supplement traditional clinical diagnostics, many of the current technologies do not provide a proven or adequate solution for NEC diagnosis [2, 9]. It follows that research should turn to less explored lines of scientific inquiry to further supplement or even replace what already exists.

The microbial milieu is increasingly considered to play a pivotal role in human health and biology [38]. Current literature documents gut dysbiosis, or microbial imbalance in the gut, as a likely contributor to NEC [2, 10, 11]. Gram-negative bacteria have been identified as possible contributors to vulnerability to injury by conditions such as oxidative stress [2, 5]. However, a more comprehensive understanding of the specific ways in which microbes contribute to NEC has yet to be developed. Our analysis of the literature reveals that many of the biomarkers listed as most promising are factors released due to cell injury and inflammation, and are perhaps approaching the issue from too narrow a viewpoint. We respectfully argue that while these factors should still be explored, not enough bacteria-related metabolites and factors have been investigated thus far. Discovery may result from researching such factors, like butyrate and other fermentation products of bacteria [12], which have been implicated in several past and recent publications [5, 10, 11]. The Sylvester Lab’s recent published data (see section 3.9 for a description) appears to be one of the first to explore butyrate in significant depth [5], but butyrate is not the only product related to bacteria. We propose widening and deepening the search area overall.

Moreover, we hold that more emphasis should be placed on strategically choosing the best source of biomarkers with future applications in mind. Blood, urine, and stool are three possible sources for NEC biomarkers. Because premature infants are so small, blood is limited in volume and difficult and invasive to sample. Parents are also hesitant to consent to sampling. Therefore, blood is perhaps not the ideal biomarker source for development into the kind of quick and easy bedside diagnostic test that would best facilitate diagnosis, despite the promise shown for blood biomarkers like C-reactive protein and inflammatory cytokines [2]. The Sylvester Lab’s work with urine biomarkers has proven successful, but because the diagnostic strategies are not fully validated yet, there is still a need to continue searching [2]. Fecal biomarkers come directly from
the disease environment. Collecting stool for screening would be simple, noninvasive, painless, and sustainable, and would allow us to study any bacterial fermentation products of interest. It offers more opportunity for pure discovery, and is useful from a humanitarian and engineering perspective.

Finally, while many of the biomarkers listed are more correlative, researchers may benefit from choosing to explore biomarker candidates that suggest a stronger causative relationship with NEC. This will accomplish two tasks simultaneously: finding a useful biomarker while also uncovering more about NEC’s mysterious etiology.

Current invasive methods and broad-spectrum antibiotic treatments might contribute to gut dysbiosis [11], doing more harm than good. Strategies targeting the gut microbiome and/or microenvironment in a less destructive and invasive way, such as pre and probiotics or damaging-molecule inhibitors [5, 7], may be a better approach; however, without etiological knowledge, developing new treatments becomes guesswork. By identifying and exploring factors of interest with causation in mind now, we can better formulate treatment strategies later on.

1.4 Project Goal

Herein we address the pressing concerns that (1) the rate limiting step for reducing disease-onset-to-treatment time is diagnosis, but the nonspecificity of current diagnostic methods impedes early and accurate diagnosis; (2) that there are no known specific and useful fecal biomarkers to aid physicians in diagnosis; and (3) that despite decades of research on the disease, much about NEC’s etiology remains unknown. To address these concerns, we aim to identify a useful fecal biomarker and help illuminate its related molecular mechanisms through preliminary research studies.

We began by plating HIEC-6 and HT-29 cells with various factors, chosen to represent an array of biomarker sources and potential disease-associated phenomena. After narrowing down specific factors of interest based on morphological and other notable changes they induce, as well as verifying that HT-29 is an acceptable choice for our study, we plated these two factors (both detectable in stool) with HT-29 cells. After choosing one factor of interest as our main focus, we plated it over varying HT-29-cell confluency to confirm that the observed phenomena are related to cellular mechanisms, collected more detailed results, and ultimately performed a more detailed analysis of these results. This proof-of-concept study will pave the way for higher-level research on our factor of interest in the future.
1.5 Backup Plan

The primary point of vulnerability in our design is identifying a factor that warrants more detailed study. Should we fail to identify a factor using the proposed method, we may need to screen a larger array of factors for evidence of disease phenomena. Another potential failure could be that HT-29 does not make a suitable substitute for HIEC-6 in the context of our study. Should we encounter this obstacle, we can look into using another human adenocarcinoma cell line isolated from epithelial intestinal tissues such as Caco-2 or HuTu-80 [15, 16]. However, it may be that in general a cancer cell line, perhaps due to fundamental metabolic differences or other factors, is not the proper cell model for our purpose; if so, we can use HIEC-6 cells despite their disadvantages for an early-stage study.

1.6 Significance

Our project has several significant implications. If successful, it may vastly benefit physicians, NEC patients and their families, and the field of medicine.

NEC risk is inversely correlated with gestation time [2]. Current neonatal healthcare techniques and technology have contributed to the incidence of NEC in the way that they have allowed for the survival of infants outside the womb at increasingly early gestation times [2, 6, 17]. Neonatal healthcare for premature infants will likely continue to improve, meaning that it will enable viability of infants with increasing susceptibility to NEC [2, 17]. As such, NEC will only become more prevalent despite the consistent percentage of premature births it affects. Given the weaknesses in current diagnostic methods and the plateau of NEC survival rate [2], a growing prevalence of NEC will mean that more and more premature infants will die of this disease. Therefore, there is a pressing need to improve early and accurate diagnosis, as well as understand the underlying mechanisms of the disease so that researchers can develop new measures to fight it. The biomarker screening process may contribute to present understanding of NEC’s molecular underpinnings, which will ultimately inform the development of new treatments [2].

Through a point-of-care diagnostic test (one that can be performed onsite or at the bedside), an easily accessible and reliable biomarker would facilitate accurate, early diagnosis, decreasing the time between disease onset and treatment and perhaps increasing NEC survival rate. Compared to other methods, sampling stool for biomarker testing is noninvasive, non-harmful, and potentially more fruitful. Thus, finding a fecal biomarker would presumably enable a superior method for NEC diagnosis.
1.7 Team and Management

1.7.1 Team Members

Table 1. Project Members

<table>
<thead>
<tr>
<th>Member</th>
<th>Degree</th>
<th>Email</th>
</tr>
</thead>
<tbody>
<tr>
<td>Daniela Campa</td>
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<td>Kyle Sullivan</td>
<td>Bioengineering - Biomolecular Track</td>
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</tr>
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Table 2. Project Advisors

<table>
<thead>
<tr>
<th>Advisor</th>
<th>School</th>
<th>Email</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dr. Jonathan Zhang</td>
<td>Santa Clara University</td>
<td><a href="mailto:zhang@scu.edu">zhang@scu.edu</a></td>
</tr>
<tr>
<td>Dr. Guo-Zhong Tao</td>
<td>Stanford University</td>
<td><a href="mailto:gtao@stanford.edu">gtao@stanford.edu</a></td>
</tr>
</tbody>
</table>

1.7.2 Project Budget

SCU Engineering generously granted us $1000 for our project. Materials for our work in the Stanford lab were graciously provided by Stanford. Since we did not have time to perform planned experiments at SCU, we used our grant to restock vital materials in SCU’s lab as summarized in Table 3 below.

1.7.3 Project Timeline

During fall quarter, our team traveled to a satellite lab of Stanford University to tour and interview for acceptance. Over the course of the weeks following our acceptance, Stanford University and Santa Clara University discussed the specifics and liabilities associated with collaboration. During this time, we began conducting our literature search on relevant subjects, which we continued for the duration of the project. Our team was officially approved week 10 of fall quarter. We spent winter break going through necessary lab safety and HIPAA training before starting our lab work. Once in the lab, we conducted routine experiments with the Stanford research team throughout winter and spring quarters. After familiarizing ourselves with the research team, lab layout, relevant subjects, and experiment protocols, we had the privilege
of contributing original experimental design to the project. We presented our work at the Senior Design Conference on May 9, and compiled the information into our thesis. A Gantt chart summarizing our project timeline can be found in Appendix A.

Table 3. Materials Ordered and Total Costs

<table>
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<th>Product Name</th>
<th>Quantity</th>
<th>Price ($)</th>
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<td>Blue 1.5mL Cuvettes (100 count)</td>
<td>3</td>
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<tr>
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<td>LB Broth (1L)</td>
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<td>Agar Medium (1kg)</td>
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Chapter 2: Plating HIEC-6 and HT-29 Cells with Various Factors

2.1 Introduction

The cell lines we used for this experiment are human intestinal epithelial cells (HIEC-6) and human colorectal adenocarcinoma cells (HT-29). HIEC-6 cells are isolated from the fetal small intestine [18] and have a doubling time of approximately 96 hours. HT-29 cells, isolated from cancerous colon epithelium [19], are hardier than HIEC-6 and have a doubling time of about 48 hours. Both of these cell lines are adherent [19, 20], meaning that if they are healthy they will appear flat and spread out on the plate under the microscope; if they are round and/or detached, they are either already dead or will die shortly.

We used several different factors to treat the cells in this experiment, including: (1) TNF-alpha, a proinflammatory protein and potential biomarker [2, 21]; (2) lipopolysaccharide (LPS), an endotoxin on the membrane surface of gram-negative bacteria [22]; (3) hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}), a strong oxidizer to induce oxidative stress [23, 24]; (4) butyrate, the anion of the short-chain fatty acid called butyric acid and a known fermentation product of certain kinds of bacteria [12]; and (5) Candidate-X, an undisclosed, soluble fermentation product of bacteria. The plated cells were visualized under an optical microscope to analyze morphology changes over time as a result of treatment. Please refer to Appendix B for a more detailed background on the first four factors listed.

2.2 Key Constraints

Our project involves \textit{in vitro} cell culture models for preliminary research. Since many of the key constraints for this project remain relatively the same throughout, this section summarizes the common key constraints in addition to the ones that are specific to this chapter.

In cell culture experiments, sterile technique must be maintained or else contamination will kill the cells and ruin the results. Cell lines chosen must be biologically relevant to the \textit{in vivo} target environment. More generally, key constraints of preliminary studies are influenced by the end goal: reaching the later-stage research phase. Specifically in terms of time, the goal is to speed up the timeline of preliminary studies to reach the later stages as soon as possible. In terms of cost, the goal is to save money for the more focused later-stage studies. The best experimental design for us is one that balances biological relevance with time and cost efficiency.

We are currently unable to obtain samples directly from the intestinal microenvironment to establish basal levels of various factors in healthy infants and test for abnormal concentrations in NEC infants, so concentrations obtained from infant stool in combination with \textit{in vitro} or animal
models are our main options. For disease modeling, *in vitro* studies are often conducted first as proof of concept. For *in vitro* models in early proof-of-concept studies, one can sacrifice certain elements of experimental design--such as fidelity of *in-vitro-to-in-vivo* comparability--in favor of speed and cost, as long as one can demonstrate the experimental design is still valid.

Ideally, for NEC disease studies we would choose a cell line that closely mimics the *in vivo* target environment: the premature gut. While HIEC-6 cells are isolated from the fetal small intestine [25], and are therefore the better cell line for mimicking our target environment, their doubling time of approximately 96 hours renders them inefficient to culture. In contrast, HT-29 cells are hardy and have a doubling time of about 48 hours, so they are easier, somewhat cheaper [25, 26], and faster to culture. HT-29 also have a lineage somewhat similar to the target environment, being isolated from colon cancer [19]. Considering all factors of experimental design for our project, HT-29 would be the better choice if we can prove biological relevance. However, if the results of our project are promising, we will transition from using HT-29 to HIEC-6 in the future.

In our project, we use visualization of cell morphology in favor of more detailed but more expensive methods. The Sylvester Lab does not have certain machines for more detailed analysis; when needed, the research team sends samples to an offsite lab and pays for content analysis. SCU has liquid-chromatography-mass-spectrometry (LC-MS) onsite, but each use costs money and transporting biological samples and materials back and forth between schools is neither quick nor simple. This kind of detailed analysis will be reserved for future work, after we can prove our factor of interest is worth exploring.

A key constraint limited to this chapter is that HIEC-6 and HT-29 are different cell lines, and therefore require somewhat different culture methods. For example, HIEC-6 is more sensitive, so we must use weaker trypsin; in contrast, HT-29 is hardier, so we can use stronger trypsin. We must be careful not to confuse these so as to not damage cells before treatment with factors.

### 2.3 Design Approach

Stanford provided the experimental design for this chapter. In this experiment, we explored biomarkers from the literature as well as the propositions we outlined in our critiques. By testing a wide array of factors, we were able to take a more comprehensive look at possible biomarkers and disease mechanisms. We cultured well-studied cell lines--one that is known to be biologically relevant and one this experiment sought to prove is biologically relevant--in well plates, and treated them with various factors. Cellular damage was assessed by examining cell death and morphology changes. By testing these cell lines in parallel, we narrowed down factors of interest while at the same time assessing the biological relevance of the cancer cell line for the
next experimental steps. We repeated the experiment at least three times to ensure our results were reproducible.

2.4 Supporting Analyses

The design successfully accomplished our objective for this experiment. We confirmed that HT-29 was biologically relevant for our next steps, as the results for both cell lines were similar. In addition, the lack of cell damage ruled out TNF-alpha, LPS, and H$_2$O$_2$ as likely causative agents in NEC, while the presence of cell damage allowed us to select butyrate and Candidate-X as factors of interest.

2.5 Expected Results

To reliably assess biological relevance of HT-29, we need to keep each treatment as consistent as possible across trials and cell lines; variations in treatment could influence our results and make it difficult to compare the two cell lines. We expect to see relatively consistent results for each cell line respectively. If we see different results across trials for a cell line, it is likely that the conditions for each treatment were not kept consistent. In addition, we must maintain sterile technique at all times during cell culture; failure to do so would force us to redo the trial and thus delay our progress. If we maintain sterile technique, we should see cells with morphology, size, and doubling time consistent with the proper cell types. Since a very small percentage of cells typically die regardless of the conditions, controls should show a small but negligible amount of cell death at time of visualization.

2.6 Backup Plan

During this process, it is possible that we will fail to identify a factor that warrants more detailed study. If this happens, we will screen a wider array of factors for evidence of disease phenomena. Based on our literature search and critiques, we would consider testing other inflammation-related factors listed, but primarily look into exploring other gut-bacteria-related metabolites like acetic acid [10]. While this is not a well-established protocol, our project involves discovery and so our lines of scientific inquiry are guided not by established methods, but by cutting-edge research and possible missed opportunities.

In addition, this experiment may show that HT-29 does not make a suitable substitute for HIEC-6 in the context of our study. Should we encounter this obstacle, we can look into using another human adenocarcinoma cell line isolated from epithelial intestinal tissues such as Caco-2 (isolated from the colon) or HuTu-80 (isolated from the duodenum), which are both well studied and widely used for similar applications [14, 15, 27]; and repeat this experiment with the new
cell line. ATCC recommends similar culture methods and materials for these cell lines compared to HT-29, so the transition would be relatively simple [11, 16]. However, it may be that in general a cancer cell line, perhaps due to fundamental metabolic differences or other factors, is not the proper cell model for our purpose; if so, we can use HIEC-6 cells for the next steps despite their disadvantages for an early-stage study.

2.7 Materials and Methods

Growth medium preparation, cell culture, and cryopreservation for each cell line were generally conducted according to ATCC’s guidelines [25, 26], but we have included below our own more detailed version of the protocol and materials needed for cell splitting and changing media.

For ATCC’s protocol on culture methods (including preparation of complete growth medium, subculture methods, cryopreservation, and culture conditions) for each cell line, please visit the following websites:

HIEC-6 [25]:
https://www.atcc.org/en/Products/Cells_and_Microorganisms/By_Tissue/Intestine_Small/CRL-3266.aspx#culturemethod

HT-29 [26]:
https://atcc.org/Products/All/HTB-38.aspx#culturemethod

2.7.1 Cell Culture Materials

General Materials
- EDTA
- Phosphate Buffered Saline (PBS) pH 7.4 (1X) - (Gibco® by Life Technologies, Ref. No. 10010-031)
- 70% Ethanol solution
- IncuSafe CO2 incubator, setpoint 37°C, 5% CO2 - (Panasonic Healthcare Co., Ltd; Model No. MCO-18ACL-PA)
- Leica DMIL microscope - (Leica Microsystems, Model No. 090-135.001)
- Sorvall ST 8R Centrifuge - (Thermo Scientific)
- SterilGARD laminar flow hood - (The Baker Company, Model No. SG 404-M)
- Fisherbrand® SureOne micropipette tips, various sizes
- Micropipettes, various sizes
- Falcon® serological pipette tips, various sizes
- Easypet 3 serological pipette gun - (Eppendorf)
- Falcon® centrifuge tubes, various sizes
- 9” Disposable Pasteur Pipettes, borosilicate glass / non-sterile - (Fisherbrand®, Catalog No. 13-678-20D)
- Costar® 10 mm, 24- and 6-well plates; tissue-culture treated / sterile - (Corning Incorporated)
- -153°C liquid nitrogen storage tank - (Thermo Fisher Scientific, Model No. 7400)
- -20°C refrigerator - (Kenmore)
- 4°C refrigerator - (Kenmore Elite)

**HIEC-6 Culture Materials**
- Complete Growth Medium (4% FBS)
  - OptiMEM 1 Reduced Serum Medium - (Gibco® by Life Technologies, Catalog No. 31985)
  - Fetal Bovine Serum (FBS) - (ATCC®, Catalog No. 30-2020)
  - 20 mM HEPES
  - 10 mM GlutaMAX (Gibco® by Life Technologies, Catalog No. 35050)
  - 10 ng/mL Epidermal Growth Factor (EGF)
- HIEC-6 cells, frozen (ATCC®, CRL-3266™)
- 0.25% Trypsin-EDTA (1X) - (VWR Life Science, Catalog No. 02-0154-0100)
- Freeze medium (95% growth medium, 5% DMSO)
  - Complete Growth Medium (4% FBS)
  - Dimethylsulfoxide (DMSO) - (ATCC® 4-X™)

**HT-29 Culture Materials**
- Complete Growth Medium (10% FBS)
  - McCoy’s 5a Medium Modified - (ATCC®, Catalog No. 30-2007)
  - Fetal Bovine Serum (FBS) - (ATCC®, Catalog No. 30-2020)
- HT-29 cells, frozen (ATCC® HTB-38™)
- TrypLE™ Express, [+] Phenol Red - (Gibco® by Life Technologies, Ref. No. 12605-010)
- Freeze medium (95% growth medium, 5% DMSO)
  - Complete Growth Medium (10% FBS)
  - Dimethylsulfoxide (DMSO)

**2.7.2 Cell Culture Methods**

**Splitting Cells**
1. Ensure all cell culture surfaces and tools have been sterilized with 70% Ethanol.
2. Retrieve cell culture plate with cells from 37°C incubator.
3. Visualize cells under bright-field microscope to ensure they are at the desired confluency (70-90%).

4. Place cell culture plate in laminar flow hood and suction out the old media.

5. Add 1X PBS to the plate in accordance to the plate size.
   a. 10 mm plate receives 3 mL of 1X PBS.
   b. 6-well plate receives 1 mL of 1X PBS.
   c. 24-well plate receives 500 µL of 1X PBS.

6. Tilt the plate back and forth to evenly mix the 1X PBS and then suction up the PBS.

7. Add Trypsin (see materials for proper type for desired cell line) to the plate and place back in the 37°C incubator for 3 to 5 minutes.

8. Hold the plate up to the ambient light to visualize the bottom of the plate for cell detachment, and visualize plate under bright-field microscope to confirm.

9. Add required amount of media (see materials for proper type for desired cell line) to each plate while tilting it, and cycle the media from top to bottom in order to ensure maximum collection of viable cells.
   a. 10 mm plate receives 3 mL of media.
   b. 6-well plate receives 1 mL of media.
   c. 24-well plate receives 500 µL of media.

10. Add the cell mixture to a 15 mL centrifuge tube and centrifuge at 200g to 300g for 3 minutes.

11. Suction out the supernatant, taking care not to suction out the pellet. Tilt the tube to make the liquid easier to suction.

12. Resuspend the cell pellet with media to the desired concentration, and add to a new, sterile plate. Make sure the plate is labeled with your name, date, and cell line.

13. Place the plate back into the incubator.

**Changing Media**

1. Ensure all cell culture surfaces and tools have been sterilized with 70% Ethanol.

2. Visualize cells under bright-field microscope to ensure they are at the desired confluency (< 70%).

3. Place cell culture plate in laminar flow hood and suction out the old media.

4. Add 1X PBS to the plate in accordance to the plate size.
   a. 10 mm plate receives 3 mL of 1X PBS.
   b. 6-well plate receives 1 mL of 1X PBS.
   c. 24-well plate receives 500 µL of 1X PBS.

5. Tilt the plate back and forth to evenly mix the 1X PBS and then suction up the PBS.

6. Add the desired amount of media (see materials for proper type for desired cell line) to the plate, which now contains only viable cells.
2.7.3 Materials for Treatment with Various Factors

- Fisherbrand® SureOne micropipette tips, various sizes
- Micropipettes, various sizes
- Falcon® serological pipette tips, various sizes
- Easypet 3 serological pipette gun - (Eppendorf)
- Phosphate Buffered Saline (PBS) pH 7.4 (1X) - (Gibco® by Life Technologies, Ref. No. 10010-031)
- Deionized Water from MilliQ system
- Fisherbrand™ Premium Microcentrifuge Tubes: 1.5 mL
- Factors obtained from Sigma-Aldrich
  - TNF-alpha
  - lipopolysaccharide (LPS)
  - hydrogen peroxide (H$_2$O$_2$)
  - Butyrate (acid form)
  - Candidate-X (acid form)

2.7.4 Methods for Treatment with Various Factors

1. Calculate the desired concentration for each factor.
2. Identify required suspension media.
   a. Most likely Deionized Water, PBS, or cell culture media for applicable cell type.
3. Weigh out desired amount of factor.
4. Add individual factors to their own 1.5mL Microcentrifuge tube.
5. Suspend factors with suspension media.
6. Calculate the volume of suspended factor must be added to cell culture wells to obtain desired final concentration.
7. Add calculated volume to the wells if the cells have had time to adhere to the bottom of the well.
8. Once factors are added, tilt the cell culture plate back and forth to homogenize the solution.
9. Visualize cells with a brightfield microscope at desired time points.
10. Repeat steps 1-9 at least three times in separate trials.

2.8 Results

Results were similar for all trials all trials. TNF-alpha, H$_2$O$_2$, and LPS had minimal to no notable effect on cells relative to the control. By visualizing the plate under the microscope, we determined that butyric acid induced a moderate amount of cell death while Candidate-X in acid
form induced widespread cell death. Both induced notably more cell death than the control. Morphology changes for butyric acid include a transition from flat to round followed by detachment, death, and lysis. Morphology changes for Candidate-X include freezing in place (remained flat with no rounding), death, and eventual detachment with no lysis. There were also unidentified granules in the cytoplasm. We observed that the effects of Candidate-X took longer to manifest compared to butyric acid. While the onset time of effects was delayed for HIEC-6 compared to HT-29, we observed the same phenomena for both cell lines.

We noted a significant color change from pink to yellow in the Candidate-X treated well. An image of this can be found in Chapter 3, Figure 1.

2.9 Discussion

Our results are most likely reliable due to their reproducibility. HIEC-6 cells took longer than HT-29 cells to reveal the changes, which may be related to the elevated metabolic demands of HT-29 as a cancer cell line. However, given that we observed the same ultimate effects across cell lines, we are able to confirm the biological relevance of HT-29 for use in our next steps. This will speed up the timeline of our project.

The lack of notable effects of the first three factors suggests that there is not a strong causative relationship between them and NEC; rather, in the context of the literature and known physiology, it is more likely these are correlative and not the ideal line of inquiry for our purpose.

Given the occurrence of cell lysis, the morphology of butyric-acid-induced cell death is consistent with necroptosis or necrosis [18, 28]. The morphology of Candidate-X-induced cell death appears distinctly different from that of the most familiar pathways [18]. These two factors are more likely to have a causative relationship with NEC due to their apparently cytotoxic effects. Therefore, we have singled these out as our factors of interest for our next steps.

Perhaps the most surprising result of all was the significant color change with Candidate-X. The growth media contains phenol red, a colorimetric pH indicator [29]. At neutral pH, the supernatant appears pink, as seen in control wells (refer to Figure 1 in section 3.8), but the yellow color indicates an acidic (i.e. low) pH. Since the color is consistently still pink immediately following the treatment, we know that the pH change is something that occurs over time. Given the pH change, widespread cell death, and puzzling morphology, we hypothesize that Candidate-X may end up being an exciting line of inquiry for this project and perhaps have implications for understanding why NEC’s pathophysiology is so devastating.
Chapter 3: Plating HT-29 Cells with Factors of Interest

3.1 Introduction

Candidate-X and butyrate are fermentation products of bacteria [12]. In gut dysbiosis, it is possible that the normal levels of these factors may be increased beyond what the preterm intestine can handle, reaching cytotoxic levels. Prior to conducting this experiment, the research team identified threshold concentrations inducing cell death for both factors of interest; this is significant because it allows us to compare the in vitro model to levels found in stool, and better mimic the stressors experienced in the in vivo environment. This will be explained further in section 3.3.

In this experiment, we use these threshold concentrations to guide our treatment of cells in well plates and study cell morphology changes over time.

3.2 Key Constraints

As touched on in section 2.2, a key constraint is that it is not currently possible to sample directly from the affected in vivo microenvironment for concentrations of factors of interest. With diluted concentrations of factors of interest obtained from stool, we must determine through trial and error what concentrations may be in the intestinal microenvironment and test this in vitro. The experimentally determined threshold concentration of Candidate-X, not the concentration found in NEC stool prior to treatment, should be the primary concentration for treating the cells. For additional key constraints, please see section 2.2.

3.3 Design Approach

Stanford provided the experimental design for this chapter. Plating different neutral-pH forms of the factors will determine if the factors’ acidity is responsible for the effects we observed. The research team had recently identified (via trial-and-error treatments in 96-well plates with varying factor concentrations) that butyrate induced cell death at a threshold concentration of 10 mM, while Candidate-X induced cell death at a threshold concentration of 50 mM. With this knowledge, we performed an experiment that tested the reproducibility of previous results in tandem with a more detailed morphologic analysis.

3.4 Supporting Analyses

This experiment confirmed that effects were reproducible for the two factors of interest in all forms, evidenced by the occurrence of similar cell death levels and color changes. The more
detailed recordkeeping and testing also gave more in-depth information on the timeline of effects and morphology. Similar results across different forms of each factor confirmed that their acidity / the initial extracellular pH were not factors in these effects.

3.5 Expected Results

To complete this experiment, we need to successfully culture and treat the cells with a known concentration of factors, maintaining sterile technique at all times. Should we successfully set up our well plate, we expect to see wells with cells and pink supernatant at time of treatment. Controls should show a small but negligible amount of cell death at time of visualization. Total cell death will be estimated again through visualization under the microscope after at least a day, and this time confirmed and quantified using Keratin-8 (K-8, or cytokeratin[CK]-8) assays.

3.6 Backup Plan

HT-29 may not be biologically relevant; this will have been established in the previous experiment, and an alternate cell line will have been validated and chosen for this experiment. Should we encounter this obstacle, we will adapt our protocol to match the chosen cell line according to ATCC’s guidelines and order any additional materials as needed.

3.7 Materials and Methods

This section includes a list of materials and new protocols used for treatment of HT-29 with butyrate and Candidate-X. For HT-29 culture materials and methods, please refer to sections 2.7.1 and 2.7.2.

3.7.1 Materials

Required cell culture and plating factor material is the same as Section 2.7.1, with the exception of the following:

- 6-well plates only
- Factors obtained from Sigma-Aldrich (acid neutralization step performed in Stanford lab)
  - Butyrate (acid and neutralized acid with NaOH)
  - Candidate-X (acid, neutralized acid with NaOH, and neutral sodium salt)
- Sodium hydroxide solution (NaOH)
3.7.2 Methods for Treatment with Factors of Interest

The method for HT-29 cell culturing and plating Candidate-X is the same as Section 2.7.2, with the exception of neutralizing the acid forms with NaOH solution to the desired physiological pH. Cell death percentages are 100 times the amount of cell death in the plate divided by the total amount of cells, dead or alive. The research team used K-8 assays for more precise quantification of total cell death for comparison to baseline cell death seen in the controls; we should note that this step was performed mostly by other lab members and was not a significant part of our work in the lab, but Appendix C1 includes the K-8 assay protocol used. Further information may be obtained by consulting the Sylvester Lab.

3.8 Results

Results were consistent across trials. The research team determined that butyrate induced about 5 to 10% cell death while Candidate-X induced about 90 to 100% cell death compared to compared to the controls.

Again, morphology changes for butyric acid include a transition from flat to round followed by detachment, death, and lysis into small parts. We were able to determine that the onset time of these effects are within 24 hours in HT-29. Morphology changes for Candidate-X include granules in the cytoplasm, freezing in place while still flat and fully intact, and over time, almost all the cells lifting off the plate. We were able to determine that the onset time was between 48 to 72 hours in HT-29. For both factors, the same effects occurred regardless of initial pH; that is to say, (1) the acid and neutralized acid forms of butyrate all produced the same outcome, and (2) the acid, neutralized acid, and neutral sodium salt forms of Candidate-X all produced the same outcome.

As before, we noted a distinct color change from pink to yellow in the Candidate-X-treated well. The control also shows a very slight color change. These results are shown below in Figure 1.
A 6-well plate was used to compare effects of neutralized acid forms of Candidate-X (50 mM; boxed in red) and butyrate (BA) (50, 10 mM; boxed in blue) on HT-29 cells to the control (boxed in black). The other wells were used at the same time for a different project, and are not relevant to this experiment. No picture for the acid forms or neutral salt form is available.

3.9 Discussion

Quantifications of cell death percentages coincide with our visual estimates in Chapter 2.

Butyrate’s effects on cells were the same for acid and neutralized acid forms, so they are not due to its acidity / the initial extracellular pH. As seen in Chapter 2, we observed a distinct color change in the Candidate-X-treated well; this further demonstrates the reproducibility of the pH change (see Figure 1). The fact that the neutralized acid and neutral salt forms of Candidate-X produced the same effects as the acid form shows that the morphology changes, cell death, and pH change over time are unrelated to its acidity / the initial pH. We hypothesize this is a cell-related phenomenon: that Candidate-X treatment might induce some kind of metabolic change in which the cell produces increased amounts of hydrogen ions and/or becomes impaired at regulating extracellular pH. We will begin testing this hypothesis in the next chapter. An issue
to note, however, is that the control does show a very slight color change; while the change is negligible, it suggests that the cells can produce small levels of an acidic substance on their own.

The morphology of Candidate-X-induced cell death does not fit apoptosis, necrosis, or necroptosis, as there were granules in the cytoplasm and no apoptotic bodies or cell swelling, shrinkage, or autolysis [18]. Because of the unique morphology along with the difference in onset time of effects compared to butyrate, we believe that cells treated with Candidate-X undergo a different cell death pathway than what we have observed with cells under other treatment conditions. In addition, our results point again to causation for NEC. As a result, we have decided to continue to the next step with Candidate-X as our primary factor of interest.

Butyrate’s cell death pathway was ultimately proven to be necroptosis in a separate publication by the Stanford research team; this represents a huge and exciting leap forward in understanding the molecular mechanisms at work in NEC, and will likely lead to NEC-specific treatments [5]. A small molecule inhibitor has already shown promise in this regard [5]. Our work with Candidate-X may have similar success in the future.
Chapter 4: Plating Candidate-X with Varying Cell Confluency of HT-29

4.1 Introduction

Candidate-X is our primary factor of interest. Recent, unpublished data from UC Davis show that Candidate-X concentration in confirmed-NEC infant stool prior to standard-of-care treatment, sampled onsite at Stanford’s hospital, is four to ten times the basal concentration as determined by analysis of healthy infant stool samples. The concentration decreases to basal levels following treatment for NEC. These results prove that an elevated concentration of Candidate-X in stool is strongly correlated with the disease.

Because the UC Davis results are unpublished, we are currently unable to include a citation for them. In the interest of protecting intellectual property we are also unable to provide more detailed background information on Candidate-X other than what has already been said, as this may reveal its identity.

We already know from the previous experiments that Candidate-X induces up to 100% cell death with unique morphology in vitro. This experiment aims to verify or disprove that the pH change we observed is correlated with cell interactions with either Candidate-X or any related intermediates in solution.

4.2 Key Constraints

As explained in section 2.2, ideally we would choose HIEC-6 to mimic the premature gut environment when studying NEC. However, in section 2.8 we outlined that the same supernatant color change, cell morphology, and widespread cell death for both HIEC-6 and HT-29 was already observed following Candidate-X treatment; the only observable difference across cell lines was the onset time of the effects, suggesting that HT-29 is an adequate substitute for HIEC-6 in the context of Candidate-X treatment. Since the literature we reviewed has yet to document Candidate-X as a major factor of interest in NEC, we can consider this experiment to be in the very early stages; the research does not yet require as stringent of a match to the in vivo environment. Also, due to time constraints, we were unable to conduct multiple trials. Therefore, we are unable to assess the reproducibility of our results. Please see section 2.2 for further detail and additional key constraints.

4.3 Design Approach

We have had the privilege of designing and implementing our own experiment to determine if the color change is related to the growth media or the cells.
In this experiment, we adapted the chemical dilution protocol to set up wells with varying cell confluency (i.e. concentration) in a uniform extracellular environment. Our design was inspired by the fact that the previous experiments have a major weakness: they do not explore Candidate-X treatment in wells with no cells, so there are no data to prove that the color change we see is directly related to a combination of Candidate-X and cells. Observing this control is imperative before taking any further steps in exploring Candidate-X as a potential contributor to the development of NEC. Moreover, even if the effects are independent of media components, previous work varied the concentration of Candidate-X to determine threshold cell death concentration but failed to assess how a constant starting extracellular environment affects cells at varying confluency. An analysis of the correlation between the cells and Candidate-X’s effects by varying the confluency will allow us to identify the direction in addition to the magnitude of the effects. This is vital to understanding what molecular mechanisms may be at work following the treatment.

4.4 Supporting Analyses

This experiment successfully ruled out media interactions and proved that Candidate-X’s effects were cell-correlated, as varying cell confluency did produce a notable pattern in Candidate-X-treated wells.

4.5 Expected Results

To complete this experiment, we need to establish a method of creating different concentrations of cells to vary cell confluency and treat the cells with a constant concentration of Candidate-X while maintaining sterile technique at all times. Should we successfully set up our well plate, we should see no cells in the first column at any point. At time of visualization, we expect to see a color change in the cells + treatment wells and negligible color change in the cells-only wells; if not, the results would be inconsistent with our previous experiments and would indicate we may have made a mistake. We should note that while the results can confirm correlation of Candidate-X’s effects with cells, the experiment does not give enough detail to distinguish between Candidate-X directly interacting with cells and Candidate-X creating an intermediate(s) that interacts with cells. We can only hypothesize what is happening mechanistically based on our knowledge of the molecule, which we are unable to fully disclose in this paper.

4.6 Backup Plan

As explained in preceding sections, HT-29 may not make a suitable substitute for HIEC-6 in the context of our study. If HT-29 is not a suitable substitute, we will instead be using for this
experiment whichever cell line has been validated and/or used in the previous experiments, whether that is HIEC-6 or a human adenocarcinoma cell line other than HT-29.

In addition, this experiment may reveal that Candidate-X’s effects are related to the media. Should we see color change with either well containing no cells, we will (1) look into different media to test with Candidate-X for the same effects, choosing to use one in the future that shows no effects; and (2) consider searching for or selecting another factor of interest for more detailed exploration, as the Candidate-X’s effects may not be correlated with the cells, and therefore, not necessarily involved in the disease mechanism.

4.7 Materials and Methods

This section includes a list of materials and new protocols used for treatment of HT-29 with Candidate-X. For HT-29 culture materials and methods, please refer to Sections 2.7.1 and 2.7.2.

4.7.1 Materials

Required cell culture and plating factor material is the same as Section 2.7.1, with the exception of the following:

- 24-well plates only
- Factors obtained from Sigma-Aldrich
  - Candidate-X (neutral sodium salt only)

4.7.2 Methods for Treatment with Candidate-X over Varying Cell Confluency

1. Estimate confluency of cells in bright-field microscope.
   a. Should be approximately 90 to 100% if the cells are ready for passage.
2. Harvest cells in the same manner as the Cell Splitting protocol.
3. Calculate the percentage of cells needed from original plate to new well by dividing the new wells surface area by the old wells surface area. Multiply value by desired confluency percentage.
4. Pipette the calculated volume of resuspended cells concentration into the new well.
   a. Set up in 8 wells of a 24-well plate with varying cell confluency (see Figure 2) as 2 rows of 4 wells
   b. Row A: + NaX (wells treated with 50 mM NaX); Row B: - NaX (control wells with no NaX)
Figure 2. Schematic of experimental layout. Wells were set up with varying cell confluency across a row, observed with (Row A) and without (Row B) NaX treatment. Column 1 contains no cells.

4.8 Results

Negligible color change was seen in the control wells (very slight color change ≥100% cell confluency), and the X-treated well containing only media did not show any color change whatsoever. The most intense color change was seen in the treated row, and intensity of yellow color increased with cell confluency. These results are shown in Figure 3.

Figure 3. Before/after comparison of experiment in Fig. 2. Panel 1 shows the plate at time of treatment. Panel 2 shows the plate at time of visualization. Controls did not exhibit notable color change, but wells with cells + NaX did. For these wells, amount of cell death and intensity of yellow color increased with cell confluency.

Morphology analysis again revealed granules in the cytoplasm, no apoptotic bodies, and no cell shrinkage, swelling, or autolysis. Cell death occurred along approximately the same timeline as
explained in section 3.8 and in the same amount, increasing with cell confluency just as the intensity of color change increased.

4.9 Discussion

The morphology of Candidate-X-induced cell death again does not fit traditional apoptosis, necrosis, or necroptosis. The lack of color change in the media-only control, the negligible color change in the untreated controls, and the pattern of color change in the treated wells with cells proves that the pH change was correlated with cell confluency and intensified with the addition of Candidate-X. As previously mentioned in section 3.9, the effects of Candidate-X involve a decrease in pH not due to the initial acidity of the treatment; the treatment triggers cytotoxicity and a pH decrease that is in some way related to cell activity. With confirmation that the effects correlate with cell activity, we can now begin considering potential avenues to explore in the future.

The pH decrease following Candidate-X treatment could mirror acid exposure in vitro. While we cannot cite sources to protect the identity of Candidate-X, poisoning with a precursor molecule is known to induce metabolic acidosis in humans as the body processes the chemical into Candidate-X, forcing the concentration of it beyond normal physiological levels. Granules we saw in the cytoplasm may be explained by acidosis [30], though we do not have the expertise to say this confidently. A study of the effects of acid on HT-29 for a disease called Barrett’s esophagus states that:

“acid has a highly variable effect on cell proliferation and differentiation depending on the pattern of acid exposure [....] Chronic acid exposure arrested cell proliferation, whereas a 1 hour acid-pulse enhanced cell proliferation [....] Serum starvation attenuated the hyperproliferative effect of an acid-pulse. In addition, the doubling time of at least the first cell cycle after an acid-pulse was shortened. These results support a role for extracellular pH on cell proliferation and differentiation of HT29 cells.” [31]

These findings indicate that the extracellular pH decrease we observed in our own experiment may have influenced the cells’ metabolism and had an effect on their ability to thrive in the plate. Since we ultimately observed the same effects as HT-29 in HIEC-6, a cell line that closely resembles the premature intestine, we can say it is also possible that intestinal cells in NEC infants experience similar effects. If premature intestinal cells are metabolically immature [5], they may be much more vulnerable to damage and death upon experiencing acid-related metabolic stress; more experiments need to be conducted before we can confidently assert this.
Metabolic acidosis can be accompanied by increased lactic acid production [33], though a study of the effects of bacterial fermentation products on the intestine of live infant mice concluded that: “overproduction/accumulation of short chain fatty acids, but not lactic acid, in the proximal colon and/or distal ileum may play a role in the pathogenesis of necrotizing enterocolitis in premature infants” [10]. We originally hypothesized (based on the slight pH drop at ≥100% confluency in the control) the damage might have something to do with lactic acid and upregulation of the Warburg effect, a metabolic phenomenon wherein tumors and proliferative tissues favor aerobic glycolysis and produce increased amounts of lactic acid [32]; the study’s findings seem to suggest lactic acid is more of a bystander. However, the tests were performed in vivo, Candidate-X was not one of the factors tested, and the noted cell death morphology (inflammation and necrosis) does not match what we observed with Candidate-X. Therefore, it is difficult to compare the findings of the study to our study of Candidate-X. Further experiments will be needed to examine the extent of the Warburg effect’s involvement.

Overall, our project leaves more questions than answers. The final experiment and continued literature search have given rise to our current hypothesis on Candidate-X: that beyond the threshold concentration of 50 mM, Candidate-X treatment in vitro may mimic the effects of in vivo poisoning with a Candidate-X precursor molecule, leading to metabolic acidosis and ultimately cell death with autophagy (a result of cell starvation and a known outcome of acidosis [34, 35, 36]). This could help explain why we see the effects much faster with the cancer cell line than HIEC-6; cancer cells have a shorter doubling time and would likely experience serum starvation more quickly. However, this hypothesis is largely built upon other hypotheses; many layers must be rigorously experimentally explored before it is seriously considered. For example, lactic acid concentrations must be determined and compared to see if there is a change in lactic acid production following treatment with Candidate-X. We may also want to conduct experiments to characterize any changes in expected doubling time. We have yet to conduct more trials and repeat the experiment with HIEC-6. Even then, the in vitro environment we would use neglects many of the dynamic aspects found in vivo, including an influx of nutrients. On a fundamental level, we do not yet understand the complete mechanism behind the pH decrease and cell death (i.e. what about Candidate-X triggers these effects, and if/how they are interrelated).
Chapter 5: Conclusion

5.1 Conclusion and Summary

This project aimed to identify a fecal biomarker for necrotizing enterocolitis (NEC). The disease affects approximately 10% of preterm neonates, or infants with a gestation period of less than 37 weeks [1, 2, 4]. About one in four NEC cases results in death [5]. Currently, diagnosis is based on nonspecific signs, some of which closely resemble sepsis and can lead to misdiagnosis [2, 6]. Our project identified several potential candidates for biomarkers, with focus on the main two detectable in stool. After scrutinizing the candidates and conducting further experiments, the list of candidates was narrowed down to only Candidate-X. Because of intellectual property concerns, this team is unable to release the molecular name of Candidate-X.

Stanford obtained several stool samples from both healthy neonates and neonates with NEC. After UC Davis ran quantitative analytical tests on stool samples, NEC neonates showed a four-fold to ten-fold increase in biomarker concentration compared to healthy (control) infants, suggesting that elevated Candidate-X concentrations in stool could be used as a parameter for the diagnosis of NEC. The experiments we conducted in the Stanford lab build on these results, and furthermore suggest that Candidate-X--given its cytotoxic effects--is involved in the causation of NEC.

Additional verification of our findings is needed and will come through continued research on the topic after this paper is released. Further reading on recent papers from the Sylvester Lab in the Stanford School of Medicine is highly encouraged to have the most up-to-date and accurate findings. So far, all experiments and research point to the validity of Candidate-X. This team was preliminarily successful in identifying a fecal biomarker for NEC; however, further research is needed to confirm specificity. Until then, we recommend that Candidate-X be used in combination with current diagnostic methods and predictive models rather than as a standalone diagnostic parameter.

5.2 Future Steps

We have identified many potential future steps for our project. The team has already begun the first steps of mouse model studies. Besides this, we would also like to obtain more stool samples and order assay kits for Candidate-X to (1) produce our own confirmation of the UC Davis findings, and (2) increase the statistical power of Candidate-X’s correlation with NEC. This would be a straightforward experiment, but it would require waiting for additional stool samples to arrive. Because of the infrequency of collection and small amount of stool sampled from each
infant, gathering enough samples may take several months. Further exploration into the feasibility and timeline of this proposition is required.

Additionally, our experiments used HT-29 cells because of their faster doubling time and should be reperformed with HIEC-6 cells in order to create the most representative in vitro model. Because of the close proximity of the small intestine and colon, a co-culture with both cell types may be a useful in vitro simulation of the native physiological environment. A non-cancerous colon-derived cell line would be more biologically relevant than HT-29 and should be considered as an alternative. However, this type of experiment could be hard to plan due to the different requirements and characteristics of the cell types.

As the previous sections delineate, the colorimetric change in the media with the addition of Candidate-X is clearly correlated with cells and not the media; however, further characterization of the supernatant via LC-MS using SCU’s equipment will provide us with a better understanding of Candidate-X’s cellular interactions. Conducting the testing at SCU will be cheaper than sending it to other offsite labs. This experiment can involve repeating the Chapter 4 experiment with HIEC-6, and collecting the supernatant from each well in both rows (see well plate layout in Figure 2) to create a graph of relative component concentrations over varying cell confluency. We would also like to determine how pH values and lactic acid concentrations change over time. The LC-MS data could give information on lactic acid content, and pH could be measured via pH meter multiple times per day to determine the pattern of acid exposure. This would help us explore the relevance of the Warburg effect and begin to illuminate the metabolic phenomena at work. If we want to study changes in doubling time, we could try to develop a way to compare the amount of cells in the controls to the amounts in the treated wells using cell pellets that would otherwise be discarded after supernatant extraction. To more closely monitor the culture environment and replicate the in vivo environment in vitro, a small perfusion bioreactor could be used instead of culture in plates, but this might be prohibitively expensive (especially since adherent cells would require special materials for culture in suspension).

The molecular underpinnings of Candidate-X in NEC remain unknown, as does the level of specificity of elevated Candidate-X in NEC stool. More in-depth and intensive research was outside the scope, funding, and time constraints of our project, but still serves as a crucial step in validating Candidate-X as a specific biomarker and causative agent in NEC. Should the biologically relevant interactions be characterized and the specificity further confirmed, an appropriate diagnostic tool can be developed through utilizing homogeneous interactions. One proposed method could be based on a colorimetric assay. If the difference in concentration of Candidate-X between healthy and NEC infants is large enough, we may perhaps be able to circumvent the need for expensive detection devices like microplate readers (likely unavailable onsite in most clinical settings) and develop an assay protocol that only requires visual
analysis/comparison. Creating a point-of-care diagnostic tool like this will provide physicians with a method to deliver quick results with sufficient sensitivity and selectivity [37], thus improving NEC patients’ prospects of survival and reallocating resources more efficiently in the healthcare system.
Chapter 6: Engineering Standards

6.1 Economical Concerns

Our project hopes to ultimately decrease the medical costs associated with diagnosing NEC. Currently, physicians order an array of tests aiming to create a differential diagnosis that may be a positive indicator for NEC, but cannot find a solution that is closer to binary; we hope to decrease the number of tests ordered by developing a biomarker detection test that offers physicians a more binary solution. Additionally, we want to be conscientious about the cost of the materials required; we want to avoid extravagant and/or unnecessary spending.

6.2 Manufacturability Concerns

Creating a detection protocol (perhaps a homogenous-phase assay, in this case) for the proposed soluble NEC biomarker is a long-term objective of our project. We hope our device is held to high standards so as to avoid negatively affecting the user. We would need to design a device with a reasonable shelf life so that physicians will not need to worry about needing to regularly discard unused materials. We want to use material that undergoes the appropriate quality assurance testing to ensure that (1) there are no issues associated with our final product and (2) that it maintains its stated efficacy over the course of its shelf life.

6.3 Ethical Concerns

Having a specific biomarker to diagnose NEC will help improve the quality of life of the stakeholders involved. The patient will benefit from more rapid and effective treatment, reducing the duration and severity of pain/suffering and potentially reducing mortality. The physicians will benefit from biomarker-assisted diagnosis because they will need to order fewer tests and be able to allocate their time more effectively. The patient’s parents will benefit because there will ideally be a decreased medical bill as a result of fewer tests being ordered, and also because their newborn will receive the treatment he/she needs more quickly with less long-term consequences. Lastly, insurance companies will benefit as a result of the decreased costs of diagnosis and treatment.

6.4 Health and Safety Concerns

Health and safety is a very important topic in research to ensure that there is no unnecessary risk associated with the chemicals used or experiments conducted. Our group underwent extensive health and safety training at both SCU and Stanford before being able to enter the laboratory
space. Additionally, we followed the proper safety policies and procedures throughout the duration of our experiments as outlined by each university. Our group kept a watchful eye throughout our time in the lab and promptly addressed any safety hazards.

6.5 Political Concerns

With our Senior Design Project addressing biomarker discovery and eventually fulfilling an unmet diagnostic need, it is unlikely that our device will initially be covered in health insurance plans. We hope that legislation is introduced that will require health insurance companies to cover the cost. Currently, there is a strong social backing to improve neonatal care and protect defenseless beings. Hopefully, this social backing can be translated into political pressure to broaden coverage for neonates with impaired conditions.

6.6 Environmental Concerns

Our Senior Design project aimed to be highly cognizant of the material we used during our experiments and aimed to not have a significant and/or toxic effect on the environment. We reused materials whenever possible to reduce waste, planning our methods around this. Part of the team’s training to work in the Stanford lab was being able to identify the characteristics of different chemicals that had indications or contraindications to store in the same container. Additionally, the team made sure to properly dispose of all liquid waste from ELISA, cell culturing, and other experiments and to never discard down a sink drain.

6.7 Sustainability

The sustainability of our project is closely related to the environmental impact it has. Our project wants to have a minimal waste footprint and hopes to use material that can be easily regenerated and replenished. In the long run, our project will promote a more sustainable treatment path for neonates with NEC because of the decreased number of ineffective tests and treatments as a result of rapid diagnosis. Finally, the source of our proposed biomarker, stool, is much more sustainable than blood-sourced.

6.8 Social Concerns

With infants being defenseless beings, there is a strong social backing to support them and promote their well-being. As such, there does not appear to be any negative social concern surrounding this project, but rather support to continue its work and improve neonatal diagnosis and care. Additionally, there is no concern surrounding withholding or delivering improper
treatment to neonates as a result of experiments as we are only aiming to create a noninvasive biomarker detection route for diagnosis.
References


Appendix

Appendix A. Gantt chart summarizing our project timeline.

<table>
<thead>
<tr>
<th>Fall Quarter</th>
<th>Winter Break</th>
<th>Winter Quarter</th>
<th>Spring Quarter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stanford interviews, selection, and approval for project</td>
<td>Complete lab safety and HIPAA training</td>
<td>Conducting experiments at Stanford’s lab (2-3 days per week)</td>
<td>Senior Design presentation</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Writing Thesis</td>
</tr>
</tbody>
</table>
Appendix B. Detailed Profile on Four Initial Factors Plated

(1) TNF-alpha:
TNF-alpha is a proinflammatory cytokine involved in many different cell signalling pathways to necrosis or apoptosis [2, 21]. With the onset of acute inflammation, macrophages and white blood cells produce and secrete TNF-alpha into the blood [21]; in the literature it is noted to be a potentially useful blood biomarker for NEC as an inflammatory disease [2].

(2) LPS:
LPS is an endotoxin existing on the surface of gram-negative bacteria to protect them from damage [22]. In humans, LPS plays a role in sepsis; in mammalian cells, it brings about a strong immune response [22].

(3) H$_2$O$_2$:
As a product of the mitochondrial respiratory chain, H$_2$O$_2$ is involved in cellular metabolism [34]. Abnormally high levels are classified as a source of oxidative stress, which can damage cells and biomolecules [23]. Thus, it can sometimes be used as a biomarker for oxidative stress, and can be detected in urine [24].

(4) Butyrate:
Butyrate, the anion of the short-chain fatty acid called butyric acid, is a fermentation product of bacteria found in the human colon. Butyrate is thought to play a role in maintaining a healthy colon, perhaps by removing damaged cells [12]; it has recently been confirmed to induce necroptosis [5]. Healthy colorectal epithelial cells easily absorb and use butyric acid as an energy source [12], but in NEC babies, who may have an abnormal fatty acid metabolism [9], this beneficial process may become dysfunctional.
Appendix C. Additional Laboratory Protocols

1. Modified ELISA to Measure Insoluble Protein, Cytokeratin-8 (CK8), in Stool

1. 96-well MaxiSorp™ Nunc-immuno module plates (Thermo Fisher Scientific) were pre-coated with HT-29 cells containing CK8 (protein concentration 10 μg/ml) in coating buffer (PBS 50 μl/well) overnight at 4°C.
2. 25 μl samples and standards were diluted in PBS/EDTA buffer and mixed with 25μl blocking buffer (5% skim milk/PBST/2% NP40: 0.25 g milk + 4.5 ml PBST + 0.5 ml 20% NP40) for 30 min with shaking.
3. 50 μl anti-CK8 antibody (Troma-1, 1:4,000, 4 ml PBS/EDTA + 1 μl Troma-1) was added to each tube and incubated at RT for 1 h with shaking.
4. The pre-coated plates (1) were washed 3 times in 1X PBS buffer. Premixed samples with antibody (2,3) were transferred to each well of the pre-coated plates and incubated for 1 h at RT with shaking (100 μl/well), followed by washing 3 times in PBST buffer.
5. 100 μl HRP (horseradish peroxidase)-conjugated goat anti-rat antibodies (Immnuoreagents Inc.) diluted 1:5,000 (v/v) in PBST/5% skim milk buffer was added per well and incubated at RT with shaking for 30 min.
6. Plates were washed 5 times and visualized by incubating in TMB substrate kit (Thermo Fisher Scientific, 100 μl/well) for 15 min.
7. Subsequently, 100 μl 2 M H₂SO₄ was added to each well to stop color development.
8. The plates were read at 450 nm with reference filter at 620 nm using the SpectraMax® i3x multi-mode microplate reader (Molecular Devices, LCC. CA, USA).
9. Final concentrations were obtained using the standard curve method.
Appendix D. Slides from Senior Design Presentation

Noninvasive Screening of a Fecal Biomarker for Human Necrotizing Enterocolitis
Daniela Campos and Kyle Sullivan
Advisors: Dr. Qiao-Zhuang Tao (Stanford) and Dr. Jonathan Zhang (UCSF)

Project Description
Identify a unique and useful fecal biomarker for NEC that can facilitate diagnosis in preterm infants (<37 weeks).

Hypothesis
The biomarker will be a pathological molecule that is found in preterm gastrointestinal epithelial cells with a detectable concentration in stool samples.

Project Significance
- NEC affects 10% of preterm infants; 1/4 cases is fatal
- Pathophysiology (diseased function)
  - Inflamed tissue = tissue death = holes in intestinal wall
- Undetermined etiology (cause)
- Main risk factors: working theory
  - Genetic factors
  - Premature intestinal environment
- Furthering the understanding of NEC underpinnings

Project Significance (cont.)
- Currently difficult to make differential diagnosis
  - NEC biomarker discovery gives a binary solution
  - Decreases time between disease onset and treatment
- Use stool instead of blood or urine samples
  - Biomarkers likely to be more readily found in stool
  - Easier with collecting blood samples
Engineering Standards

- Economic
  - Reduce total neutral costs and enhance research regulatory costs

- Manoeuvrability
  - A longer-term option: Direct route to create a profoundly connected device

- Environmental
  - Impact on automation (SOC/LOC: cell culture, random tests, and other significant determinants)

- Sustenability
  - Use stocks to testing material: Follow Standards for acceptable doses

- Safety
  - Standard of automation and SOC patients used, track facilities

- Health and Safety
  - Use the Lab: mechanisms in place

- Social Aspects
  - Strengthen social support for improving the societal context

- Political Aspects
  - Health policy, increasing coverage of health, an area to consider in future

Preliminary Data

Cell Lines Used

- HIEC-6: Human Intestinal Epithelial Cells
  - Doubling time: ~96h

- HT-29: Human Colon Cancer
  - Doubling time: ~48h

Plating Factors and Studying Effects

- Plated HIEC-6 and HT-29 cells in 96-, 24- well plates (approx. 90% confluence) with factors such as:
  - TNF-alpha, LPS, HGF
  - Butyrate (BA) in acid form, in acid form neutralized with NaOH
  - Candidate-X in acid form, in acid form neutralized with NaOH, and in neutral form salt form
- Cells visualized using optical microscope to analyze morphology over time

Plating Factors of Interest

- 6-well plate with media and factors of interest to see effects on HT-29 cells
  - BA: 10, 50 mM
  - Candidate-X: 50 mM
  - Control
- Reproducible results
Results & Discussion

- Identified Candidate-X as a factor of interest
- Strong correlation between NCC and [Candidate-X]
- Whole samples collected at Stanford but tested at UC Davis (graph is a conceptual summary)

Identifying Factors of Interest

- Studied effects of various factors
  - NCC-6 and HT-29 cells
  - BA resulted in necrotic cell death at 50 mM
  - Candidate-X resulted in most extensive cell death (unknown mode) at 50 mM
  - Not dependent on pH
- Comparison of Candidate-X effects
  - HEC-6 vs. HT-29 (time)
  - Candidate-X vs. BA (time + magnitude)

Morphology of Candidate-X-induced Cell Death

- The morphology of Candidate-X-induced cell death does not fit apoptosis, necrosis, or necroptosis
  - No apoptotic bodies
  - No cell shrinkage, swelling, or autolysis
  - Granules in the cytoplasm
Conclusion

Our results suggest that elevated Candidate-X in stool could be used as a parameter for the diagnosis of NEC.

Future Directions

Discovery Leaves More Questions
- Identify mechanism of Candidate-X-induced cell death
  - Run LC-MS on supernatant (Candidate-X-treated and control)
  - Look into detection methods for models of cell death
  - Consider Warburg effect (cancer cells and proliferative tissues favor aerobic glycolysis) vs. normal gut vs. proliferating environment
- Determine if elevated Candidate-X in infant stool is a result of gut dysbiosis

Thank You

Special thanks to Dr. Zhang and Dr. Tao, Dr. Wang, and Dr. Wei, as well as to Stanford’s School of Medicine.

Questions?

References
