

UNITED STATES  
SECURITIES AND EXCHANGE COMMISSION  
Washington, D.C. 20549

FORM 10-K

ANNUAL REPORT PURSUANT TO SECTION 13 OR 15(d) OF THE SECURITIES EXCHANGE ACT OF 1934

For the Fiscal Year Ended December 31, 2019

TRANSITION REPORT PURSUANT TO SECTION 13 OR 15(d) OF THE SECURITIES EXCHANGE ACT OF 1934

For the transition period from \_\_\_\_\_ to \_\_\_\_\_

Commission File Number: 001-36833

**VOLITIONRX LIMITED**

(Exact name of registrant as specified in its charter)

**Delaware**  
(State or other jurisdiction of  
incorporation or organization)

**13215 Bee Cave Parkway**  
**Suite 125, Galleria Oaks B**  
**Austin, Texas 78738**  
(Address of principal executive offices)  
**+1 (646) 650-1351**  
(Registrant's telephone number, including area code)

**91-1949078**  
(I.R.S. Employer Identification No.)

**Securities registered pursuant to Section 12(b) of the Act:**

<u>Title of Each Class:</u>	<u>Trading Symbol(s)</u>	<u>Name of Each Exchange on Which Registered:</u>
<b>Common Stock, par value \$0.001 per share</b>	<b>VNRX</b>	<b>NYSE American, LLC</b>

**Securities registered pursuant to Section 12(g) of the Act: None**

Indicate by check mark if the registrant is a well-known seasoned issuer, as defined in Rule 405 of the Securities Act.

Yes  No

Indicate by check mark if the registrant is not required to file reports pursuant to Section 13 or Section 15(d) of the Act.

Yes  No

Indicate by check mark whether the registrant (1) has filed all reports required to be filed by Section 13 or 15(d) of the Securities Exchange Act of 1934 during the preceding 12 months (or for such shorter period that the registrant was required to file such reports) and (2) has been subject to such filing requirements for the past 90 days. Yes  No

Indicate by check mark whether the registrant has submitted electronically every Interactive Data File required to be submitted pursuant to Rule 405 of Regulation S-T (§ 232.405 of this chapter) during the preceding 12 months (or for such shorter period that the registrant was required to submit such files). Yes  No

Indicate by check mark whether the registrant is a large accelerated filer, an accelerated filer, a non-accelerated filer, a smaller reporting company, or an emerging growth company. See the definitions of "large accelerated filer," "accelerated filer," "smaller reporting company" and "emerging growth company" in Rule 12b-2 of the Exchange Act:

Large accelerated filer	<input type="checkbox"/>	Accelerated filer	<input type="checkbox"/>
Non-accelerated filer	<input checked="" type="checkbox"/>	Smaller reporting company	<input checked="" type="checkbox"/>
Emerging growth company	<input type="checkbox"/>		

If an emerging growth company, indicate by check mark if the registrant has elected not to use the extended transition period for complying with any new or revised financial accounting standards provided pursuant to Section 13(a) of the Exchange Act.

Indicate by check mark whether the registrant is a shell company (as defined in Rule 12b-2 of the Act). Yes  No

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As of June 28, 2019, the last trading day of the registrant's most recently completed second fiscal quarter, the aggregate market value of the voting common stock held by non-affiliates of the registrant was \$72,468,073 (based upon the \$3.14 per share closing price for the registrant's common stock as reported by the NYSE American on such date). This calculation does not reflect a determination that persons deemed to be affiliates for this purpose are affiliates for any other purpose.

As of February 17, 2020, there were 41,204,685 shares of the registrant's \$0.001 par value common stock issued and outstanding.

**Documents incorporated by reference:**

Portions of the registrant's Proxy Statement for its 2020 Annual Meeting of Stockholders, to be filed on or before April 29, 2020 are incorporated by reference into Part III, Items 10-14 of this Annual Report on Form 10-K.

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## CAUTIONARY NOTE REGARDING FORWARD-LOOKING STATEMENTS

*This Annual Report on Form 10-K for the fiscal year ended December 31, 2019, which we refer to as this Report, contains “forward-looking statements” within the meaning of Section 27A of the Securities Act of 1933, as amended, or the Securities Act, and Section 21E of the Securities Exchange Act of 1934, as amended, or the Exchange Act, which statements are subject to considerable risks and uncertainties. These forward-looking statements are intended to qualify for the safe harbor from liability established by the Private Securities Litigation Reform Act of 1995. All statements other than statements of historical fact included in this Report or incorporated by reference into this Report are forward-looking statements. Throughout this Report, we have attempted to identify forward-looking statements by using words such as “may,” “believe,” “will,” “could,” “project,” “anticipate,” “expect,” “estimate,” “should,” “continue,” “potential,” “plans,” “forecasts,” “goal,” “aim,” “seek,” “intend,” other forms of these words or similar words or expressions or the negative thereof (although not all forward-looking statements contain these words). In particular, forward-looking statements contained in this Report relate to, among other things, any predictions of earnings, revenues, expenses or other financial items; plans or expectations with respect to our development activities or business strategy, including commercialization and market acceptance; statements concerning industry trends and industry size; statements regarding anticipated demand for our products and market opportunity, or the products of our competitors; statements relating to manufacturing forecasts, and the potential impact of our relationship with contract manufacturers and original equipment manufacturers on our business; assumptions regarding the future cost and potential benefits of our research and development efforts; the effect of critical accounting policies; forecasts of our liquidity position or available cash resources; statements relating to the impact of pending litigation; and statements relating to the assumptions underlying any of the foregoing.*

*We have based our forward-looking statements on our current expectations and projections about trends affecting our business and industry and other future events. Although we do not make forward-looking statements unless we believe we have a reasonable basis for doing so, we cannot guarantee their accuracy. Forward-looking statements are subject to substantial risks and uncertainties that could cause our future business, financial condition, results of operations or performance to differ materially from our historical results or those expressed or implied in any forward-looking statement contained in this Report. We discuss these risks and uncertainties in greater detail in the section entitled “Risk Factors” in Part I, Item 1A of this Report, and the other documents that we have filed with the Securities and Exchange Commission, or the SEC.*

*In addition, actual results may differ as a result of additional risks and uncertainties of which we are currently unaware or which we do not currently view as material to our business. For these reasons, readers are cautioned not to place undue reliance on any forward-looking statements.*

*You should read this Report in its entirety, including the documents that we file as exhibits to this Report and the documents that we incorporate by reference into this Report, with the understanding that our future results may be materially different from what we currently expect. The forward-looking statements we make speak only as of the date on which they are made. We expressly disclaim any intent or obligation to update any forward-looking statements after the date hereof to conform such statements to actual results or to changes in our opinions or expectations. If we do update or correct any forward-looking statements, readers should not conclude that we will make additional updates or corrections.*

### **Use of Terms**

Except as otherwise indicated by the context, references in this Report to “Company,” “VolitionRx,” “Volition,” “we,” “us,” and “our” are references to VolitionRx Limited and its wholly-owned subsidiaries, Singapore Volition Pte. Limited, Belgian Volition SPRL, Volition Diagnostics UK Limited and Volition America, Inc., as well as majority-owned subsidiary Volition Veterinary Diagnostics Development LLC. Additionally, unless otherwise specified, all references to “\$” refer to the legal currency of the United States of America.

Nucleosomics<sup>™</sup> and Nu.Q<sup>™</sup> and their respective logos are trademarks and/or service marks of VolitionRx and its subsidiaries. All other trademarks, service marks and trade names referred to in this Report are the property of their respective owners.

## PART I

### ITEM 1. BUSINESS

#### *Overview*

VolitionRx is a multi-national epigenetics company that applies its Nucleosomics™ platform through its subsidiaries to develop simple, easy to use, cost-effective blood tests to help diagnose a range of cancers and other diseases. We hope that through earlier diagnosis we can help save and improve the quality of many people's and animal's lives throughout the world.

#### *Our Solution/ Science*

Our assays are based on the science of Nucleosomics™, which is the practice of identifying and measuring nucleosomes in the bloodstream or other bodily fluid – since changes in these parameters are an indication that disease is present.

#### *Background to Genetics Epigenetics and Cancer*

Human genetics, the sequence of our DNA, is essentially a “recipe book” containing details of how to make each of the thousands of different proteins in the human body; simply put, there is a different gene (or recipe) for each protein. However, just because a recipe is in the book, doesn't mean you have to make it, and nobody makes all the proteins in their DNA. For example, men have all the genes necessary to make ovarian and uterine proteins but do not do so. Similarly, muscle cells do not make liver proteins or kidney proteins. This is because the genes for liver and kidney proteins are “switched off” in muscle cells. The mechanisms for the control of which genes are active or inactive in a cell are collectively known as epigenetics.

There are many different types of cancers but generally the primary cause of each cancer is the mutation within a cell of the DNA encoding or regulating the expression of one or more specific genes called oncogenes. While many mutations can have no consequence, some can lead to the uncontrolled expansion of the mutated cells and their dissemination to other parts of the body from the tissue of origin in a process called metastasis. Another consequence of these mutations is an alteration in the epigenetic regulation of many other genes and this, in turn, can create a unique epigenetic signature in the cancer cells.

Epigenetic control is therefore a critical factor in biology and medicine. A number of epigenetic cancer drugs have been in routine clinical use for more than a decade and the altered epigenetic signature seen in cancer underpins Volition's diagnostic approaches.

A major mechanism for epigenetic control is mediated through chromosome and nucleosome structure. Each chromosome contains a long, single molecule of DNA which is coated by a complex array of proteins, mostly in the form of nucleosomes, giving the stretched-out, unwound DNA/protein core (or chromatin) the appearance of “beads on a string”. Unwound chromatin is accessible for reading (or transcribing) and “unwound” genes may be active. However, genes whose nucleosomes are coiled or supercoiled are inaccessible and inactive.

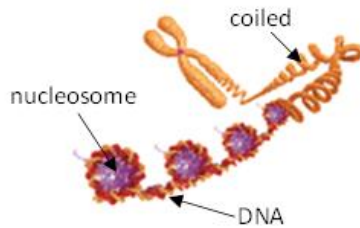


Figure 1 – A chromosome

Each nucleosome consists of a disc of eight histone proteins wrapped by a short length of DNA. Nucleosome structure has a dual role: first, it allows the compact storage and protection of the genetic material (or DNA), and second, it modulates the epigenetic regulation (or transcription) of that DNA. This regulation is achieved through reversible chemical changes to both the DNA and protein components as well as through the binding of specific regulatory proteins to the DNA.

### ***Volition's Epigenetic Approach***

Volition's approach is to investigate the epigenetic structure of chromatin and nucleosomes rather than investigating only the DNA sequence. We are continuously developing new technologies including:

- ① A suite of low cost Nu.Q™ immunoassays that can accurately measure nucleosomes containing numerous epigenetic signals or structure.
- ① Nu.Q™ Capture technology to isolate or enrich nucleosomes containing particular epigenetic signals or structures for a wide range of potential scientific and medical applications. For example, the enrichment of nucleosomes of tumor origin in blood samples taken from cancer patients.
- ① We plan to develop an ability to produce synthetic (recombinant) nucleosomes containing exact defined epigenetic signals and structures. These are used to ensure exquisite accuracy of Nu.Q™ immunoassay tests but also have many other applications including use as tools in epigenetic drug development.

### ***Improving Outcomes for Cancer Patients***

The prospects for cancer patients vary greatly depending on whether the disease is detected at an early localized stage when effective treatment options are available, or at an advanced stage when the disease may have spread, and treatment is much more difficult. Unfortunately, most cancers are symptomless at early stage and most patients are not diagnosed until the disease has spread to other organs in the body and the likely outcome is poor. Simple low-cost immunoassay blood tests to detect cancer at an early stage leading to earlier treatment would greatly improve patient outcomes.

### ***The Limitations of DNA Sequencing in Cancer***

The advent of next generation sequencing has revolutionized medical research and led to a host of medical and other innovations. For example, sequencing the DNA of tumor tissue removed by surgery or biopsy uncovers cancer DNA mutations present in the tumor and is used to direct patient treatment selection, but tissue biopsy cannot be used routinely for cancer detection.

However, small fragments of cancer DNA from dead tumor cells are also found in the blood of cancer patients so it is possible to sequence circulating tumor DNA (ctDNA) in a blood sample taken from a patient to test for any cancer DNA mutations (e.g., mutated P53, KRAS, EGFR). Unfortunately, these ctDNA blood tests, often called liquid biopsy tests, have thus far also proved ineffectual for early stage cancer detection.

The main reasons why ctDNA tests alone have not proved useful for early cancer detection include:

- ① The level of DNA fragments circulating in the blood is very low.
- ① Only a small proportion of the circulating DNA fragments are of tumor origin and the proportion is especially low in early stage cancer (usually less than 1%). The remaining "healthy" DNA fragments originate mainly from dead white blood cells.
- ① A DNA sequence mutation will occur on only one in several million (up to 20 million) of the circulating DNA fragments that do originate from cancer cells.
- ① This means that cancer mutations are found in one in millions of a small percentage of a very low level of circulating DNA fragments, with the result that ctDNA is undetectable in most early stage cancer patients.
- ① Many cancer-like mutations have recently been found to be present in the blood of healthy elderly people through a process known as clonal hematopoiesis. Any DNA released from these cells could lead to false positive readings.

### ***Volition's Epigenetic Approach to Cancer***

Cancer is in essence a disease of genetic and epigenetic mis-regulation of oncogenes and tumor suppressor genes in the chromosomes of affected cells, leading to uncontrolled cell division and eventually to uncontrolled tumor growth and spread. Thus, the epigenetic signaling structures of chromosomes and nucleosomes are different in cancer cells and healthy cells of the same tissue.

When a cancer cell dies, its chromosomes are digested into nucleosomes as shown in the figure below. Most nucleosomes are metabolized, but some are released into the blood stream as circulating nucleosomes. The DNA attached to these nucleosomes is ctDNA.

However, liquid biopsy companies extract only the DNA and discard the remainder of the nucleosome.

Volition analyzes whole circulating nucleosomes containing particular epigenetic signals and structures using our low cost, but highly accurate Nu.Q™ nucleosome immunoassay tests.



Figure 2 – Digestion of a chromosome into nucleosomes.

The epigenetic structure of nucleosomes of cancer origin is known to differ from that of nucleosomes from healthy cells. These epigenetic changes occur early and drive the development of cancer, for example by inappropriately activating oncogenes that promote cell division or inactivating tumor suppressor genes that repress cell division. However, the structural epigenetic changes that occur are not restricted to “1 in 20 million” nucleosomes or even to oncogenes and tumor suppressor genes, but are widely distributed, providing a larger cancer signal, enabling earlier detection of cancer. We use our Nu.Q™ immunoassay tests to detect a variety of early stage cancers.

Circulating cancer nucleosomes also differ from nucleosomes of healthy origin in other ways. For example, the DNA fragments in cancer nucleosomes are approximately 20 base pairs (or about 14%) shorter than the DNA fragments in nucleosomes originating in healthy cells. This structural difference is used as the basis of one of Volition’s Nu.Q™ Capture technologies to separate or enrich cancer nucleosomes by removing nucleosomes of healthy origin. Volition expects that Nu.Q™ Capture technology will further increase the accuracy of its Nu.Q™ immunoassay tests to detect early stage cancers and will also be useful to ctDNA companies to decrease the cost and increase the accuracy of liquid biopsy tests.

### Research and Development

We are developing Nucleosomics™ technologies in a number of areas including:

Adaptation and optimization of Nu.Q™ immunoassay tests across multiple clinical platforms worldwide for the rapid quantification of epigenetic changes in blood and other biofluids. Volition’s Nu.Q™ assays for use in clinical studies operate on an FDA-approved random access immunoassay autoanalyzer using a chemiluminescent magnetic particle-based assay format, a format which has enhanced analytical performance.

Nu.Q™ assays are used for the development of Nu.Q™ blood tests for the most prevalent cancers focusing initially on colorectal cancer, lung cancer and hematological cancers using our Nucleosomics™ biomarker discovery platform. Our development platform includes assays to be used for asymptomatic (screening) subjects, high-risk populations and symptomatic patients. We are developing blood based Nu.Q™ assays to detect specific biomarkers that can be used individually or in combination to generate a profile which forms the basis of a product for a particular cancer or disease.

Nu.Q™ Capture technology to isolate or enrich nucleosomes containing particular epigenetic signals or structures for complete analysis by mass spectrometry, DNA sequencing, immunoassays or other methods for a wide range of potential scientific and medical applications. For example, the enrichment of nucleosomes of tumor origin in blood samples taken from cancer patients for biomarker discovery.

More widespread analysis of circulating chromatin fragments that include epigenetically active chromatin proteins.

In addition to human diagnostics, we are also developing the use of the Nu.Q™ technology in veterinary applications. An initial proof-of-concept study demonstrated that nucleosomes can be detected in dogs and therefore have the potential to differentiate cancer from other diseases. We will now test the Nu.Q™ platform in larger trials in veterinary medicine. Our extensive intellectual property portfolio includes the coverage of veterinary applications.

## **Commercialization Strategy**

We believe that given the global prevalence of cancer and the low-cost, accessible and routine nature of our tests, Nu.Q™ could potentially be used throughout the world. Our launch sequence is determined to a large extent by regulatory hurdles - consequently, we aim to initially launch in Europe and Asia, and subsequently in the United States. We plan to work with partners and/or distributors to commercialize Nu.Q™ worldwide. Additionally, we are working on complete nucleosome analysis in our Nu.Q™ Capture technology. The goal of this project is to investigate ways to specifically target ctDNA. The ability to enrich ctDNA will allow us to use mass spectrometry to analyze histone and DNA modifications and moreover to sequence the DNA present around the nucleosomes. This information might enable cancer diagnosis to identify the tissue of origin of that given cancer.

Commercialization will take multiple forms in various markets and opportunities including, but not limited to:

- ① Licensing of intellectual property for Research Use Only (RUO) sale of Nu.Q™ assays and/or Nu.Q™ Capture reagents;
- ① Licensing of intellectual property for laboratory developed patient testing services utilizing Nu.Q™ assays and/or Nu.Q™ Capture reagents;
- ① Licensing of intellectual property for clinical products utilizing Nu.Q™ assays and/or Nu.Q™ Capture reagents;
- ① Sale of clinical products utilizing Nu.Q™ assays and/or Nu.Q™ Capture reagents through distributor networks;
- ① Direct research services in Nu.Q™ assays and/or Nu.Q™ Capture technology;
- ① Direct veterinary clinical services in Nu.Q™ assays; and
- ① Sale of veterinary clinical products utilizing Nu.Q™ Vet assays and/or Nu.Q™ Capture reagents through distributor networks.

If we do not have enough funds to fully implement our business plan, we will be forced to scale back our plan of operations and our business activities, increase our anticipated timeframes to complete each milestone or seek additional funding. In the event that additional financing is delayed, we will prioritize the maintenance of our research and development personnel and facilities, primarily in Belgium.

## **The Market Opportunity**

Cancer is one of the leading causes of death worldwide, accounting for around 9.5 million annual deaths globally. There are over 18 million new cases of cancer diagnosed each year and given the aging population this is expected to grow rapidly to over 29.5 million new cases annually by 2040. Currently, in the United States there are more than three new cases of cancer diagnosed and one person dies from a cancer-related cause every minute. Statistically, the chances of surviving cancer are greatly improved by early detection and treatment. However, there are currently very few blood tests for diagnosis of cancer in common clinical use.

We believe that early, non-invasive, accurate cancer diagnosis remains a significant unmet medical need and a significant commercial opportunity. For these reasons, cancer diagnostics is an active field of research and development both academically and commercially.

The global in vitro diagnostic medical device, or IVD, market was \$64.5 billion in 2017 and is forecasted to reach \$93.6 billion by 2025, registering a compound annual growth rate, or CAGR, of 4.8% from 2018 to 2025. The forecasted growth is due primarily to the increasing health care demands of an aging population.

The United States is currently the largest veterinary market in the world and has a clearly defined regulatory pathway through the U.S. Department of Agriculture (USDA), requiring fewer and smaller clinical studies than the FDA process for human diagnostics. This generally allows for a much faster route to revenue for veterinary products as compared to human products.

We anticipate that because of their ease of use and cost efficiency, our tests have the potential to become the first method of choice for cancer diagnostics, allowing detection of a range of cancers at an earlier stage than typically occurs currently, and testing of individuals who, for reasons such as time, cost or aversion to current methods, are not currently being tested.

## **Competition**

We anticipate facing competition primarily from healthcare, pharmaceutical and diagnostic companies such as Exact Sciences Corporation, Guardant Health, GRAIL Inc., Freenome Holdings Inc., CellMax Life, Archer DX Inc., Thrive Earlier Detection Corp., Foundation Medicine Inc., Oncocyte Corporation, OpKo Health Inc., MDNA Life Sciences Inc., Oncimmune Holdings Plc, Abbott Laboratories Inc., Cepheid Inc., Koninklijke Philips N.V., GE Healthcare, Siemens, Gen-Probe Incorporated, EpiGenomics AG, MDxHealth SA, and Roche Diagnostics. There may also be other companies developing products competitive with ours of which we are unaware.



We predict that our future products will have a competitive edge compared to those offered by competitors on the basis that our tests are being developed to be accurate, cost-effective and attractive from a government reimbursement perspective, easy to use, non-invasive, technologically advanced, and compatible with immunoassay systems, based on strong intellectual property and to be used for mass screenings.

Many of our competitors have substantially greater financial, technical, and other resources and larger, more established marketing, sales and distribution systems than we have. Many of our competitors also offer broad product lines outside of the diagnostic testing market and have brand recognition. Moreover, our competitors may make rapid technological developments that may result in our intended technologies and products becoming obsolete before we are able to enter the market, recover the expenses incurred to develop them or generate significant revenue. Our success will depend, in part, on our ability to develop our intended products in a timely manner, keep our future products current with advancing technologies, achieve market acceptance of our future products, gain name recognition and a positive reputation in the healthcare industry, and establish successful marketing, sales and distribution efforts.

### ***Government Regulations***

The health care industry, and thus our business, is subject to extensive federal, state, local and foreign regulation. Some of the pertinent laws have not been definitively interpreted by the regulatory authorities or the courts, and their provisions are open to a variety of subjective interpretations. In addition, these laws and their interpretations are subject to change.

Both United States federal and state governmental agencies continue to subject the health care industry to intense regulatory scrutiny, including heightened civil and criminal enforcement efforts. As indicated by work plans and reports issued by these agencies, the federal government will continue to scrutinize, among other things, the marketing, labeling, promotion, manufacturing and export of diagnostic health care products. Our diagnostic products fall within the IVD medical device category and are subject to FDA clearance or approval in the United States.

The federal government also has increased funding in recent years to fight health care fraud, and various agencies, such as the United States Department of Justice, the Office of Inspector General of the Department of Health and Human Services, or OIG, and state Medicaid fraud control units, are coordinating their enforcement efforts.

In Europe, medical devices are regulated by self-certification through the CE mark system. Under the system, developers and manufacturers must operate a Quality System and validate medical devices in a limited clinical trial to demonstrate the manufacturer has met analytical and clinical performance criteria. We have implemented an International Organization for Standardization standard - ISO 13485 - quality management system for the design and manufacture of medical devices. ISO 13485 addresses managerial awareness of regulatory requirements, control systems, inspection and traceability, device design, risk and performance criteria as well as verification for corrective and preventative measures for device failure. Medical device companies such as ours are subject to pre-market compliance assessments from Notified Bodies, a certification organization which the national authority (the competent authority) of a European Union member state designates to carry out one or more of the conformity assessment procedures. ISO 13485 certification establishes conformity to specific European Union directives related to medical devices and allows CE marking and sale of the device.

As of May 25, 2017, the new European In Vitro Diagnostic Regulation (IVDR - 2017/746), or the EU IVDR, became effective, marking the start of a transition period for manufacturers selling IVD devices into Europe. The EU IVDR, which replaces IVD Directive 98/79/EC, has a transition period of five years, after which the EU IVDR will apply in full, and no new applications pursuant to the former Directive will be accepted. Manufacturers have the duration of the five-year transition period to update their technical documentation and processes to meet the new, more stringent European Union regulatory requirements. We believe the most challenging changes under the EU IVDR will be those regarding the classification of products, which will bring almost all IVDs under the direct review and control of Notified Bodies, and the performance evaluation of IVDs, which will require extensive clinical and analytical performance studies but also demonstration of scientific validity. Additional requirements will be applied to reinforce the safety of the products such as extended responsibilities of the economic actors of the supply chain, increased post marketing surveillance activities, unannounced audits from Notified Bodies, implementation of an improved traceability and transparency of the devices with, in particular, the introduction of the Unique Device Identification (UDI) system and an expanded European Database on Medical Devices (referred to as EUDAMED).

Notified Bodies can begin auditing to the EU IVDR once they have been designated as a Notified Body under the EU IVDR by their Competent Authority. For now, we expect the first Notified Bodies to be notified according to the EU IVDR by the end of 2019 and we anticipate that TÜV SÜD will be one of these. In practice, it will not be possible to CE mark a product according to the EU IVDR beforehand. For Class C devices (we expect that our devices will be Class C), the conformity assessment procedure will be a combination of the Quality Management System audits and Technical Documentation assessments. The assumed assessment time needed for a Technical Documentation assessment of a Class C device is expected to last from about 2 months to 6 months. We have already begun discussions with the TÜV SÜD in order to ensure compliance with the EU IVDR as soon as possible.

We will also be required to comply with numerous other federal, state, and local laws relating to matters such as safe working conditions, industrial safety, and labor laws. We may incur significant costs to comply with such laws and regulations in the future, and lack of compliance could have material adverse effects on our operations.

We believe that we have structured our business operations to comply with applicable legal requirements. However, it is possible that governmental entities or other third parties could interpret these laws differently and assert otherwise, which could have a material adverse impact on our business.

### ***Regulatory Approach***

Commercialization of our future products in the clinical IVD market (e.g. for patient diagnosis in hospitals, clinics, etc.) requires government approval (CE marking in Europe, FDA approval in the United States, and Chinese Food and Drug Administration (CFDA) approval in China).

In the United States, we anticipate that our tests will have to be cleared through the FDA's premarket notification or 510(k), process or its premarket approval, or PMA, process. The determination of whether a 510(k) or a PMA is necessary will depend in part on the proposed indications for use and the FDA's assessment of the risk associated with the use of the IVD for a particular indication. A similar system operates in China through the CFDA. In the European Union, our tests can be marketed after a declaration and marking that the test conforms to the essential requirements of the relevant European health, safety and environmental protection legislation, or CE marking. The CE mark is also recognized in certain Asian territories, including India, for the private payer market.

### ***Intellectual Property***

We are working on the development of clinical products based on the enrichment and analysis of epigenetically modified circulating nucleosomes using immunoassay, mass spectrometry, DNA sequencing and other methods. We have used this position to build a patent portfolio around the ability to profile the epigenetic environment surrounding circulating chromosome fragments from diseased cells including the epigenetic signaling status of nucleosomes, DNA, and other epigenetic chromatin proteins.

Our patent portfolio includes 23 patent families and a total of 44 patents granted related to our diagnostic tests (including veterinary applications), with 8 patents granted in the United States, 9 patents granted in Europe and a further 27 patents granted worldwide. Additionally, we have a total of 105 patent applications currently pending, with 13 patent applications in the United States, 10 in Europe and a further 82 worldwide.

We intend to continue our development of the Nucleosomics<sup>TM</sup> technologies and will continue to apply for patents for future product developments. Our strategy is to protect the technologies and gain market exclusivity with patents in Europe and the United States and in other strategic countries. The patents on the technologies underlying our products should provide broad coverage for each product, including protection through at least 2031 for products developed using the Nu.Q-X, Nu.Q-V and Nu.Q-A technologies.

### ***Employees***

As of December 31, 2019, we (including our subsidiaries) had 50 full-time equivalents compared to 44 as of December 31, 2018.

### ***Corporate History***

The Company was incorporated on September 24, 1998 in the State of Delaware under the name "Standard Capital Corporation". On September 22, 2011, the Company filed a Certificate for Renewal and Revival of Charter with the Secretary of State of Delaware. Pursuant to Section 312 of Delaware General Corporation Law, the Company was revived under the new name of "VolitionRX Limited" (which name was subsequently amended to reflect "VolitionRx Limited"). The Company acquired its wholly owned operating subsidiary, Singapore Volition Pte. Limited, a Singapore registered company, or Singapore Volition, on October 6, 2011. Singapore Volition currently has one subsidiary, Belgian Volition SPRL, a Belgium private limited liability company, or Belgian Volition, which it acquired on September 22, 2010. Belgian Volition has three subsidiaries, Volition Diagnostics UK Limited, which was formed on November 13, 2015, Volition America, Inc., which was formed on February 3, 2017, and Volition Veterinary Diagnostics Development LLC, which was formed on June 3, 2019.

Our principal executive office is located at 13215 Bee Cave Parkway, Suite 125, Galleria Oaks B, Austin, Texas 78738. Our telephone number is +1 (646) 650-1351. Our website is located at [www.volition.com](http://www.volition.com). The information that can be accessed through our website is not incorporated by reference into this Report and should not be considered to be a part hereof.

***Financial Information***

See our Consolidated Financial Statements and accompanying Notes to the Consolidated Financial Statements included in this Report.

**WHERE YOU CAN GET ADDITIONAL INFORMATION**

We file Annual Reports on Form 10-K, Quarterly Reports on Form 10-Q, and Current Reports on Form 8-K pursuant to Section 13(a) or 15(d) of the Exchange Act electronically with the SEC. You can access these reports and other filings electronically on the SEC's web site, [www.sec.gov](http://www.sec.gov).

## ITEM 1A. RISK FACTORS

*An investment in our securities involves certain risks, including those set forth below and elsewhere in this Report. In addition to the risks set forth below and elsewhere in this Report, other risks and uncertainties may exist that could adversely affect our business and financial condition. If any of the following risks actually materialize, our business, financial condition and/or operations could suffer. In such event, the value of our common stock could decline, and you could lose all or a substantial portion of your investment. You should carefully consider the risks described below as well as other information and data included in this Report.*

### **Risks Associated with our Company**

***We have not generated any significant revenue since our inception, and we may never achieve profitability.***

We are a clinical stage company and have incurred losses since our formation. As of December 31, 2019, we have an accumulated total deficit of approximately \$89.8 million. As we continue the discovery and development of our future diagnostic products, our expenses are expected to increase significantly. Even as we begin to market and sell our intended products, we expect our losses to continue as a result of ongoing research and development expenses, as well as increased manufacturing, sales and marketing expenses. These losses, among other things, have had and will continue to have an adverse effect on our working capital, total assets and stockholders' equity. Because of the numerous risks and uncertainties associated with our product development and commercialization efforts, we are unable to predict when or if we will become profitable. Even if we do achieve profitability, we may not be able to sustain or increase profitability on a quarterly or annual basis. If we are unable to achieve and then maintain profitability, our business, financial condition and results of operations will be negatively affected, and the market value of our common stock will decline.

***We may need to raise additional capital in the future. If we are unable to secure adequate funds on terms acceptable to us, we may be unable to execute our plan of operations.***

We will require additional capital to fully fund our current strategic plan, which includes successfully commercializing our Nu.Q™ cancer pipeline and developing future products. If we incur delays in commencing commercialization of our Nu.Q™ cancer pipeline or other future products or in achieving significant product revenue, or if we encounter other unforeseen adverse business developments, we may exhaust our capital resources prior to the commencement of commercialization.

We cannot be certain that additional capital will be available when needed or that our actual cash requirements will not be greater than anticipated. Financing opportunities may not be available to us, or if available, may not be available on favorable terms. The availability of financing opportunities will depend on various factors, such as market conditions and our financial condition and outlook. In addition, if we raise additional funds through the issuance of equity or convertible debt securities, the percentage ownership of our stockholders could be significantly diluted, and these newly issued securities may have rights, preferences or privileges senior to those of existing stockholders. If we obtain debt financing, a substantial portion of our operating cash flow may be dedicated to the payment of principal and interest on such indebtedness, and the terms of the debt securities issued could impose significant restrictions on our operations. If we are unable to obtain financing on terms favorable to us, we may be unable to execute our plan of operations and we may be required to cease or reduce development or commercialization of any future products, sell some or all of our technology or assets or merge with another entity.

***It is difficult to forecast our future performance, which may cause our financial results to fluctuate unpredictably.***

Our limited operating history and the rapid evolution of the market for diagnostic products make it difficult for us to predict our future performance. A number of factors, many of which are outside of our control, may contribute to fluctuations in our financial results, such as:

- ① our ability to develop or procure antibodies for clinical use in our future products;
- ② our ability to translate preliminary clinical results to larger prospective symptomatic and screening populations;
- ③ the demand for our intended products;
- ④ our ability to obtain any necessary financing;
- ⑤ our ability to market and sell our future products;
- ⑥ market acceptance of our future products and technology;
- ⑦ performance of any future strategic business partners;
- ⑧ our ability to obtain regulatory clearances or approvals;
- ⑨ our success in collecting payments from third-party payer and customers;
- ⑩ changes in technology that may render our future products uncompetitive or obsolete;
- ⑪ competition with other cancer diagnostics companies; and
- ⑫ adverse changes in the healthcare industry.

***Our future success depends on our ability to retain our officers and directors, scientists, and other key employees and to attract, retain and motivate qualified personnel.***

Our success depends on our ability to attract, retain and motivate highly qualified management and scientific personnel. In particular, we are highly dependent on Cameron Reynolds, our President and Chief Executive Officer, our other officers and directors, scientists and key employees. The loss of any of these persons or their expertise would be difficult to replace and could have a material adverse effect on our ability to achieve our business goals. In addition, the loss of the services of any one of these persons may impede the achievement of our research, development and commercialization objectives by diverting management's attention to the identification of suitable replacements, if any. There can be no assurance that we will be successful in hiring or retaining qualified personnel and our failure to do so could have a material adverse effect on our business, financial condition and results of operations.

Recruiting and retaining qualified scientific personnel and, in the future, sales and marketing personnel will also be critical to our success. We may not be able to attract and retain these personnel on acceptable terms given the competition among pharmaceutical, biotechnology and diagnostic companies for similar personnel. We also experience competition for the hiring of scientific personnel from universities and research institutions. We do not maintain "key person" insurance on any of our employees. In addition, we rely on consultants and advisors, including scientific and clinical advisors, to assist us in formulating our research, development and commercialization strategies. Our consultants and advisors, however, may have other commitments or employment that may limit their availability to us.

***We expect to expand our product development, research and sales and marketing capabilities, and as a result, we may encounter difficulties in managing our growth, which could disrupt our operations.***

We are focused on developing our pipeline for future products. Our efforts will result in significant growth in the number of our consultants, advisors, and employees and the scope of our operations. In order to manage our anticipated future growth, we must continue to implement and improve our managerial, operational and financial systems, expand our facilities, and continue to recruit and train additional qualified personnel. Due to our limited resources, we may not be able to effectively manage the expansion of our operations or recruit and train additional qualified personnel. The expansion of our operations may lead to significant costs and may divert our management and business development resources. Any inability to manage growth could delay the execution of our business plan or disrupt our operations.

***We have limited experience with direct sales and marketing and any failure to build and manage a direct sales and marketing team effectively, or to successfully engage third party providers for such services, could have a material adverse effect on our business.***

Our products will require several dynamic and evolving sales models tailored to different worldwide markets, users and products. In 2015, we decided to focus our sales strategy on the clinical IVD market with the CE marking of our first product in Europe. Following CE marking of our first product in Europe we intend to enter the European markets and, following the completion of any necessary regulatory clearances, certain Asian markets. Even when we have received a CE mark, we must still seek regulatory clearance in other jurisdictions. A failure to obtain these regulatory clearances in other jurisdictions could negatively affect our business. Pending completion of our review of the regulatory environment in the United States, including the effect of recent pronouncements regarding Laboratory Developed Tests, or LDTs, by the FDA, we may decide to enter the United States market through a CLIA certified laboratory located in the United States. We remain firmly committed to pursuing FDA approval as our primary objective. FDA approval can consist of PMA or 510(k) clearance depending on the test complexity and risk posed to patients. We intend to pursue the most appropriate approval pathway for each individual product developed. We intend to progressively grow to large volumes of tests sold to centralized laboratories and eventually reach the mass diagnostics testing market. The exact nature of the ideal sales strategy will evolve as we continue to develop our intended products and seek entry into the IVD markets. We have limited experience with direct sales and marketing and we currently intend to engage a network of distributors to help commercialize our products worldwide. Any failure to build and manage a direct sales and marketing team effectively, or to successfully engage third party providers for such services, could have a material adverse effect on our business.

There are significant risks involved in building and managing our sales and marketing organization, as well as identifying and negotiating deals with the right sales and distribution partners, including risks related to our ability to:

- ① identify appropriate partners;
- ① negotiate beneficial partnership and distribution agreements;
- ① hire qualified individuals as needed;
- ① generate sufficient leads within our targeted market for our sales force;
- ① provide adequate training for effective sales and marketing;
- ① protect intellectual property rights;
- ① retain and motivate our direct sales and marketing professionals; and
- ① effectively oversee geographically dispersed sales and marketing teams.

Our failure to adequately address these risks could have a material adverse effect on our ability to increase sales and use of our future products, which would cause our revenues to be lower than expected and harm our results of operations.

***Our Second Amended and Restated Certificate of Incorporation exculpates our officers and directors from certain liability to our Company and our stockholders.***

Our Second Amended and Restated Certificate of Incorporation contains a provision limiting the liability of our officers and directors for their acts or failures to act, except for acts involving intentional misconduct, fraud or a knowing violation of law. This limitation on liability may reduce the likelihood of derivative litigation against our officers and directors and may discourage or deter our stockholders from suing our officers and directors based upon breaches of their duties to our Company.

***We have identified material weaknesses in our internal control over financial reporting that have not yet been remediated, and the failure to address these material weaknesses, or the identification of any others, could impact the reliability of our financial reporting and harm investors' views of us, which could adversely impact our stock price.***

Our management is responsible for establishing and maintaining adequate internal control over financial reporting. As defined in Exchange Act Rule 13a-15(f), internal control over financial reporting is a process designed by, or under the supervision of, the principal executive and principal financial officer and effected by the board of directors, management and other personnel, to provide reasonable assurance regarding the reliability of financial reporting and the preparation of financial statements for external purposes in accordance with generally accepted accounting principles and includes those policies and procedures that:

- ① pertain to the maintenance of records that in reasonable detail accurately and fairly reflect our transactions and dispositions of assets;
- ① provide reasonable assurance that transactions are recorded as necessary to permit preparation of financial statements in accordance with generally accepted accounting principles, and that our receipts and expenditures are being made only in accordance with authorizations of our management and/or directors; and
- ① provide reasonable assurance regarding prevention or timely detection of unauthorized acquisition, use or disposition of our assets that could have a material effect on the financial statements.

We have determined that we have material weaknesses in our internal control over financial reporting as of December 31, 2019. See *Item 9A. Controls and Procedures* of this Report for a complete discussion of these material weaknesses in our internal control over financial reporting and remediation efforts. Although we are undertaking steps to address these material weaknesses, the existence of a material weakness is an indication that there is more than a remote likelihood that a material misstatement of our financial statements will not be prevented or detected in the current or any future period. There can be no assurance that we will be able to fully implement our plans and controls, as further described in *Item 9A*, to address these material weaknesses, or that the plans and controls, if implemented, will be successful in fully remediating these material weaknesses. In addition, we may in the future identify further material weaknesses in our internal control over financial reporting that we have not discovered to date. If we fail to successfully remediate the identified material weaknesses, or we identify further material weaknesses in our internal controls, the market's confidence in our financial statements could decline and the market price of our common stock could be adversely impacted. Additionally, for so long as we remain as a smaller reporting company, under current rules our accounting firm will not be required to provide an opinion regarding our internal controls over financial reporting.

***We have a "going concern" opinion from our auditors, indicating the possibility that we may not be able to continue to operate.***

Our independent registered public accountants have expressed substantial doubt about our ability to continue as a going concern. This opinion could materially limit our ability to raise additional funds by issuing new debt or equity securities or otherwise. If we fail to raise sufficient capital when needed, we will not be able to complete our proposed business plan. As a result, we may have to liquidate our business and investors may lose their investments. Our ability to continue as a going concern is dependent upon our ability to successfully accomplish our plan of operations described herein, obtain financing and eventually attain profitable operations. Investors should consider our independent registered public accountant's comments when deciding whether to invest in the Company.

***Our management has broad discretion over the use of our available cash and might not spend available cash in ways that increase the value of your investment.***

As of December 31, 2019, we had \$16,966,168 in combined cash and cash equivalents compared to \$13,427,222 as of December 31, 2018. Our management currently expects to deploy these resources primarily to expand our commercialization activities, to fund our product development efforts and for general corporate and working capital purposes. However, our management has broad discretion to pursue other objectives. You will be relying on the judgment of our management regarding the application and prioritization of our resources. Our management might not apply our cash in ways that increase or permit any return of your investment.

## Risks Associated with our Business

***Failure to successfully develop, manufacture, market, and sell our future products will have a material adverse effect on our business, financial condition, and results of operations.***

We are in the process of developing a suite of diagnostic tests as well as additional products. The successful development and commercialization of our intended products is critical to our future success. Our ability to successfully develop, manufacture, market, and sell our future products is subject to a number of risks, many of which are outside our control. There can be no assurance that we will be able to develop and manufacture products in commercial quantities at acceptable costs, successfully market any products, or generate revenues from the sale of any products. Failure to achieve any of the foregoing would have a material adverse effect on our business, financial condition, and results of operations.

***Our business is dependent on our ability to successfully develop and commercialize diagnostic products. If we fail to develop and commercialize diagnostic products, we may be unable to execute our plan of operations.***

Our current business strategy focuses on discovering, developing and commercializing diagnostic products. The success of our business will depend on our ability to fully develop and commercialize the diagnostic products in our current development pipeline as well as continue the discovery and development of other diagnostics products.

Prior to commercializing the Nu.Q™ tests and other diagnostic products, we will be required to undertake time-consuming and costly development activities with uncertain outcomes, including conducting clinical studies and obtaining regulatory clearance or approval in the United States, Asia and in Europe. Delays in obtaining approvals and clearances could have material adverse effects on us and our ability to fully carry out our plan of operations. We have limited experience in taking products through these processes and there are considerable risks involved in these activities. The science and methods that we are employing are innovative and complex, and it is possible that our development programs will ultimately not yield products suitable for commercialization or government approval. Products that appear promising in early development may fail to be validated in subsequent studies, and even if we achieve positive results, we may still fail to obtain the necessary regulatory clearances or approvals. Few research and development projects result in commercial products, and perceived viability in early clinical studies often is not replicated in later studies. At any point, we may abandon development of a product, or we may be required to expend considerable resources obtaining additional clinical and nonclinical data, which would adversely impact the timing for generating potential revenue from those products. Further, our ability to develop and launch diagnostic tests is dependent on our receipt of substantial additional funding. If our discovery and development programs yield fewer commercial products than we expect, we may be unable to execute our business plan, and our business, financial condition and results of operations may be adversely affected.

***The results of pre-clinical studies and completed clinical trials are not necessarily predictive of future results, and our current product candidates may not have favorable results in later studies or trials which, in turn, could have a material adverse effect on our business.***

As described above, we must conduct extensive testing of our product candidates and new indications of our marketed products before we can obtain regulatory approval to market and sell them. Success in pre-clinical studies or completed clinical trials does not ensure that later studies or trials, including continuing pre-clinical studies and large-scale clinical trials, will be successful nor does it necessarily predict future results. Favorable results in early studies or trials may not be repeated in later studies or trials, and product candidates in later stage trials may fail to show acceptable safety and efficacy despite having progressed through earlier trials. We may be required to demonstrate through large, long-term outcome trials that our product candidates are safe and effective for use in a broad population prior to obtaining regulatory approval. The failure of clinical trials to demonstrate the safety and effectiveness of our clinical candidates for the desired indication(s) would preclude the successful development of those candidates for such indication(s), in which event our business, prospects, results of operations and financial condition may be adversely affected.

***Our failure to obtain necessary regulatory clearances or approvals on a timely basis would significantly impair our ability to distribute and market our future products on the clinical IVD market.***

We are subject to regulation by the FDA in the United States, the Conformité Européenne in Europe, the CFDA in China, and other regulatory bodies in other countries where we intend to sell our future products. Before we are able to place our intended products in the clinical IVD markets in the United States, China and Europe, we will be required to obtain clearance or approval of our future products from the FDA and the CFDA with respect to the United States and China, respectively, and receive a CE mark with respect to Europe.

The European Union has recently adopted regulations that may impose additional requirements to obtain a CE mark, which could result in delays and further expense, in terms of staff costs to us as compared to the current CE mark process. The new regulations will require each product submission to be thoroughly audited by Notified Bodies, instead of the current self-certification process. The European Medical Device Regulations (EU MDR) will be fully applicable in 2020 and the EU IVDR will be fully applicable in 2022.

Additionally, even if we receive the required government clearance or approval of our intended products, we are still subject to continuing regulation and oversight. Under the FDA, diagnostics are considered medical devices and are subject to ongoing controls and regulations, including inspections, compliance with established manufacturing practices, device-tracking, record-keeping, advertising, labeling, packaging, and compliance with other standards. The process of complying with such regulations with respect to current and new products can be costly and time-consuming. Failure to comply with these regulations could have a material adverse effect on our business, financial condition, and results of operations. Furthermore, any FDA regulations governing our future products are subject to change at any time, which may cause delays and have material adverse effects on our operations. In Europe, IVD companies are currently able to self-certify that they meet the appropriate regulatory requirements (which are subject to change with the EU MDR and the EU IVDR noted above) but are subject to inspection for enforcement. European national agencies, such as customs authorities and/or the Departments of Health, Industry and Labor, conduct market surveillance to ensure the applicable requirements have been met for products marketed within the European Union.

***Reductions or changes in reimbursement policies could limit our ability to sell our products.***

Market acceptance and sales of our products will depend, in part, on reimbursement policies and may be affected by healthcare reform measures. Government authorities and third-party payers, such as private health insurers and health maintenance organizations, decide which products they will pay for and establish reimbursement levels for those products. To manage healthcare costs, many governments and third-party payers in the United States increasingly scrutinize the pricing of new products and require greater levels of evidence of favorable clinical outcomes and cost-effectiveness before extending coverage. We cannot be sure that reimbursement will be available for our products and, if reimbursement is available, the level of such reimbursement. Reimbursement may impact the demand for, or the price of, our products. If reimbursement is not available or is available only at limited levels, we may not be able to successfully commercialize our future products.

***If the marketplace does not accept the products in our development pipeline or any other diagnostic products we might develop, we may be unable to generate sufficient revenue to sustain and grow our business.***

Our intended products may never gain significant acceptance in the research or clinical marketplace and therefore may never generate substantial revenue or profits. Physicians, hospitals, clinical laboratories, researchers or others in the healthcare industry may not use our future products unless they determine that they are an effective and cost-efficient means of detecting and diagnosing cancer. If our research and studies do not satisfy providers, payors and others as to the reliability and effectiveness, we may experience reluctance or refusal on the part of the physician to use our future products. In addition, we will need to expend a significant amount of resources on marketing and educational efforts to create awareness of our future products and to encourage their acceptance and adoption. If the market for our future products does not develop sufficiently or the products are not accepted, our revenue potential will be harmed.

***The cancer diagnostics market is highly competitive and subject to rapid technological change; accordingly, we will face fierce competition and our intended products may become obsolete.***

The cancer diagnostics market is extremely competitive and characterized by evolving industry standards and new product enhancements. Cancer diagnostic tests are technologically innovative and require significant planning, design, development, and testing at the technological, product, and manufacturing process levels. These activities require significant capital commitments and investment. There can be no assurance that our intended products or proprietary technologies will remain competitive following the introduction of new products and technologies by competing companies within the industry. Furthermore, there can be no assurance that our competitors will not develop products that render our future products obsolete or that are more effective, accurate or can be produced at lower costs. There can be no assurance that we will be successful in the face of increasing competition from new technologies or products introduced by existing companies in the industry or by new companies entering the market.

***We expect to face intense competition from companies with greater resources and experience than us, which may increase the difficulty for us to achieve significant market penetration.***

The market for cancer diagnostics is intensely competitive, subject to rapid change, and significantly affected by new product introductions and other market activities of industry participants. Our competitors include large multinational corporations and their operating units, including Exact Sciences Corporation, Guardant Health, GRAIL Inc., Freenome Holdings Inc., CellMax Life, Archer DX Inc., Thrive Earlier Detection Corp., Foundation Medicine Inc., Oncocyte Corporation, OpKo Health Inc., MDNA Life Sciences Inc., Oncimmune Holdings Plc, Abbott Laboratories Inc., Cepheid Inc., Koninklijke Philips N.V., GE Healthcare, Siemens, Gen-Probe Incorporated, EpiGenomics AG, MDxHealth SA, and Roche Diagnostics. There may also be other companies developing products competitive with ours of which we are not aware. Many of our competitors have greater resources than us and may enjoy several competitive advantages, including:



- ① significantly greater name recognition;
- ② established relationships with healthcare professionals, companies and consumers;
- ③ additional lines of products, and the ability to offer rebates or bundle products to offer higher discounts or incentives to gain a competitive advantage;
- ④ established supply and distribution networks; and
- ⑤ greater resources for product development, sales and marketing, and intellectual property protection.

Many of these other companies have developed and will continue to develop new products that will compete directly with our future products. In addition, many of our competitors spend significantly greater funds for the research, development, promotion, and sale of new and existing products. These resources may allow them to respond more quickly to new or emerging technologies and changes in consumer requirements. We also face competition in our search for third parties to assist us with sales and marketing of our product candidates, which may negatively impact our ability to enter into favorable sales and marketing arrangements. For all the foregoing reasons, we may not be able to compete successfully against our competitors.

***Declining global economic or business conditions may have a negative impact on our business.***

Concerns over United States healthcare reform legislation and energy costs, geopolitical issues, the availability and cost of credit and government stimulus programs in the United States and other countries may contribute to increased volatility and diminished expectations for the global economy. If the economic climate deteriorates, our business, including our access to the Research Use Only, or RUO, or clinical IVD markets for diagnostic tests, could be adversely affected, resulting in a negative impact on our business, financial condition and results of operations.

On June 23, 2016, the United Kingdom held a referendum in which voters approved an exit from the European Union, commonly referred to as “Brexit”. On March 29, 2017, the country formally notified the European Union of its intention to withdraw pursuant to Article 50 of the Lisbon Treaty, and withdrawal negotiations began in June 2017. The United Kingdom’s withdrawal from the European Union rules became effective on January 31, 2020. Existing trade rules will continue to apply through December 31, 2020 (subject to extension), during which the United Kingdom and the European Union will negotiate the rules that will govern their economic relationship following such period. The negotiations between the parties have yet to produce an overall structure for their ongoing relationship following Brexit. Although it is unknown what those terms will be, it is possible that there will be greater restrictions on imports and exports between the European Union countries and the United Kingdom and increased regulatory complexities. These changes may adversely affect our ability to market our future products in the United Kingdom which could have an adverse effect on our business, financial condition, and results of operations.

***We will rely on third parties to manufacture and supply our intended products. Any problems experienced by these third parties could result in a delay or interruption in the supply of our intended products to our customers, which could have a material negative effect on our business.***

We will rely on third parties to manufacture and supply our intended products. The manufacture of our intended diagnostic products will require specialized equipment and utilize complicated production processes that would be difficult, time-consuming and costly to duplicate. If the operations of third-party manufacturers are interrupted or if they are unable to meet our delivery requirements due to capacity limitations or other constraints, we may be limited in our ability to fulfill our future sales orders. Any prolonged disruption in the operations of third-party manufacturers could have a significant negative impact on our ability to sell our future products, could harm our reputation and could cause us to seek other third-party manufacturing contracts, thereby increasing our anticipated development and commercialization costs. In addition, if we are required to change manufacturers for any reason, we will be required to verify that the new manufacturer maintains facilities and procedures that comply with quality standards required by the FDA and with all applicable regulations and guidelines. The delays associated with the verification of a new manufacturer could negatively affect our ability to develop products or receive approval of any products in a timely manner.

***The manufacturing operations of our future third-party manufacturers will likely be dependent upon third-party suppliers, making us vulnerable to supply shortages and price fluctuations, which could harm our business.***

The operations of our future third party manufacturers will likely be dependent upon third-party suppliers. A supply interruption or an increase in demand beyond a supplier’s capabilities could harm the ability of our future manufacturers to manufacture our intended products until new sources of supply are identified and qualified.

Reliance on these suppliers could subject us to a number of risks that could harm our business, including:

- ① interruption of supply resulting from modifications to or discontinuation of a supplier's operations;
- ① delays in product shipments resulting from uncorrected defects, reliability issues, or a supplier's variation in a component;
- ① a lack of long-term supply arrangements for key components with our suppliers;
- ① inability to obtain adequate supply in a timely manner, or to obtain adequate supply on commercially reasonable terms;
- ① difficulty and cost associated with locating and qualifying alternative suppliers for components in a timely manner;
- ① production delays related to the evaluation and testing of products from alternative suppliers, and corresponding regulatory qualifications;
- ① delay in delivery due to suppliers prioritizing other customer orders over ours;
- ① damage to our brand reputation caused by defective components produced by the suppliers; and
- ① fluctuation in delivery by the suppliers due to changes in demand from us or their other customers.

Any interruption in the supply of components of our future products or materials, or our inability to obtain substitute components or materials from alternate sources at acceptable prices in a timely manner, could impair our ability to meet the demand of our future customers, which would have an adverse effect on our business.

***We will depend on third-party distributors in the future to market and sell our future products which will subject us to a number of risks.***

We will depend on third-party distributors to sell, market, and service our future products in our intended markets. We are subject to a number of risks associated with reliance upon third-party distributors including:

- ① lack of day-to-day control over the activities of third-party distributors;
- ① third-party distributors may not commit the necessary resources to market and sell our future products to our level of expectations;
- ① third-party distributors may terminate their arrangements with us on limited or no notice or may change the terms of these arrangements in a manner unfavorable to us; and
- ① disagreements with our future distributors could result in costly and time-consuming litigation or arbitration which we could be required to conduct in jurisdictions with which we are not familiar.

If we fail to establish and maintain satisfactory relationships with our future third-party distributors, our revenues and market share may not grow as anticipated, and we could be subject to unexpected costs which could harm our results of operations and financial condition.

***If the patents that we rely on to protect our intellectual property prove to be inadequate, our ability to successfully commercialize our future products will be harmed and we may never be able to operate our business profitably.***

Our success depends, in large part, on our ability to protect proprietary methods, discoveries and technologies that we develop under the patents and intellectual property laws of the United States, Europe and other countries, so that we can seek to prevent others from unlawfully using our inventions and proprietary information. Our patent portfolio includes 23 patent families related to our diagnostic tests, with 8 patents granted in the United States, 9 patents granted in Europe and a further 27 patents granted worldwide. Additionally, we have 13 patent applications currently pending in the United States, 10 in Europe and a further 82 worldwide.

If we are not able to protect our proprietary technology and information, our competitors may use our inventions to develop competing products. We cannot assure you that any of the pending patent applications will result in patents being issued. In addition, due to technological changes that may affect our future products or judicial interpretation of the scope of our patents, our intended products might not, now or in the future, be adequately covered by our patents.

***If third parties assert that we have infringed their patents and proprietary rights or challenge the validity of our patents and proprietary rights, we may become involved in intellectual property disputes and litigation that would be costly, time consuming, and delay or prevent the development or commercialization of our future products.***

Our ability to commercialize our intended products depends on our ability to develop, manufacture, market and sell our future products without infringing the proprietary rights of third parties. Third parties may allege that our future products or our methods or discoveries infringe their intellectual property rights. Numerous United States and foreign patents and pending patent applications, which are owned by third parties, exist in fields that relate to our intended products and our underlying methodologies, discoveries and technologies. A third party may sue us for infringing its patent rights.

Our ability to successfully commercialize our intended products depends on our ability to protect our proprietary technology and information. Likewise, we may need to resort to litigation to enforce a patent issued or licensed to us or to determine the scope and validity of third-party proprietary rights. In addition, a third party may claim that we have improperly obtained or used its confidential or proprietary information. The cost to us of any litigation or other proceeding relating to intellectual property rights, even if resolved in our favor, could be substantial, and the litigation could divert our management's attention from other aspects of our business. Some of our competitors may be able to sustain the costs of complex patent litigation more effectively than we can because they have substantially greater resources. Uncertainties resulting from the initiation and continuation of any litigation could limit our ability to continue our operations. Additionally, we cannot be certain of the level of protection, if any that will be provided by our patents if they are challenged in court, where our competitors may raise defenses such as invalidity, unenforceability or possession of a valid license.

If we are found to infringe upon intellectual property rights of third parties, we might be forced to pay damages, potentially including triple damages. In addition to any damages we might have to pay, a court could require us to stop the infringing activity or obtain a license. Any license required under any patent may not be made available on commercially acceptable terms, if at all. In addition, such licenses are likely to be non-exclusive and, therefore, our competitors may have access to the same technology licensed to us. If we fail to obtain a required license and are unable to design around a patent, we may be unable to effectively market some or all of our future products, which could limit our ability to generate revenue or achieve profitability and possibly prevent us from generating revenue sufficient to sustain our operations.

***If we are unable to protect our trade secrets, we may be unable to protect our interests in proprietary technology, processes and know-how that is not patentable or for which we have elected not to seek patent protection.***

In addition to patented technology, we rely upon trade secret protection to protect our interests in proprietary know-how and for processes for which patents are difficult or impossible to obtain or enforce. We may not be able to protect our trade secrets adequately. Although we use reasonable efforts to protect our trade secrets, our employees, consultants, contractors and outside scientific advisors may unintentionally or willfully disclose our information to competitors. Enforcing a claim that a third party illegally obtained and is using any of our trade secrets is expensive and time consuming, and the outcome is unpredictable. In addition, courts outside the United States are sometimes less willing to protect trade secrets. We rely, in part, on non-disclosure and confidentiality agreements with our employees, consultants and other parties to protect our trade secrets and other proprietary technology. These agreements may be breached, and we may not have adequate remedies for any breach. Moreover, others may independently develop equivalent proprietary information, and third parties may otherwise gain access to our trade secrets and proprietary knowledge. Any disclosure of confidential information into the public domain or to third parties could allow our competitors to learn our trade secrets and use the information in competition against us, which could adversely affect our competitive advantage.

***Defects in our products may subject us to substantial damages which could materially harm our business or financial condition.***

The products we develop could lead to product liability claims based on allegations that one or more of our products contained a design or manufacturing defect which resulted in the failure to detect the disease for which it was designed. A product liability claim could result in substantial damages and be costly and time consuming to defend, either of which could materially harm our business or financial condition. We cannot assure you that our product liability insurance would protect our assets from the financial impact of defending a product liability claim. Any product liability claim brought against us, with or without merit, could increase our product liability insurance rates or prevent us from securing insurance coverage in the future.

#### **Risks Associated with our Common Stock**

***The market prices and trading volume of our stock may be volatile.***

The market price of our common stock is likely to be highly volatile and the trading volume may fluctuate and cause significant price variation to occur. We cannot assure you that the market prices of our common stock will not fluctuate or decline significantly in the future. Some of the factors that could negatively affect the prices of our shares or result in fluctuations in those prices or in trading volume of our common stock could include the following, many of which will be beyond our control:

- ④ competition;
- ④ comments by securities analysts regarding our business or prospects;
- ④ additions or departures of key personnel;
- ④ our ability to execute our business plan;
- ④ issuance of common stock or other securities;
- ④ operating results that fall below expectations;
- ④ loss of any strategic relationship;
- ④ industry developments;
- ④ economic and other external factors; and
- ④ period-to-period fluctuations in our financial results.

In addition, the securities markets have from time to time experienced significant price and volume fluctuations that are unrelated to the operating performance of particular companies. These market fluctuations may also materially and adversely affect the market price and trading volume of our common stock.

***Share ownership by our executive officers and directors make it more difficult for third parties to acquire us or effectuate a change of control that might be viewed favorably by other stockholders.***

As of February 17, 2020, our executive officers and directors beneficially owned, in the aggregate, approximately 14.9% of our outstanding shares. As a result, if the executive officers and directors were to oppose a third party's acquisition proposal for, or a change in control of, the Company, such officers and directors may have sufficient voting power to be able to block or at least delay such an acquisition or change in control from taking place, even if other stockholders would support such a sale or change of control.

***Our corporate governance documents, and certain corporate laws applicable to us, could make a takeover attempt, which may be beneficial to our stockholders, more difficult.***

Our Board of Directors, or Board, has the power, under our charter documents to:

- ① issue additional shares of common stock without having to obtain stockholder approval for such action;
- ① fill vacant directorships except for vacancies created by the removal of a director;
- ① amend our bylaws without stockholder approval subject to certain exceptions; and
- ① require compliance with an advance notice procedure with regard to business to be brought by a stockholder before an annual or special meeting of stockholders and with regard to the nomination by stockholders of candidates for election as directors.

These provisions may discourage potential acquisition proposals and could delay or prevent a change of control, including under circumstances in which our stockholders might otherwise receive a premium over the market price of our common stock.

***We do not expect to pay dividends in the foreseeable future.***

We have never declared or paid cash dividends on our common stock. We do not intend to declare dividends for the foreseeable future, as we anticipate that we will reinvest any future earnings in the development and growth of our business. Therefore, investors will not receive any funds unless they sell their common stock, and stockholders may be unable to sell their shares on favorable terms or at all. We cannot assure you of a positive return on investment or that you will not lose the entire amount of your investment in our common stock.

***We may in the future issue additional shares of our common stock which would reduce investors' ownership interests in the Company, and which may cause our stock price to decline.***

Our Second Amended and Restated Certificate of Incorporation authorizes the issuance of 100,000,000 shares of common stock, par value \$0.001 per share. The future issuance of all or part of our remaining authorized common stock may result in substantial dilution in the percentage of our common stock held by our then existing stockholders. We may value any common stock issued in the future on an arbitrary basis. The issuance of common stock for future services or acquisitions or other corporate actions may have the effect of diluting the percentage ownership of our stockholders and, depending upon the prices at which such shares are sold or issued, on their investment in our common stock and, therefore, could have an adverse effect on any trading market for our common stock.

***Future sales of our common stock could depress the market price of our common stock.***

Sales of a substantial number of shares of our common stock in the public market or the perception that large sales of our shares could occur, could cause the market price of our common stock to decline or limit our future ability to raise capital through an offering of equity securities.

***If equity research analysts do not publish research or reports about our business, or if they do publish such reports but issue unfavorable commentary or downgrade our common stock, the price and trading volume of our common stock could decline.***

The trading market for our common stock could be affected by whether and to what extent equity research analysts publish research or reports about us and our business. If one or more equity analysts cover us and publish research reports about our common stock, the price of our stock could decline rapidly if one or more securities analysts downgrade our stock or if those analysts' issue or offer unfavorable commentary or cease publishing reports about us. If any of these analysts ceases coverage of us, we could lose visibility in the market, which in turn could cause our common stock price or trading volume to decline and our common stock to be less liquid.

*We are a smaller reporting company and a non-accelerated filer and we cannot be certain if the reduced disclosure requirements applicable to our filing status, as well as the exemption from the requirement to provide an auditor's attestation report regarding the effectiveness of our internal controls, will make our common stock less attractive to investors.*

We are currently a "smaller reporting company," meaning that we are not an investment company, an asset-backed issuer, or a majority-owned subsidiary of a parent company that is not a smaller reporting company and have a public float of less than \$250 million measured as of the last business day of our most recently completed second fiscal quarter. "Smaller reporting companies" are able to provide simplified executive compensation disclosures in their filings and have certain other decreased disclosure obligations in their SEC filings, including, among other things, only being required to provide two years of audited financial statements in annual reports. We are also a "non-accelerated filer," meaning we have a public float of less than \$75 million measured as of the last business day of our most recently completed second fiscal quarter. As a "non-accelerated filer," we are exempt from the provisions of Section 404(b) of the Sarbanes-Oxley Act requiring that independent registered public accounting firms provide an attestation report on the effectiveness of internal control over financial reporting. Decreased disclosures in our SEC filings due to our status as a "smaller reporting company" and as a "non-accelerated filer" may make it harder for investors to analyze our results of operations and financial prospects and may make our common stock a less attractive investment.

**ITEM UNRESOLVED STAFF COMMENTS**

**1B.**

None.

**ITEM 2. PROPERTIES**

Listed below are our current facilities as of December 31, 2019:

<u>Location</u>	<u>Primary Function</u>	<u>Approx. Square Feet</u>	<u>Leased or Owned</u>
Namur, Belgium <sup>(1)</sup>	Research and development	17,300	Owned
London, UK <sup>(2)</sup>	Sales and marketing	690	Leased, expiring 2021
Shaw Centre, Singapore <sup>(3)</sup>	Sales and marketing	150	Leased, expiring 2020
Austin, Texas <sup>(4)</sup>	Executive suite	1,228	Leased, expiring 2022

- (1) Belgian Volition purchased property located in Namur, Belgium, in October 2016, to be used as a laboratory facility for R&D. The purchase price for the property was €1.2 million Euros, exclusive of any closing costs.
- (2) Volition Diagnostics UK signed a two-year lease for this property located at 93-95 Gloucester Place, London, W1U 6JQ, United Kingdom, commencing January 30, 2019, at an annual rent of £118,800 GBP.
- (3) Singapore Volition signed a one-year lease for this property, commencing August 1, 2019, located at 1 Scotts Road, #24-05 Shaw Centre, Singapore 228208, at an annual rent of SGD 29,508.
- (4) VolitionRx Limited signed a three-year lease for this property, commencing on June 1, 2019, located at 13215 Bee Cave Parkway, Suite 125, Galleria Oaks B, Austin, Texas 78738, at an annual rent of \$34,384.

**ITEM 3. LEGAL PROCEEDINGS**

In the ordinary course of business, we may be subject to claims, counter claims, suits and other litigation of the type that generally arise from the conduct of our business. We are not aware of any threatened or pending litigation that we expect will have a material adverse effect on our business operations, financial condition or results of operations.

**ITEM 4. MINE SAFETY DISCLOSURES**

Not Applicable.

## PART II

### ITEM 5. MARKET FOR THE REGISTRANT'S COMMON EQUITY, RELATED STOCKHOLDER MATTERS AND ISSUER PURCHASES OF EQUITY SECURITIES

#### *Market Information*

Our common stock is currently traded on the NYSE American under the symbol "VNRX".

#### *Holders*

As of February 17, 2020, there were 41,204,685 shares of our common stock outstanding held by 153 holders of record, based on information provided by our transfer agent. The actual number of stockholders is greater than this number of record holders and includes stockholders who are beneficial owners but whose shares are held in street name by brokers and other nominees.

#### *Dividends*

We have not declared or paid any cash dividends on our common stock since inception and presently anticipate that all earnings, if any, will be retained for development of our business and that no dividends on our common stock will be declared in the foreseeable future. Any future dividends will be subject to the discretion of our board of directors and will depend upon, among other things, future earnings, operating and financial conditions, capital requirements, general business conditions and other pertinent facts. Therefore, there can be no assurance that any dividends on our common stock will be paid in the future.

#### *Securities Authorized for Issuance Under Equity Compensation Plans*

The information required under this item is incorporated by reference from our definitive proxy statement related to our 2020 Annual Meeting of Stockholders, to be filed pursuant to Regulation 14A, on or before April 29, 2020.

#### *Recent Sales of Unregistered Securities*

From October 1, 2019 through December 31, 2019, we sold the following securities on an unregistered basis for which disclosure under Item 701 of Regulation S-K was not previously provided in a Form 10-Q or Form 8-K filed with the SEC:

On November 15, 2019, 4,167 stock options were exercised to purchase shares of our common stock at \$5.00 per share in a cashless exercise that resulted in the issuance of 371 shares of our common stock.

From November 25, 2019 to November 27, 2019, warrants to purchase 29,392 shares of our common stock were exercised at a price of \$2.40 per share, for gross proceeds to the Company of \$70,541.

We did not utilize any underwriters for any of the sales of securities on an unregistered basis. We relied on an exemption to the registration requirements of the federal securities laws pursuant to Section 4(a)(2) of the Securities Act and Regulation D promulgated thereunder for each of the sales of securities on an unregistered basis. At the time of their issuance, unless registered for resale under an effective registration statement filed with the SEC, the shares were deemed to be restricted securities for purposes of the Securities Act and the certificates representing the shares, if any, and the transfer agent's books shall bear legends to that effect.

#### *Repurchase of Equity Securities*

None.

### ITEM 6. SELECTED FINANCIAL DATA

We are currently a smaller reporting company and are not required to disclose this information.

**ITEM 7. MANAGEMENT'S DISCUSSION AND ANALYSIS OF FINANCIAL CONDITION AND RESULTS OF OPERATIONS**

**Overview**

We have identified the specific processes and resources required to achieve the near and medium-term objectives of our business plan, including personnel, facilities, equipment, research and testing materials including antibodies and clinical samples, and the protection of intellectual property. To date, operations have proceeded satisfactorily in relation to our business plan. However, it is possible that some resources will not readily become available in a suitable form or on a timely basis or at an acceptable cost. It is also possible that the results of some processes may not be as expected, and that modifications of procedures and materials may be required. Such events could result in delays to the achievement of the near and medium-term objectives of our business plan, in particular the progression of clinical validation studies and regulatory approval processes for the purpose of bringing products to the IVD market.

Our future as an operating business will depend on our ability to obtain sufficient capital contributions, financing and/or generate revenues as may be required to sustain our operations. Management plans to address the above as needed by: (a) securing additional grant funds; (b) obtaining additional equity or debt financing; (c) granting licenses to third parties in exchange for specified up-front and/or back end payments; and (d) developing and commercializing our products on an accelerated timeline. Management continues to exercise tight cost controls to conserve cash.

Our ability to continue as a going concern is dependent upon our accomplishment of the plans described in the preceding paragraph and eventually to attain profitable operations. The accompanying consolidated financial statements do not include any adjustments that might be necessary if we are unable to continue as a going concern. If we are unable to obtain adequate capital, we could be forced to cease operations.

**Liquidity and Capital Resources**

We have financed our operations since inception primarily through private placements and public offerings of our common stock. As of December 31, 2019, we had cash and cash equivalents of \$16,966,168.

Net cash used in operating activities was \$12.7 million and \$14.7 million for the years ended December 31, 2019 and December 31, 2018, respectively. The decrease in cash used in operating activities during 2019 was primarily due to reduced research and development activities together with lower charges for stock-based compensation offset by increased personnel expenses.

Net cash used in investing activities was \$0.5 million and \$0.3 million for the years ended December 31, 2019 and December 31, 2018, respectively. The increase in cash used in investing activities during 2019 was primarily a result of increased purchases of laboratory equipment for our research and development facility in Belgium.

Net cash provided by financing activities was \$16.9 million and \$18.0 million for the years ended December 31, 2019 and December 31, 2018, respectively. The decrease in cash provided by financing activities during 2019 was due to less capital raised from debt and equity financing as well as reduced debt payments during such period. During 2019, the Company received \$16.6 million in net proceeds from the issuance of common stock plus debt funding of \$0.9 million, offset by debt payments of \$0.4 million.

The following table summarizes our approximate contractual payments due by year as of December 31, 2019.

**Approximate Payments (Including Interest) Due by Years**

Description	Total	2020	2021 - 2024	2025 +
	\$	\$	\$	\$
Financing lease liabilities	812,497	114,649	252,517	445,331
Operating lease liabilities	418,906	281,965	136,941	-
Grants repayable	337,286	52,879	166,046	118,361
Long-term debt	3,164,547	777,648	2,212,861	174,038
Collaborative agreements obligations	2,688,267	1,699,767	988,500	-
Total	<u>7,421,503</u>	<u>2,926,908</u>	<u>3,756,865</u>	<u>737,730</u>

We intend to use our cash reserves to predominantly fund further research and development activities. We do not currently have any substantial source of revenues and expect to rely on additional future financing, through the sale of equity or debt securities, or the sale of licensing rights, to provide sufficient funding to execute our strategic plan. There is no assurance that we will be successful in raising further funds.

In the event that additional financing is delayed, we will prioritize the maintenance of our research and development personnel and facilities, primarily in Belgium, and the maintenance of our patent rights. In such instance, the completion of clinical validation studies and regulatory approval processes for the purpose of bringing products to the IVD market would be delayed. In the event of an ongoing lack of financing, it may be necessary to discontinue operations, which will adversely affect the value of our common stock.

We have not attained profitable operations and are dependent upon obtaining financing to pursue any extensive activities. For these reasons, our auditors stated in their report on our audited financial statements for the fiscal year ended December 31, 2019 an explanatory paragraph regarding factors that raise substantial doubt that we will be able to continue as a going concern.

### **Results of Operations**

#### **Comparison of the Years Ended December 31, 2019 and December 31, 2018**

The following table sets forth our results of operations for the years ended on December 31, 2019 and December 31, 2018, respectively (expressed in United States Dollars, except outstanding share numbers and percentages).

	<b>2019</b>	<b>2018</b>	<b>Increase (Decrease)</b>	<b>Percentage Increase (Decrease)</b>
	<b>\$</b>	<b>\$</b>	<b>\$</b>	<b>%</b>
Service	16,204	-	16,204	100%
Royalty	892	-	892	100%
<b>Total Revenues</b>	<b>17,096</b>	<b>-</b>	<b>17,096</b>	<b>100%</b>
Research and development	10,363,253	10,906,871	(543,618)	(5%)
General and administrative	4,731,054	5,821,072	(1,090,018)	(19%)
Sales and marketing	965,713	1,169,756	(204,043)	(17%)
Total Operating Expenses	16,060,020	17,897,699	(1,837,679)	(10%)
Grant income	155,031	-	(155,031)	100%
Interest income	112,367	-	(112,367)	100%
Interest expense	(126,572)	(110,924)	15,648	14%
Other expenses	(196,957)	-	196,957	100%
Total Other Income (Expenses)	(56,131)	(110,924)	(54,793)	(49%)
Net Loss	(16,099,055)	(18,008,623)	(1,909,568)	(11%)
Net Loss per Share – Basic and Diluted	(0.41)	(0.57)	(0.16)	(28%)
Weighted Average Shares Outstanding - Basic and Diluted	39,180,369	31,389,220	7,791,149	25%

### **Revenues**

Our operations are still predominantly in the research and development stage and we had minimal revenues of \$17,096 and \$Nil during the years ended December 31, 2019 and December 31, 2018, respectively.



## Operating Expenses

Total operating expenses decreased to \$16.1 million from \$17.9 million for the years ended December 31, 2019 and December 31, 2018, respectively, as a result of the factors described below.

## Research and Development Expenses

Research and development expenses decreased to \$10.4 million from \$10.9 million for the years ended December 31, 2019 and December 31, 2018, respectively. The decrease in overall research and development expenditures during 2019 was primarily related to lower research and collaborative expenditures, lower chemical and biological costs partly offset by increased laboratory costs.

	2019	2018	Change
	\$	\$	\$
Personnel expenses	3,833,289	2,917,147	916,142
Stock based compensation	410,178	811,902	(401,724)
Direct research and development expenses	4,619,515	5,309,172	(689,657)
Other research and development	809,585	1,265,967	(456,382)
Depreciation and amortization	690,686	602,683	88,003
Total research and development expenses	<u>10,363,253</u>	<u>10,906,871</u>	<u>(543,618)</u>

## General and Administrative Expenses

General and administrative expenses decreased to \$4.7 million from \$5.8 million for the years ended December 31, 2019 and December 31, 2018, respectively. The decrease in overall general and administrative expenditures during 2019 were primarily due to favorable foreign exchange costs, reduced legal costs in relation to our capital raises and reduced stock-based compensation expenses.

	2019	2018	Change
	\$	\$	\$
Personnel expenses	2,185,349	2,199,866	(14,517)
Stock-based compensation	868,762	1,505,900	(637,138)
Legal and professional fees	1,180,876	1,188,554	(7,678)
Other general and administrative	284,341	889,519	(605,178)
Depreciation and amortization	211,726	37,233	174,493
Total general and administrative expenses	<u>4,731,054</u>	<u>5,821,072</u>	<u>(1,090,018)</u>

## Sales and Marketing Expenses

Sales and marketing expenses decreased to \$1.0 million from \$1.2 million for the years ended December 31, 2019 and December 31, 2018, respectively. The decrease in overall sales and marketing expenditures was primarily due to reduced stock-based compensation and personnel expenses.

	2019	2018	Change
	\$	\$	\$
Personnel expenses	586,207	673,430	(87,223)
Stock-based compensation	188,173	275,069	(86,896)
Direct marketing and professional fees	191,333	221,257	(29,924)
Total sales and marketing expenses	<u>965,713</u>	<u>1,169,756</u>	<u>(204,043)</u>

## Other Expenses

Other expenses decreased to \$56,131 compared to \$110,924 for the years ended December 31, 2019 and December 31, 2018, respectively. This decrease was primarily due the exercise of warrants to purchase approximately 1.7 million shares of our common stock by Cotterford Company Limited during 2019 at an amended exercise price of \$2.90 per share, which resulted in a \$196,957 expense, offset by interest income received from cash deposited in an overnight money market account and grant income received.

## **Net Loss**

For the year ended December 31, 2019, the Company's net loss was \$16.1 million, a decrease of approximately \$1.9 million, or 11%, in comparison to a net loss of \$18.0 million for the year ended December 31, 2018. The change was a result of the factors described above.

## ***Going Concern***

We have not attained profitable operations and are dependent upon obtaining external financing to continue to pursue our operational and strategic plans. For these reasons, management has determined that there is substantial doubt that the business will be able to continue as a going concern without further financing.

## ***Off-Balance Sheet Arrangements***

We have no significant off-balance sheet arrangements that have or are reasonably likely to have a current or future effect on our financial condition, changes in financial condition, revenues or expenses, results of operations, liquidity, capital expenditures or capital resources that are material to stockholders.

## ***Future Equity or Debt Financings***

We may seek to obtain additional capital through the sale of debt or equity securities, if we deem it desirable or necessary. However, we may be unable to obtain such additional capital when needed, or on terms favorable to us or our stockholders, if at all. If we raise additional funds by issuing equity securities, the percentage ownership of our stockholders will be reduced, stockholders may experience additional dilution, or such equity securities may provide for rights, preferences or privileges senior to those of the holders of our common stock. If additional funds are raised through the issuance of debt securities, the terms of such securities may place restrictions on our ability to operate our business.

## ***Critical Accounting Policies***

Our financial statements and accompanying notes have been prepared in accordance with United States generally accepted accounting principles, or U.S. GAAP, applied on a consistent basis. The preparation of consolidated financial statements in conformity with U.S. GAAP requires management to make estimates and assumptions that affect the reported amounts of assets and liabilities, the disclosure of contingent assets and liabilities at the date of the financial statements and the reported amounts of revenues and expenses during the reporting periods.

We regularly evaluate the accounting policies and estimates that we use to prepare our consolidated financial statements. A complete summary of these policies is included in the notes to our consolidated financial statements. In general, management's estimates are based on historical experience, on information from third party professionals, and on various other assumptions that are believed to be reasonable under the facts and circumstances. Actual results could differ from those estimates made by management.

We consider the following accounting policies to be critical:

## ***Stock-Based Compensation***

The Company records stock-based compensation in accordance with ASC 718, "*Compensation – Stock Compensation*" and ASC 505-50, "*Equity-Based Payments to Non-Employees*". All transactions in which goods or services are the consideration received for the issuance of equity instruments are accounted for based on the fair value of the consideration received or the fair value of the equity instrument issued, whichever is more reliably measurable. Equity instruments issued to employees and the cost of the services received as consideration are measured and recognized based on the fair value of the equity instruments issued and are recognized over the employees required service period, which is generally the vesting period.

### ***Impairment of Long-Lived Assets***

In accordance with ASC 360, “*Property Plant and Equipment*”, the Company tests long-lived assets or asset groups for recoverability when events or changes in circumstances indicate that their carrying amount may not be recoverable. Circumstances which could trigger a review include, but are not limited to: significant decreases in the market price of the asset; significant adverse changes in the business climate or legal factors; accumulation of costs significantly in excess of the amount originally expected for the acquisition or construction of the asset; current period cash flow or operating losses combined with a history of losses or a forecast of continuing losses associated with the use of the asset; and current expectation that the asset will more likely than not be sold or disposed of significantly before the end of its estimated useful life. Recoverability is assessed based on the carrying amount of the asset and its fair value which is generally determined based on the sum of the undiscounted cash flows expected to result from the use and the eventual disposal of the asset, as well as specific appraisal in certain instances. An impairment loss is recognized when the carrying amount is not recoverable and exceeds fair value. Impairment losses of \$nil and \$nil were recognized during the years ended December 31, 2019 and December 31, 2018, respectively.

### ***Foreign Currency Translation***

The Company has functional currencies in the Euro, the United States Dollar and British Pounds Sterling and its reporting currency is the United States Dollar. Management has adopted ASC 830-20, “*Foreign Currency Matters – Foreign Currency Transactions*”. All assets and liabilities denominated in foreign currencies are translated using the exchange rate prevailing at the balance sheet date. For revenues and expenses, the weighted average exchange rate for the period is used. Gains and losses arising on translation of foreign currency denominated transactions are included in Other Comprehensive Income.

### ***Use of Estimates***

The Company bases its estimates and assumptions on current facts, historical experiences and various other factors that it believes to be reasonable under the circumstances, the results of which form the basis for making judgments about the carrying values of assets and liabilities and the accrual of costs and expenses that are not readily apparent from other sources. The actual results experienced by the Company may differ materially and adversely from the Company’s estimates. To the extent there are material differences between the estimates and the actual results, future results of operations could be affected.

### ***Recently Issued Accounting Pronouncements***

The Company has implemented all applicable new accounting pronouncements that are in effect. The Company does not believe that there are any other applicable new accounting pronouncements that have been issued that might have a material impact on its financial position or results of operations.

### **ITEM 7A. QUANTITATIVE AND QUALITATIVE DISCLOSURES ABOUT MARKET RISK**

We are currently a smaller reporting company and are not required to disclose this information.

**VOLITIONRX LIMITED**

**Consolidated Financial Statements**

For the Years Ended December 31, 2019 and 2018

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## **REPORT OF INDEPENDENT REGISTERED PUBLIC ACCOUNTING FIRM**

To the Board of Directors and Shareholders of VolitionRx Limited:

### **Opinion on the Financial Statements**

We have audited the accompanying consolidated balance sheets of VolitionRx Limited (“the Company”) as of December 31, 2019 and 2018, the related consolidated statements of operations and comprehensive loss, stockholders’ equity, and cash flows for each of the years in the two-year period ended December 31, 2019 and the related notes (collectively referred to as the “financial statements”). In our opinion, the financial statements referred to above present fairly, in all material respects, the financial position of the Company as December 31, 2019 and 2018, and the results of its operations and its cash flows for each of the years in the two-year period ended December 31, 2019, in conformity with accounting principles generally accepted in the United States of America.

### **Explanatory Paragraph Regarding Going Concern**

The accompanying financial statements have been prepared assuming that the Company will continue as a going concern. As discussed in Note 2 to the financial statements, the Company has incurred losses since inception, has negative cash flows from operations, and has generated minimal revenues. These factors raise substantial doubt about its ability to continue as a going concern. Management's plans in regard to these matters are also described in Note 2. The financial statements do not include any adjustments that might result from the outcome of this uncertainty.

### **Basis for Opinion**

These financial statements are the responsibility of the Company’s management. Our responsibility is to express an opinion on the Company’s financial statements based on our audits. We are a public accounting firm registered with the Public Company Accounting Oversight Board (United States) (“PCAOB”) and are required to be independent with respect to the Company in accordance with the U.S. federal securities laws and the applicable rules and regulations of the Securities and Exchange Commission and the PCAOB.

We conducted our audits in accordance with the standards of the PCAOB. Those standards require that we plan and perform the audit to obtain reasonable assurance about whether the financial statements are free of material misstatement, whether due to error or fraud. The Company is not required to have, nor were we engaged to perform, an audit of its internal control over financial reporting. As part of our audits, we are required to obtain an understanding of internal control over financial reporting, but not for the purpose of expressing an opinion on the effectiveness of the Company’s internal control over financial reporting. Accordingly, we express no such opinion.

Our audits included performing procedures to assess the risks of material misstatement of the financial statements, whether due to error or fraud, and performing procedures that respond to those risks. Such procedures included examining on a test basis, evidence regarding the amounts and disclosures in the financial statements. Our audits also included evaluating the accounting principles used and significant estimates made by management, as well as evaluating the overall presentation of the financial statements. We believe that our audits provide a reasonable basis for our opinion.

*/s/ Sadler Gibb & Assoc.*

We have served as the Company’s auditor since 2011.

Salt Lake City, UT  
February 20, 2020

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**VOLITIONRX LIMITED**  
Consolidated Balance Sheets  
(Expressed in United States Dollars, except share numbers)

	<b>December 31, 2019</b>	<b>December 31, 2018</b>
	<u>\$</u>	<u>\$</u>
<b>ASSETS</b>		
<u>Current Assets</u>		
Cash and cash equivalents	16,966,168	13,427,222
Prepaid expenses	267,518	245,441
Other current assets	322,593	229,755
<b>Total Current Assets</b>	<u>17,556,279</u>	<u>13,902,418</u>
Property and equipment, net	2,981,225	3,119,643
Operating lease right-of-use assets	381,483	-
Intangible assets, net	372,305	466,905
<b>Total Assets</b>	<u>21,291,292</u>	<u>17,488,966</u>
<b>LIABILITIES AND STOCKHOLDERS' EQUITY</b>		
<u>Current Liabilities</u>		
Accounts payable	627,253	807,162
Accrued liabilities	2,168,588	923,034
Management and directors' fees payable	21,979	1,200
Current portion of long-term debt	647,569	416,553
Current portion of financing lease liabilities	97,946	145,150
Current portion of operating lease liabilities	257,244	-
Current portion of grant repayable	39,295	40,094
<b>Total Current Liabilities</b>	<u>3,859,874</u>	<u>2,333,193</u>
Long-term debt, net of current portion	2,195,278	1,984,262
Financing lease liabilities, net of current portion	607,708	720,013
Operating lease liabilities, net of current portion	131,875	-
Grant repayable, net of current portion	297,991	311,042
<b>Total Liabilities</b>	<u>7,092,726</u>	<u>5,348,510</u>
<b>STOCKHOLDERS' EQUITY</b>		
Common Stock		
Authorized: 100,000,000 shares of common stock, at \$0.001 par value		
Issued and outstanding: 41,125,303 shares and 35,335,378 shares, respectively	41,125	35,335
Additional paid-in capital	103,853,627	85,604,271
Accumulated other comprehensive income	125,670	223,651
Accumulated deficit	(89,821,856)	(73,722,801)
<b>Total Stockholders' Equity</b>	<u>14,198,566</u>	<u>12,140,456</u>
<b>Total Liabilities and Stockholders' Equity</b>	<u>21,291,292</u>	<u>17,488,966</u>

(The accompanying notes are an integral part of these consolidated financial statements)

**VOLITIONRX LIMITED**  
Consolidated Statements of Operations and Comprehensive Loss  
(Expressed in United States Dollars, except share numbers)

	For the year ended	
	December 31, 2019	December 31, 2018
	\$	\$
<b>Revenues</b>		
Service	16,204	-
Royalty	892	-
<b>Total Revenues</b>	17,096	-
<b>Operating Expenses</b>		
Research and development	10,363,253	10,906,871
General and administrative	4,731,054	5,821,072
Sales and marketing	965,713	1,169,756
<b>Total Operating Expenses</b>	16,060,020	17,897,699
<b>Operating Loss</b>	(16,042,924)	(17,897,699)
<b>Other Income (Expenses)</b>		
Grant income	155,031	-
Interest income	112,367	-
Interest expense	(126,572)	(110,924)
Other expenses	(196,957)	-
<b>Total Other Income (Expenses)</b>	(56,131)	(110,924)
<b>Net Loss</b>	(16,099,055)	(18,008,623)
<b>Other Comprehensive Income (Loss)</b>		
Foreign currency translation adjustments	(97,981)	352,994
<b>Net Comprehensive Loss</b>	(16,197,036)	(17,655,629)
<b>Net Loss per Share – Basic and Diluted</b>	(0.41)	(0.57)
<b>Weighted Average Shares Outstanding – Basic and Diluted</b>	39,180,369	31,389,220

(The accompanying notes are an integral part of these consolidated financial statements)

**VOLITIONRX LIMITED**  
Consolidated Statement of Stockholders' Equity  
For the Years Ended December 31, 2019 and 2018  
(Expressed in United States Dollars, except share numbers)

	Common Stock		Additional Paid-in Capital \$	Accumulated Other Comprehensive Income (Loss) \$	Accumulated Deficit \$	Total \$
	Shares #	Amount \$				
Balance, December 31, 2017	26,519,394	26,519	65,774,870	(129,343)	(55,714,178)	9,957,868
Common stock issued for cash, net	8,804,153	8,804	17,236,542	-	-	17,245,346
Common stock issued for cashless exercise of warrants	11,831	12	(12)	-	-	-
Employee stock options granted for services	-	-	2,570,095	-	-	2,570,095
Warrants granted for services	-	-	22,776	-	-	22,776
Foreign currency translation	-	-	-	352,994	-	352,994
Net loss for the year	-	-	-	-	(18,008,623)	(18,008,623)
Balance, December 31, 2018	35,335,378	35,335	85,604,271	223,651	(73,722,801)	12,140,456
Common stock issued for cash, net	5,787,067	5,787	16,585,289	-	-	16,591,076
Common stock issued for cashless exercise of stock options	2,858	3	(3)	-	-	-
Employee stock options granted for services	-	-	1,458,607	-	-	1,458,607
Warrants granted for services	-	-	8,506	-	-	8,506
Modification of financing warrants	-	-	196,957	-	-	196,957
Foreign currency translation	-	-	-	(97,981)	-	(97,981)
Net loss for the year	-	-	-	-	(16,099,055)	(16,099,055)
Balance, December 31, 2019	41,125,303	41,125	103,853,627	125,670	(89,821,856)	14,198,566

(The accompanying notes are an integral part of these consolidated financial statements)



**VOLITIONRX LIMITED**  
Consolidated Statements of Cash Flows  
(Expressed in United States Dollars)

	For the year ended	
	December 31, 2019	December 31, 2018
	\$	\$
Operating Activities:		
Net loss	(16,099,055)	(18,008,623)
Adjustments to reconcile net loss to net cash used in operating activities:		
Depreciation and amortization	676,815	636,380
Amortization of operating lease right-of-use assets	225,597	-
Loss on disposal of property and equipment	-	403
Stock based compensation	1,458,607	2,570,095
Warrants issued for services	8,506	22,776
Financing costs for warrants modified	196,957	-
Changes in operating assets and liabilities:		
Prepaid expenses	(22,080)	10,012
Other current assets	(92,838)	(29,910)
Accounts payable and accrued liabilities	1,105,211	100,037
Management and directors' fees payable	20,779	(34,197)
Operating leases liabilities	(217,954)	-
Net Cash Used In Operating Activities	<u>(12,739,455)</u>	<u>(14,733,027)</u>
Investing Activities:		
Purchases of property and equipment	(511,266)	(301,805)
Net Cash Used In Investing Activities	<u>(511,266)</u>	<u>(301,805)</u>
Financing Activities:		
Net proceeds from issuance of common shares	16,591,076	17,245,346
Proceeds from grants repayable	32,795	177,079
Proceeds from long-term debt	838,039	1,159,836
Payments on long-term debt	(351,009)	(436,784)
Payments on grants repayable	(39,335)	(40,877)
Payments on financing leases	(142,039)	(137,513)
Net Cash Provided By Financing Activities	<u>16,929,527</u>	<u>17,967,087</u>
Effect of foreign exchange on cash and cash equivalents	<u>(139,860)</u>	<u>378,704</u>
Net Change in Cash and Cash Equivalents	3,538,946	3,310,959
Cash and Cash Equivalents – Beginning of Year	<u>13,427,222</u>	<u>10,116,263</u>
Cash and Cash Equivalents – End of Year	<u><u>16,966,168</u></u>	<u><u>13,427,222</u></u>
Supplemental Disclosures of Cash Flow Information:		
Interest paid	126,572	110,924
Income tax paid	<u>-</u>	<u>-</u>
Non - Cash Financing Activities:		
Common Stock issued on cashless exercises of stock options and warrants	3	12
Finance lease obligations	-	28,605
Offering costs from issuance of common stock	-	872,571

(The accompanying notes are an integral part of these consolidated financial statements)

**VOLITIONRX LIMITED**  
**Notes to Consolidated Financial Statements**  
**For Years Ended December 31, 2019 and 2018**  
**(\$ expressed in United States Dollars)**

**Note 1 - Nature of Operations**

The Company was incorporated under the laws of the State of Delaware on September 24, 1998. On September 22, 2011, the Company filed a Certificate for Renewal and Revival of Charter with the Secretary of State of Delaware. Pursuant to Section 312(1) of the Delaware General Corporation Law, the Company was revived under the new name of "VolitionRX Limited" and the name change became effective on October 11, 2011. On October 7, 2016, the Company amended its Certificate of Incorporation to reflect a name change to "VolitionRx Limited."

On October 6, 2011, the Company entered into a share exchange agreement with Singapore Volition Pte. Limited, a Singapore corporation incorporated on August 5, 2010 ("Singapore Volition"), and the shareholders of Singapore Volition. Pursuant to the terms of the share exchange agreement, the former shareholders of Singapore Volition held 85% of the issued and outstanding common shares of the Company. The issuance was deemed to be a reverse acquisition for accounting purposes and as such, Singapore Volition is regarded as the predecessor of the Company. The number of shares outstanding and per share amounts of the Company have been restated to recognize the foregoing recapitalization.

The Company's principal business objective through its subsidiaries is to develop and bring to market simple, easy to use, cost effective blood tests designed to help diagnose a range of cancers and other diseases. The tests are based on the science of Nucleosomics, which is the practice of identifying and measuring nucleosomes in the bloodstream or other bodily fluid – an indication that disease is present. The Company has one wholly owned subsidiary, Singapore Volition. Singapore Volition has one wholly owned subsidiary, Belgian Volition SPRL, a Belgium private limited liability company formerly known as ValiBioSA ("Belgian Volition"), which it acquired as of September 22, 2010. Belgian Volition has three subsidiaries, Volition Diagnostics UK Limited ("Volition Diagnostics"), which was formed as of November 13, 2015, Volition America, Inc. ("Volition America"), which was formed as of February 3, 2017, as well as its majority-owned subsidiary Volition Veterinary Diagnostics Development LLC ("Volition Vet"), which was formed as of June 3, 2019. Following the acquisition of Singapore Volition in 2011, the Company's fiscal year end was changed from August 31 to December 31.

**Note 2 - Going Concern**

The Company's Consolidated Financial Statements are prepared using accounting principles generally accepted in the United States of America ("U.S. GAAP") applicable to a going concern which contemplates the realization of assets and liquidation of liabilities in the normal course of business. The Company has incurred losses since inception of \$89.8 million, has negative cash flows from operations, and has minimal revenues, which creates substantial doubt about its ability to continue as a going concern for a period at least one year from the date of issuance of these Consolidated Financial Statements.

The future of the Company as an operating business will depend on its ability to obtain sufficient capital contributions, financing and/or generate revenues as may be required to sustain its operations. Management plans to address the above as needed by, (a) securing additional grant funds, (b) obtaining additional financing through debt or equity transactions; (c) granting licenses to third parties in exchange for specified up-front and/or back end payments, and (d) developing and commercializing its products on an accelerated timeline. Management continues to exercise tight cost controls to conserve cash.

The ability of the Company to continue as a going concern is dependent upon its ability to successfully accomplish the plans described in the preceding paragraph and to eventually attain profitable operations. The accompanying Consolidated Financial Statements do not include any adjustments that might be necessary if the Company is unable to continue as a going concern. If the Company is unable to obtain adequate capital, it could be forced to cease operations.

**VOLITIONRX LIMITED**  
**Notes to Consolidated Financial Statements**  
**For Years Ended December 31, 2019 and 2018**  
**(\$ expressed in United States Dollars)**

**Note 3 - Summary of Significant Accounting Policies**

**Basis of Presentation**

The consolidated financial statements of the Company have been prepared in accordance with U.S. GAAP and are expressed in United States dollars. The Company's fiscal year end is December 31.

**Use of Estimates**

The preparation of financial statements in conformity with U.S. GAAP requires management to make estimates and assumptions that affect the reported amounts of assets and liabilities, the disclosure of contingent assets and liabilities at the date of the financial statements and the reported amounts of revenues and expenses during the reporting period. The Company also regularly evaluates estimates and assumptions related to deferred income tax asset valuation allowances, impairment analysis of intangible assets and valuations of stock-based compensation.

The Company bases its estimates and assumptions on current facts, historical experiences and various other factors that it believes to be reasonable under the circumstances, the results of which form the basis for making judgments about the carrying values of assets and liabilities and the accrual of costs and expenses that are not readily apparent from other sources. The actual results experienced by the Company may differ materially and adversely from the Company's estimates. To the extent there are material differences between the estimates and the actual results, future results of operations could be affected.

**Principles of Consolidation**

The accompanying consolidated financial statements for the year ended December 31, 2019 include the accounts of the Company and its wholly owned subsidiaries, Singapore Volition, Belgian Volition, Volition Diagnostics UK Limited and Volition America, as well as its majority-owned subsidiary Volition Vet. All significant intercompany balances and transactions have been eliminated in consolidation.

**Cash and Cash Equivalents**

The Company considers all highly liquid instruments with a maturity of three months or less at the time of issuance to be cash equivalents. At December 31, 2019 and December 31, 2018, the Company had \$16,966,168 and \$13,427,222, respectively, in cash and cash equivalents. At December 31, 2019 and December 31, 2018, the Company had \$16,499,679 and \$12,899,095, respectively, in its domestic accounts in excess of Federal Deposit Insurance Corporation insured limits. At December 31, 2019 and December 31, 2018, the Company had \$2,887,483 and \$451,468, respectively, in its foreign accounts in excess of the Belgian Deposit Guarantee insured limits. At December 31, 2019 and December 31, 2018, the Company had \$170,387 and \$76,665, respectively, in its foreign accounts in excess of the Singapore Deposit Insurance Scheme. At December 31, 2019 and December 31, 2018, the Company had \$777,432 and \$55,398, respectively, in its foreign accounts in excess of the UK Deposit Protection Scheme.

**Basic and Diluted Net Loss Per Share**

The Company computes net loss per share in accordance with Accounting Standards Codification ("ASC") 260, "Earnings Per Share," which requires presentation of both basic and diluted earnings per share ("EPS") on the face of the income statement. Basic EPS is computed by dividing net loss available to common stockholders (numerator) by the weighted average number of shares outstanding (denominator) during the period. Diluted EPS gives effect to all dilutive potential common shares outstanding during the period using the treasury stock method. In computing diluted EPS, the average stock price for the period is used in determining the number of shares assumed to be purchased from the exercise of stock options or warrants. As of December 31, 2019, 4,359,301 potential common shares equivalents from warrants and options were excluded from the diluted EPS calculations as their effect is anti-dilutive.

**VOLITIONRX LIMITED**  
**Notes to Consolidated Financial Statements**  
**For Years Ended December 31, 2019 and 2018**  
**(\$ expressed in United States Dollars)**

**Note 3 - Summary of Significant Accounting Policies (Continued)**

**Foreign Currency Translation**

The Company has functional currencies in the Euro, the United States Dollar and British Pounds Sterling and its reporting currency is the United States Dollar. Management has adopted ASC 830-20, “*Foreign Currency Matters – Foreign Currency Transactions*”. All assets and liabilities denominated in foreign currencies are translated using the exchange rate prevailing at the balance sheet date. For revenues and expenses, the weighted average exchange rate for the period is used. Gains and losses arising on translation of foreign currency denominated transactions are included in other comprehensive income (loss).

**Financial Instruments**

Pursuant to ASC 820, “*Fair Value Measurements and Disclosures*”, an entity is required to maximize the use of observable inputs and minimize the use of unobservable inputs when measuring fair value. ASC 820 establishes a fair value hierarchy based on the level of independent, objective evidence surrounding the inputs used to measure fair value. A financial instrument’s categorization within the fair value hierarchy is based upon the lowest level of input that is significant to the fair value measurement. ASC 820 prioritizes the inputs into three levels that may be used to measure fair value:

*Level 1*

Level 1 applies to assets or liabilities for which there are quoted prices in active markets for identical assets or liabilities.

*Level 2*

Level 2 applies to assets or liabilities for which there are inputs other than quoted prices that are observable for the assets or liabilities such as quoted prices for similar assets or liabilities in active markets; quoted prices for identical assets or liabilities in markets with insufficient volume or infrequent transactions (less active markets); or model-derived valuations in which significant inputs are observable or can be derived principally from, or corroborated by, observable market data.

*Level 3*

Level 3 applies to assets or liabilities for which there are unobservable inputs to the valuation methodology that are significant to the measurement of the fair value of the assets or liabilities.

The Company’s financial instruments consist principally of cash, accounts payable, accrued liabilities, loans payable, and amounts due to related parties. Pursuant to ASC 820, the fair value of cash is determined based on “Level 1” inputs, which consists of quoted prices in active markets for identical assets. The Company believes that the recorded values of all of our other financial instruments approximate their current fair values because of their nature and respective maturity dates or durations.

**Income Taxes**

Potential benefits of income tax losses are not recognized in the accounts until realization is more likely than not. The Company has adopted ASC 740, “*Accounting for Income Taxes*” as of its inception. Pursuant to ASC 740, the Company is required to compute tax asset benefits for net operating losses carried forward. The potential benefits of net operating losses have not been recognized in these consolidated financial statements because the Company cannot be assured it is more likely than not it will utilize the net operating losses carried forward in future years.

**Other Comprehensive Income (Loss)**

ASC 220, “*Other Comprehensive Income/(Loss)*”, establishes standards for the reporting and display of other comprehensive loss and its components in the financial statements. As of December 31, 2019, the Company had \$125,670 of accumulated other comprehensive loss, relating to foreign currency translation.

**VOLITIONRX LIMITED**  
**Notes to Consolidated Financial Statements**  
**For Years Ended December 31, 2019 and 2018**  
**(\$ expressed in United States Dollars)**

**Note 3 - Summary of Significant Accounting Policies (Continued)**

**Revenue Recognition**

Beginning in 2014, the Financial Accounting Standards Board (“FASB”) issued several Accounting Standards Updates establishing Accounting Standards Codification (“ASC”) Topic 606, “*Revenue from Contracts with Customers*” (“ASC 606”). ASC 606 replaces most industry-specific revenue recognition guidance in U.S. GAAP with a new principles-based, five-step revenue recognition model. The Company adopted ASC 606 effective January 1, 2019. Under ASC 606, the Company recognizes revenues when the customer obtains control of promised goods or services, in an amount that reflects the consideration which the Company expects to receive in exchange for those goods or services. The Company recognizes revenues following the five step model prescribed under ASC 606: (i) identify contract(s) with a customer; (ii) identify the performance obligations in the contract; (iii) determine the transaction price; (iv) allocate the transaction price to the performance obligations in the contract; and (v) recognize revenues when (or as) we satisfy the performance obligation(s).

The Company generates revenue from its license agreement with Active Motif, Inc. (“Active Motif”) for the sale of Research Use Only (“ROU”) kits from which the Company receives royalties. In addition, revenue is received from external third parties for services the Company performs for them in its laboratory.

Revenues, and their respective treatment for financial reporting purposes under ASC 606, are as follows:

*Royalty*

The Company receives royalty revenues on the net sales recognized during the period in which the revenue is earned, and the amount is determinable from the licensee. These are presented under “Royalty” in the consolidated statements of operations. The Company does not have future performance obligations under this revenue stream. In accordance with ASC 606, the Company records these revenues based on estimates of the net sales that occurred during the relevant period from the licensee. The relevant period estimates of these royalties are based on preliminary gross sales data provided by Active Motif and analysis of historical gross-to-net adjustments. Differences between actual and estimated royalty revenues are adjusted for in the period in which they become known.

*Services*

The Company includes revenue recognized from laboratory services performed in the Company’s laboratory on behalf of third parties under “Services” in the consolidated statements of operations.

For each development and/or commercialization agreement that results in revenues, the Company identifies all performance obligations, aside from those that are immaterial, which may include a license to intellectual property and know-how, development activities and/or transition activities. In order to determine the transaction price, in addition to any upfront payment, the Company estimates the amount of variable consideration at the outset of the contract either utilizing the expected value or most likely amount method, depending on the facts and circumstances relative to the contract. The Company constrains the estimates of variable consideration such that it is probable that a significant reversal of previously recognized revenue will not occur throughout the life of the contract. When determining if variable consideration should be constrained, management considers whether there are factors outside the Company’s control that could result in a significant reversal of revenue. In making these assessments, the Company considers the likelihood and magnitude of a potential reversal of revenue. These estimates are re-assessed each reporting period as required.

**Research and Development**

In accordance with ASC 730, the Company follows the policy of expensing its research and development costs in the period in which they are incurred. The Company incurred research and development expenses of \$10.4 million and \$10.9 million during the years ended December 31, 2019 and December 31, 2018, respectively.

**VOLITIONRX LIMITED**  
**Notes to Consolidated Financial Statements**  
**For Years Ended December 31, 2019 and 2018**  
**(\$ expressed in United States Dollars)**

**Note 3 - Summary of Significant Accounting Policies (Continued)**

**Impairment of Long-Lived Assets**

In accordance with ASC 360, “*Property Plant and Equipment*”, the Company tests long-lived assets or asset groups for recoverability when events or changes in circumstances indicate that their carrying amount may not be recoverable. Circumstances which could trigger a review include, but are not limited to: significant decreases in the market price of the asset; significant adverse changes in the business climate or legal factors; accumulation of costs significantly in excess of the amount originally expected for the acquisition or construction of the asset; current period cash flow or operating losses combined with a history of losses or a forecast of continuing losses associated with the use of the asset; and current expectation that the asset will more likely than not be sold or disposed of significantly before the end of its estimated useful life. Recoverability is assessed based on the carrying amount of the asset and its fair value which is generally determined based on the sum of the undiscounted cash flows expected to result from the use and the eventual disposal of the asset, as well as specific appraisal in certain instances. An impairment loss is recognized when the carrying amount is not recoverable and exceeds fair value. Impairment losses of \$nil and \$nil were recognized during the years ended December 31, 2019 and December 31, 2018, respectively.

**Stock-Based Compensation**

The Company records stock-based compensation in accordance with ASC 718, “*Compensation – Stock Compensation*” and ASC 505-50, *Equity-Based Payments to Non-Employees*. All transactions in which goods or services are the consideration received for the issuance of equity instruments are accounted for based on the fair value of the consideration received or the fair value of the equity instrument issued, whichever is more reliably measurable. Equity instruments issued to employees and the cost of the services received as consideration are measured and recognized based on the fair value of the equity instruments issued and are recognized over the employees required service period, which is generally the vesting period.

**Leases**

In February of 2016, FASB issued Accounting Standards Update No. 2016-02 – Leases (“Topic 842”), which significantly amends the way companies are required to account for leases. Under the updated leasing guidance, some leases that did not have to be reported previously are now required to be presented as an asset and liability on the balance sheet. In addition, for certain leases, what was previously classified as an operating expense must now be allocated between amortization expense and interest expense. The Company adopted Topic 842 as of January 1, 2019 using the modified retrospective transition method and prior periods have not been restated. Upon implementation, the Company recognized an initial operating lease right-of-use asset of \$110,630 and operating lease liability of \$110,630. Due to the simplistic nature of the Company's leases, no retained earnings adjustment was required.

**Grants received**

The Company receives funding from public bodies for a proportion of the costs of specific projects. Funds are received in line with claims submitted for the agreed expenditure. The Company recognizes grant income once claims submitted are approved and funds are received. General working capital funding received at the commencement of a project is treated as deferred income until it has been utilized for the expenditure claimed. Funding received that is repayable is shown as a liability.

**Recent Accounting Pronouncements**

The Company has implemented all new accounting pronouncements that are in effect. The Company does not believe that there are any other new accounting pronouncements that have been issued that might have a material impact on its financial position or results of operations.

**VOLITIONRX LIMITED**  
**Notes to Consolidated Financial Statements**  
**For Years Ended December 31, 2019 and 2018**  
**(\$ expressed in United States Dollars)**

**Note 4 - Property and Equipment**

The Company's property and equipment consist of the following amounts as of December 31, 2019 and December 31, 2018:

	Useful Life	December 31,		
		2019	2018	
		Cost	Accumulated Depreciation	Net Carrying Value
		\$	\$	\$
Computer hardware and software	3 years	426,461	280,554	145,907
Laboratory equipment	5 years	2,052,348	1,256,637	795,711
Office furniture and equipment	5 years	217,545	114,242	103,303
Buildings	30 years	1,472,211	139,021	1,333,190
Building improvements	5-15 years	630,824	117,526	513,298
Land	Not amortized	89,816	-	89,816
		<u>4,889,205</u>	<u>1,907,980</u>	<u>2,981,225</u>
		Cost	Accumulated Depreciation	Net Carrying Value
		\$	\$	\$
Computer hardware and software	3 years	344,383	166,750	177,633
Laboratory equipment	5 years	1,673,215	928,841	744,374
Office furniture and equipment	5 years	204,129	75,137	128,992
Buildings	30 years	1,502,171	91,785	1,410,386
Building improvements	5-15 years	643,663	77,049	566,614
Land	Not amortized	91,644	-	91,644
		<u>4,459,205</u>	<u>1,339,562</u>	<u>3,119,643</u>

The majority of capital expenditures in 2019 are related to €0.4 million Euros for software and laboratory equipment.

During the years ended December 31, 2019 and December 31, 2018, the Company recognized \$589,532 and \$548,005, respectively, in depreciation expense.

**VOLITIONRX LIMITED**  
**Notes to Consolidated Financial Statements**  
**For Years Ended December 31, 2019 and 2018**  
**(\$ expressed in United States Dollars)**

**Note 5 - Intangible Assets**

The Company's intangible assets consist of patents, mainly acquired in the acquisition of Belgian Volition. The patents are being amortized over the assets' estimated useful lives, which range from 8 to 20 years.

	Cost	Accumulated Amortization	December 31, 2019 Net Carrying Value
	\$	\$	\$
	<u>          </u>	<u>          </u>	<u>          </u>
Patents	1,147,391	775,086	372,305

	Cost	Accumulated Amortization	December 31, 2018 Net Carrying Value
	\$	\$	\$
	<u>          </u>	<u>          </u>	<u>          </u>
Patents	1,167,383	700,478	466,905

During the years ended December 31, 2019, and December 31, 2018, the Company recognized \$87,285 and \$91,911, respectively, in amortization expense.

The Company amortizes the long-lived assets on a straight-line basis with terms ranging from 8 to 20 years. The annual estimated amortization schedule over the next five years is as follows:

2020	\$	87,539
2021	\$	87,539
2022	\$	87,539
2023	\$	87,539
2024	\$	22,149
<b>Total Intangible Assets</b>	<b>\$</b>	<b><u>372,305</u></b>

The Company periodically reviews its long-lived assets to ensure that their carrying value does not exceed their fair market value. The Company carried out such a review in accordance with ASC 360 as of December 31, 2019. The result of this review confirmed that the ongoing value of the patents was not impaired as of December 31, 2019.

**Note 6 - Related Party Transactions**

See Note 7 for common stock issued to related parties and Note 8 for stock options and warrants issued to related parties. The Company has agreements with related parties for consultancy services which are accrued under management and directors' fees payable (see consolidated balance sheet).



**VOLITIONRX LIMITED**  
**Notes to Consolidated Financial Statements**  
**For Years Ended December 31, 2019 and 2018**  
**(\$ expressed in United States Dollars)**

**Note 7 - Common Stock**

As of December 31, 2019, the Company was authorized to issue 100 million shares of common stock par value \$0.001 per share, of which 41,125,303 and 35,335,378 shares were issued as of December 31, 2019 and December 31, 2018, respectively.

On June 14, 2019, an amendment to the 2015 Stock Incentive Plan (the “2015 Plan”) was approved by the stockholders at the annual meeting to increase the number of shares of common stock available for issuance under the 2015 Plan by 1,000,000 shares to an aggregate maximum of 4,250,000 shares.

**2019**

**Issuances Upon Warrant and Option Exercises**

On August 10, 2018, the Company issued to Cotterford Company Limited (“Cotterford”) in a private placement offering (PIPE) 5.0 million shares of common stock at a price of \$1.80 per share, for aggregate gross proceeds of \$9.0 million. In connection with the transaction, approximately \$0.1 million was incurred for legal and other fees resulting in net proceeds of approximately \$8.9 million. Additionally, the Company issued to Cotterford a warrant to purchase up to an additional 5.0 million shares of common stock at an exercise price of \$3.00 per share payable in cash. This transaction resulted in Cotterford becoming a significant stockholder and therefore a related party in accordance with U.S. GAAP. The shares of common stock (including the shares underlying the warrant) were subsequently registered for resale on Form S-3 (declared effective by the SEC on October 15, 2018, File No. 333-227731).

From January 30, 2019 to February 26, 2019, warrants to purchase 754,475 shares of our common stock were exercised at a price of \$2.20 per share, for gross proceeds to the Company of approximately \$1.66 million.

On March 8, 2019, Cotterford partially exercised its warrant and purchased 1,724,138 shares of our common stock at a price of \$2.90 per share, for gross proceeds to the Company of \$5.0 million.

On May 3, 2019, Cotterford partially exercised its warrant and purchased 1,666,667 shares of our common stock at a price of \$3.00 per share, for gross proceeds to the Company of \$5.0 million.

On July 24, 2019, Cotterford exercised the remainder of its warrant and purchased 1,609,195 shares of our common stock at a price of \$3.00 per share, for gross proceeds to the Company of approximately \$4.8 million.

From August 20, 2019 to September 20, 2019, 6,166 stock options were exercised to purchase shares of our common stock at \$2.35 per share in a cashless exercise that resulted in the issuance of 2,487 shares of our common stock.

On November 15, 2019, 4,167 stock options were exercised to purchase shares of our common stock at \$5.00 per share in a cashless exercise that resulted in the issuance of 371 shares of our common stock.

From November 25, 2019 to November 27, 2019, warrants to purchase 29,392 shares of our common stock were exercised at a price of \$2.40 per share, for gross proceeds to the Company of \$70,541.

**Equity Distribution Agreement**

On September 7, 2018, the Company entered into an equity distribution agreement with Oppenheimer & Co. Inc. (“Oppenheimer”), which agreement allows it to offer and sell shares of its common stock having an aggregate offering price of up to \$10.0 million from time-to-time pursuant to a shelf registration statement on Form S-3 (declared effective by the SEC on September 28, 2018, File No. 333-227248) through Oppenheimer acting as the Company’s agent and/or principal. Through December 31, 2019, the Company raised aggregate net proceeds (net of broker commissions and fees) of \$16,547 under the equity distribution agreement through the sale of 3,200 shares of its common stock. All of such shares were sold during the quarter ended December 31, 2019. The Company used the net proceeds raised to date for continued product development, clinical studies, product commercialization, working capital and other general corporate purposes.

**2018**

From February 5 to June 4, 2018, 29,375 warrants were exercised to purchase shares of our common stock at a price of \$2.00 per share in a cashless exercise that resulted in the issuance of 11,831 shares of our common stock.

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**Note 7 - Common Stock (Continued)**

On March 13, 2018, the Company issued 3.5 million shares of common stock in a registered public offering at a price of \$2.40 per share, for aggregate gross proceeds of \$8.4 million. In connection with the transaction, approximately \$0.8 million was incurred for legal and underwriting fees resulting in net proceeds of approximately \$7.6 million. Pursuant to this offering, the underwriters had the option to purchase up to an additional 525,000 shares of our common stock for 30 days following the pricing of the initial closing, which option was not exercised.

On August 10, 2018, the Company issued to Cotterford in a private placement offering (“PIPE”) 5.0 million shares of our common stock at a price of \$1.80 per share, for aggregate gross proceeds of \$9.0 million.

On October 16, 2018, 243,903 warrants were exercised at a price of \$2.40 per share, for gross cash proceeds to the Company of \$585,367. As a result, a total of 243,903 shares of our common stock were issued.

On October 16, 2018, 60,250 warrants were exercised at a price of \$2.20 per share, for gross cash proceeds to the Company of \$132,550. As a result, a total of 60,250 shares of our common stock were issued.

**Note 8 - Warrants and Options**

**a) Warrants**

The following table summarizes the changes in warrants outstanding of the Company during the years ended December 31, 2019 and December 31, 2018:

	<u>Number of Warrants</u>	<u>Weighted Average Exercise Price (\$)</u>
Outstanding at December 31, 2017	1,731,680	2.36
Granted	5,000,000	3.00
Exercised	(333,528)	2.33
Expired	(290,535)	2.54
Outstanding at December 31, 2018	6,107,617	2.88
Granted	-	-
Exercised	(5,783,867)	2.86
Expired	(133,750)	2.20
Outstanding at December 31, 2019	190,000	2.90
Exercisable at December 31, 2019	65,000	4.53

**2019**

Effective March 5, 2019, the Company entered into an amendment to an outstanding warrant to purchase up to an aggregate of 5.0 million shares of our common stock, originally issued to Cotterford, a significant stockholder, in connection with an equity financing completed on or about August 10, 2018. The amendment temporarily reduced the exercise price of such warrant from \$3.00 per share to \$2.90 per share through the close of business on March 8, 2019. As a result of this amendment, \$196,957 of financing costs were recorded in other expenses.

On March 8, 2019, Cotterford partially exercised its warrant and purchased 1,724,138 shares of our common stock at \$2.90 per share resulting in gross proceeds to the Company of \$5.0 million.

On May 3, 2019, Cotterford partially exercised its warrant and purchased 1,666,667 shares of our common stock at \$3.00 per share resulting in gross proceeds of \$5.0 million to the Company.

On July 1, 2019, the Company modified the performance criteria for certain vesting milestones on a warrant held by an officer of the Company and as a result the Company re-measured warrants held by the officer, to purchase 125,000 shares of our common stock at an exercise price of \$2.47 per share, resulting in \$11,829 of additional warrant expense to be recorded over the vesting period. These warrants vest on achievement of certain business objectives and expire 3 years from the date of vesting.

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**Note 8 - Warrants and Options (Continued)**

On July 24, 2019, Cotterford exercised the remainder of its warrant and purchased 1,609,195 shares of our common stock at \$3.00 per share resulting in gross proceeds of \$4.8 million to the Company.

During the year 2019, warrants to purchase an aggregate of 5,783,067 shares of our common stock were exercised (including the exercises by Cotterford referenced above) for gross cash proceeds to the Company of approximately of \$16.6 million.

**2018**

On August 10, 2018, in conjunction with the PIPE transaction the Company issued to Cotterford a warrant to purchase up to 5.0 million shares of common stock at an exercise price of \$3.00 per share payable in cash (subject to adjustment pursuant to the terms of the warrant). The warrant has an expiration date of August 10, 2019 and is exercisable for a period of 6 months commencing on February 10, 2019.

On November 13, 2018, the Board of Directors amended the terms of an aggregate of 29,392 outstanding warrants to purchase common stock of the Company originally issued in connection with an equity financing completed on or about December 31, 2013 to extend the expiration date from December 31, 2018 to December 31, 2019.

During 2018, 333,528 warrants were exercised for gross cash proceeds to the Company of \$717,917. Refer to Note 7 for the details of the exercises.

Below is a table summarizing the warrants issued and outstanding as of December 31, 2019, which have a weighted average exercise price of \$2.90 per share and an aggregate weighted average remaining contractual life of 2.91 years.

Number Outstanding	Number Exercisable	Exercise Price (\$)	Weighted Average Remaining Contractual Life (Years)	Proceeds to Company if Exercised (\$)
150,000	25,000	2.47	3.25	370,500
40,000	40,000	4.53	0.87	181,200
<b>190,000</b>	<b>65,000</b>			<b>551,700</b>

Warrant expense of \$8,506 and \$22,776 was recorded in the years ended December 31, 2019, and December 31, 2018, respectively. Total remaining unrecognized compensation cost related to non-vested warrants is \$20,335 and is expected to be recognized over a period of 1.0 years. As of December 31, 2019, the total intrinsic value of warrants was \$348,900.

**b) Options**

The Company currently has options outstanding under both its 2011 Equity Incentive Plan (the "2011 Plan") (for option issuances prior to 2016) and its 2015 Plan (for option issuances commencing in 2016). Effective as of January 1, 2016, no additional awards were or may be made under the 2011 Plan.

The 2015 Plan was adopted by the Board of Directors on August 18, 2015 and approved by the stockholders at an annual meeting held on October 30, 2015. On August 5, 2016, the Board of Directors adopted an amendment to the 2015 Plan to increase the number of shares of common stock available for issuance under such Plan by 750,000 shares to an aggregate maximum of 1,750,000 shares, which amendment was approved by the stockholders at an annual meeting held on October 7, 2016. On June 13, 2017, the Board of Directors adopted a subsequent amendment to the 2015 Plan to increase the number of shares of common stock available for issuance under such Plan by 750,000 shares to an aggregate maximum of 2,500,000 shares, which amendment was approved by the stockholders at an annual meeting held on September 8, 2017. On June 15, 2018, the Board of Directors adopted a subsequent amendment to the 2015 Plan to increase the number of shares of common stock available for issuance under such Plan by 750,000 shares to an aggregate maximum of 3,250,000 shares, which amendment was approved by the stockholders at an annual meeting held on September 7, 2018. On March 27, 2019, the Board of Directors adopted a subsequent amendment to the 2015 Plan to increase the number of common stock available for issuance under the Plan by 1,000,000 shares to an aggregate maximum of 4,250,000 shares, which amendment was approved by the stockholders at an annual meeting held on June 14, 2019.

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**Note 8 - Warrants and Options (Continued)**

The 2015 Plan permits the grant of incentive stock options, non-statutory stock options, restricted stock awards, stock bonus awards, stock appreciation rights, restricted stock units and performance awards. The primary purpose of the 2015 Plan is to enhance the Company's ability to attract and retain the services of qualified employees, officers, directors, consultants and other service providers upon whose judgment, initiative and efforts the successful conduct and development of the Company's business largely depends, and to provide additional incentives to such persons or entities to devote their utmost effort and skill to the advancement and betterment of the Company, by providing them an opportunity to participate in the ownership of the Company that is tied to the Company's performance, thereby giving them an interest in the success and increased value of the Company. The 2015 Plan is administered by the Compensation Committee comprised solely of members of the Board of Directors or by the Board of Directors as a whole.

The following table summarizes the changes in options outstanding of the Company during the years ended December 31, 2019 and 2018:

	<b>Number of Options</b>	<b>Weighted Average Exercise Price (\$)</b>
Outstanding at December 31, 2017	2,939,134	4.09
Granted	805,000	4.00
Exercised	-	-
Expired/Cancelled	(245,333)	4.98
Outstanding at December 31, 2018	3,498,801	4.00
Granted	730,000	3.25
Exercised	(10,333)	3.42
Expired/Cancelled	(49,167)	3.31
Outstanding at December 31, 2019	4,169,301	3.88
Exercisable at December 31, 2019	3,484,301	4.01

**2019**

Effective February 11, 2019, the Company granted stock options to purchase 730,000 shares of our common stock to various Company personnel (including directors, executives, members of management and employees) for services to the Company. These options vested on February 11, 2020 and expire 5 years after the vesting date, with an exercise price of \$3.25 per share. The Company has calculated the estimated fair market value of these options at \$1,569,816, using the Black-Scholes model and the following assumptions: term 6 years, stock price \$3.16, exercise price \$3.25, 77.86% volatility, 2.52% risk free rate, and no forfeiture rate.

Subsequent to the February 2019 grant, stock options to purchase 45,000 shares of our common stock subject to the grant were forfeited.

**2018**

Effective January 23, 2018, the Company granted stock options to purchase 780,000 shares of our common stock to various Company personnel (including directors, executives, members of management and employees) for services to the Company. These options vested on January 23, 2019 and expire 5 years after the vesting date, with an exercise price of \$4.00 per share. The Company has calculated the estimated fair market value of these options at \$1,930,265, using the Black-Scholes model and the following assumptions: term 6 years, stock price \$3.75, exercise price \$4.00, 75.4% volatility, 2.55% risk free rate, and no forfeiture rate.

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**Note 8 - Warrants and Options (Continued)**

Effective September 28, 2018, the Company granted stock options to purchase 25,000 shares of our common stock to the Company controller for services to the Company. These options vested on September 28, 2019 and expire 5 years after the vesting date, with an exercise price of \$4.00 per share. The Company has calculated the estimated fair market value of these options at \$39,733, using the Black-Scholes model and the following assumptions: term 6 years, stock price \$2.59, exercise price \$4.00, 77.59% volatility, 3.01% risk free rate, and no forfeiture rate.

In December 2018, the Board of Directors amended the terms of certain outstanding options such that (i) the expiration date for outstanding options to purchase up to an aggregate of 645,000 shares of the Company's common stock, granted on August 18, 2014 under the 2011 Plan, was extended for both vesting installments from four (4) years from the vesting date of each installment to a single expiration date of August 18, 2020, (ii) the expiration date for outstanding options to purchase up to an aggregate of 20,000 shares of the Company's common stock, granted on May 18, 2015 under the 2011 Plan, was extended from four (4) years after the vesting date to May 18, 2021, and (iii) the expiration date for outstanding options to purchase up to an aggregate of 317,000 shares of the Company's common stock, granted July 23, 2015 under the 2011 Plan, was extended from four (4) years after vesting to five years and six months after vesting, or July 23, 2021.

Below is a table summarizing the options issued and outstanding as of December 31, 2019, all of which were issued pursuant to the 2011 Plan (for option issuances prior to 2016) or the 2015 Plan (for option issuances commencing in 2016) and which have a weighted average exercise price of \$3.88 per share and an aggregate weighted average remaining contractual life of 2.97 years.

As of December 31, 2019, an aggregate of 1,114,000 shares of common stock remained available for future issuance under the 2015 Plan.

Number Outstanding	Number Exercisable	Exercise Price (\$)	Weighted Average Remaining Contractual Life (Years)	Proceeds to Company if Exercised (\$)
11,599	11,599	2.35	0.35	27,258
322,500	322,500	2.50	0.63	806,250
322,500	322,500	3.00	0.63	967,500
685,000	-	3.25	5.12	2,226,250
17,767	17,767	3.35	1.20	59,519
20,000	20,000	3.80	1.38	76,000
1,817,837	1,817,837	4.00	3.03	7,271,348
89,163	89,163	4.38	3.03	390,534
17,768	17,768	4.35	2.20	77,291
50,000	50,000	4.80	3.01	240,000
815,167	815,167	5.00	2.51	4,075,836
<b>4,169,301</b>	<b>3,484,301</b>			<b>16,217,786</b>

Stock option expense of \$1,458,607 and \$2,570,095 was recorded in the year ended December 31, 2019 and December 31, 2018, respectively. Total remaining unrecognized compensation cost related to non-vested stock options is \$165,465 and is expected to be recognized over a period of 0.12 years. As of December 31, 2019, the total intrinsic value of stock options was \$3,759,645.

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**Note 9 - Income Taxes**

The Company has estimated net operating losses for the years ended December 31, 2019 and 2018 of \$17.3 million and \$12.4 million, respectively, available to offset taxable income in future years.

The significant components of deferred income taxes and assets as of December 31, 2019 and December 31, 2018 are as follows:

<u>Net Deferred Tax Liability</u>	<u>December 31, 2019</u>	<u>December 31, 2018</u>
	<u>\$</u>	<u>\$</u>
Excess of tax over book depreciation and amortization	(3,901)	(10,761)
ROU Asset	(41,250)	
Lease Liability	43,896	
Prepaid expenses	-	-
Allowance for doubtful accounts	-	-
Accrued expenses	1,154	1,154
Stock-based compensation	-	-
Net Operating Losses carry-forward	17,326,179	12,437,561
Research and development tax credits	231,243	337,507
Gross deferred tax assets	17,557,321	12,765,461
Valuation allowance	(17,557,321)	(12,765,461)
Net deferred tax asset	<u>-</u>	<u>-</u>
Change in Valuation Allowance	(4,791,860)	

<u>Summary Rate Reconciliation</u>	<u>December 31, 2019</u>	<u>December 31, 2018</u>
	<u>%</u>	<u>%</u>
Federal statutory rate	21.0	21.0
State income taxes, net of federal benefit	-	-
Permanent Differences	4.1	(15.1)
Stock based compensation	(2.4)	(3.2)
Federal Research & Development Credits	0.6	0.4
Foreign taxes	6.7	6.2
Federal Deferred Rate Decrease	(0.2)	-
Increase/(decrease) in valuation reserve	(29.8)	(9.3)
Total	<u>-</u>	<u>-</u>

<u>Disclosure Amounts</u>	<u>December 31, 2019</u>
	<u>\$</u>
Net Operating Losses - United States	18,214,929
Net Operating Losses - Foreign	50,464,000
Credit Carryforward - United States	-
Credit Carryforward - Foreign	231,243
Increase in Valuation Allowance	4,791,860

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**Note 10 - Commitments and Contingencies**

**a) Financing Lease Obligations**

In 2015, the Company entered into an equipment financing lease to purchase three Tecan machines (automated liquid handling robots) for €550,454 Euros, maturing May 2020. As of December 31, 2019, the balance payable was \$44,477.

In 2016, the Company entered into a real estate financing lease with ING Asset Finance Belgium S.A. (“ING”) to purchase a property located in Belgium for €1.12 million Euros, maturing May 2031. As of December 31, 2019, the balance payable was \$641,513.

In 2018, the Company entered into a financing lease with BNP Paribas leasing solutions to purchase a freezer for the Belgium facility for €25,000 Euros, maturing January 2022. As of December 31, 2019, the balance payable was \$19,664.

The following is a schedule showing the future minimum lease payments under financing leases by years and the present value of the minimum payments as of December 31, 2019.

2020	\$	114,649
2021	\$	69,946
2022	\$	61,798
2023	\$	60,387
2024	\$	60,386
Greater than 5 years	\$	445,331
Total	\$	812,497
Less: Amount representing interest	\$	<u>(106,843)</u>
<b>Present value of minimum lease payments</b>	<b>\$</b>	<b><u>705,654</u></b>

**b) Operating Lease Right-of-Use Liabilities**

The Company adopted Topic 842 on January 1, 2019. The Company elected to adopt this standard using the optional modified retrospective transition method and recognized a cumulative-effect adjustment to the consolidated balance sheet on the date of adoption. Comparative periods have not been restated. With the adoption of Topic 842, the Company’s consolidated balance sheet now contains the following line items: Operating lease right-of-use assets, Current portion of operating lease liabilities and Operating lease liabilities, net of current portion.

As all the existing leases subject to the new lease standard were previously classified as operating leases by the Company, they were similarly classified as operating leases under the new standard. The Company has determined that the identified operating leases did not contain non-lease components and require no further allocation of the total lease cost. Additionally, the agreements in place did not contain information to determine the rate implicit in the leases, so we used our incremental borrowing rate as the discount rate. Our weighted average discount rate is 4.47% and the weighted average remaining lease term is 21 months.

As of December 31, 2019, operating lease right-of-use assets and liabilities arising from operating leases were \$381,483 and \$389,119, respectively. During the year ended December 31, 2019, cash paid for amounts included for the measurement of lease liabilities was \$242,656 and the Company recorded operating lease expense of \$224,283.

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**Note 10 – Commitments and Contingencies (Continued)**

The following is a schedule showing the future minimum lease payments under operating leases by years and the present value of the minimum payments as of December 31, 2019.

2020	\$ 269,215
2021	\$ 91,671
2022	\$ 34,497
2023	\$ <u>10,773</u>
Total Operating Lease Liabilities	\$ 406,156
Less: Amount representing interest	\$ (17,037)
<b>Present Value of minimum lease payments</b>	<b>\$ <u>389,119</u></b>

The Company's office space leases are short term and the Company has elected under the short-term recognition exemption not to recognize them on the balance sheet. During the year ended December 31, 2019, \$22,096 was recognized in short-term lease costs associated with the office lease in Singapore. The annual payments remaining for such short-term office lease were as follows:

2020	\$ 12,750
2021	\$ <u>-</u>
<b>Total Operating Lease Liabilities</b>	<b>\$ <u>12,750</u></b>

**c) Grants Repayable**

In 2010, the Company entered into an agreement with the Walloon Region government in Belgium for a colorectal cancer research grant for €1.05 million Euros. Per the terms of the agreement, €314,406 Euros of the grant is to be repaid by installments over the period from June 30, 2014 to June 30, 2023. The Company has recorded the balance of €733,614 Euros to other income in previous years as there is no obligation to repay this amount. In the event that the Company receives revenue from products or services as defined in the agreement, it is due to pay a 6% royalty on such revenue to the Walloon Region. The maximum amount payable to the Walloon Region, in respect of the aggregate of the amount repayable of €314,406 Euros and the 6% royalty on revenue, is twice the amount of funding received. As of December 31, 2019, the grant balance repayable was \$137,425.

In 2018, the Company entered into an agreement with the Walloon Region government in Belgium for a colorectal cancer research grant for €605,000 Euros. Per the terms of the agreement, €181,500 Euros of the grant is to be repaid by instalments over 12 years commencing in 2020. In the event that the Company receives revenue from products or services as defined in the agreement, it is due to pay a 3.53% royalty on such revenue to the Walloon Region. The maximum amount payable to the Walloon Region, in respect of the aggregate of the amount repayable of €181,500 Euros and the 3.53% royalty on revenue, is equal to the amount of funding received. As of December 31, 2019, the grant balance repayable was \$199,861.

As of December 31, 2019, the total balance for grant repayable was \$337,286 and the annual payments remaining were as follows:

2020	\$ 52,879
2021	\$ 49,967
2022	\$ 47,266
2023	\$ 48,436
2024	\$ 20,377
Greater than 5 years	\$ <u>118,361</u>
<b>Total Grants Repayable</b>	<b>\$ <u>337,286</u></b>



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**Note 10 – Commitments and Contingencies (Continued)**

**d) Long-Term Debt**

In 2016, the Company entered into a 7-year loan agreement with Namur Invest for €440,000 Euros with a fixed interest rate of 4.85%, maturing December 2023. As of December 31, 2019, the principal balance payable was \$322,128.

In 2016, the Company entered into a 15-year loan agreement with ING for €270,000 Euros with a fixed interest rate of 2.62%, maturing December 2031. As of December 31, 2019, the principal balance payable was \$252,629.

In 2017, the Company entered into a 4-year loan agreement with Namur Invest for €350,000 Euros with a fixed interest rate of 4.00%, maturing June 2021. As of December 31, 2019, the principal balance payable was \$175,150.

In 2017, the Company entered into a 7-year loan agreement with SOFINEX for up to €1 million Euros with a fixed interest rate of 4.50%, maturing September 2024. As of December 31, 2019, €1 million Euros has been drawn down under this agreement and the principal balance payable was \$1,122,701.

In 2018, the Company entered into a 4-year loan agreement with Namur Innovation and Growth for €500,000 Euros with fixed interest rate of 4.00%, maturing June 2022. As of December 31, 2019, the principal balance payable was \$408,888.

On November 28, 2019, the Company entered into a 4-year loan agreement with Namur Innovation and Growth for €500,000 Euros with fixed interest rate of 4.80%, maturing September 2024. As of December 31, 2019, the principal balance payable was \$561,351.

As of December 31, 2019, the total balance for long-term debt payable was \$2,842,847 and the payments remaining were as follows:

2020	\$	777,648
2021	\$	735,546
2022	\$	622,760
2023	\$	526,585
2024	\$	327,970
Greater than 5 years	\$	174,038
Total	\$	3,164,547
Less: Amount representing interest	\$	(321,700)
<b>Total Long-Term Debt</b>	<b>\$</b>	<b><u>2,842,847</u></b>

**e) Collaborative Agreement Obligations**

In 2015, the Company entered into a research sponsorship agreement with the German Cancer Research Center, or DKFZ, in Germany for a 3-year period for €338,984 Euros. As of December 31, 2019, \$224,540 is still to be paid by the Company under this agreement.

In 2016, the Company entered into a research co-operation agreement with DKFZ, in Germany for a 5-year period for €400,000 Euros. As of December 31, 2019, \$84,203 is still to be paid by the Company under this agreement.

In 2016, the Company entered into a collaborative research agreement with Munich University, in Germany for a 3-year period for €360,000 Euros. As of December 31, 2019, \$110,025 is still to be paid by the Company under this agreement.

In 2017, the Company entered into a clinical study research agreement with the Regents of the University of Michigan for a 3-year period for up to \$3.0 million. As of December 31, 2019, up to \$388,000 is still to be paid by the Company under this agreement. This agreement was amended in February 2020 to redefine a new clinical study. See Note 11.

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**Note 10 – Commitments and Contingencies (Continued)**

In 2018, the Company entered into a research collaboration agreement with the University of Taiwan for a 3-year period for a cost to the Company of up to \$2.55 million payable over such period. As of December 31, 2019, \$1.66 million is still to be paid by the Company under this agreement.

On May 1, 2019, the Company entered into a research collaboration agreement with the University of Taiwan to collect a total of 1,200 samples for a 2-year period for a cost to the Company of up to \$320,000 payable over such period. As of December 31, 2019, \$224,000 is still to be paid by the Company under this agreement.

As of December 31, 2019, the total amount to be paid for future research and collaboration commitments was \$2.69 million and the annual payments remaining were as follows:

2020	\$	1,699,767
2021	\$	988,500
<b>Total Collaborative Agreement Obligations</b>	<b>\$</b>	<b><u>2,688,267</u></b>

**f) Legal Proceedings**

There are no legal proceedings which the Company believes will have a material adverse effect on its financial position.

**Note 11 - Subsequent Events**

On January 7, 2020, a former director of the Company exercised 60,000 stock options to purchase shares of our common stock at prices ranging from \$2.50 to \$4.00 per share in a cashless exercise that resulted in the issuance of 17,483 shares of our common stock.

On January 10, 2020, the Company through its wholly owned subsidiary Belgian Volition, acquired an epigenetic reagent company Octamer GmbH, based in Munich, Germany, for a total purchase price of approximately \$725,000, of this amount \$400,000 was in cash and the balance was paid with 73,263 restricted shares of our common stock. This strategic acquisition helps secure the supply of one of the key components of Volition's Nu.Q<sup>TM</sup> tests, the recombinant nucleosome used as the calibrant.

On January 14, 2020, the Company purchased from its Chief Medical Officer 11,364 shares of our common stock at \$4.79 per share, for a total cost to the Company of \$54,434. These shares were subsequently retired.

On February 17, 2020, Volition America entered into an amendment, or the Amendment, to that certain Clinical Study Agreement, or the CSA, by and between Volition America and the Regents of the University of Michigan, or the Regents, with regards to Volition America's participation with the Regents and the National Cancer Institute, or NCI, Early Detection Research Network in a clinical study. Pursuant to the terms of the Amendment, the parties acknowledged that, although not fully-completed, the requirements of the original clinical study had been satisfied, including any and all payment obligations by Volition America. Further, the Amendment provided that a new clinical study would be undertaken at no additional cost to Volition America. The remaining terms of the CSA remain unchanged.

**END NOTES TO FINANCIALS**

**ITEM 9. CHANGES IN AND DISAGREEMENTS WITH ACCOUNTANTS ON ACCOUNTING AND FINANCIAL DISCLOSURE**

None.

**ITEM 9A. CONTROLS AND PROCEDURES**

***Disclosure Controls and Procedures***

Disclosure controls and procedures are controls and procedures that are designed to ensure that information required to be disclosed in our reports filed under the Exchange Act is recorded, processed, summarized and reported, within the time periods specified in the SEC's rules and forms. Disclosure controls and procedures include, without limitation, controls and procedures designed to ensure that information required to be disclosed by our company in the reports that it files or submits under the Exchange Act is accumulated and communicated to our management, including our Principal Executive and Principal Financial Officers, or persons performing similar functions, as appropriate to allow timely decisions regarding required disclosure.

Our management carried out an evaluation under the supervision and with the participation of our Principal Executive Officer and Principal Financial Officer, of the effectiveness of the design and operation of our disclosure controls and procedures pursuant to Rules 13a-15(e) and 15d-15(e) under the Exchange Act. Based upon that evaluation, our Principal Executive Officer and Principal Financial Officer have concluded that, as of December 31, 2019, our disclosure controls and procedures were not effective because of material weakness in our internal control over financial reporting.

***Management's Report on Internal Control Over Financial Reporting***

Management is responsible for establishing and maintaining adequate internal control over financial reporting, as defined in Exchange Act Rule 13a-15(f). The Company's internal control over financial reporting is a process designed to provide reasonable assurance regarding the reliability of financial reporting and the preparation of financial statements for external purposes in accordance with accounting principles U.S GAAP.

Because of its inherent limitations, internal control over financial reporting may not prevent or detect misstatements. Also, projections of any evaluation of effectiveness to future periods are subject to the risk that controls may become inadequate because of changes in conditions, or that the degree of compliance with the policies or procedures may deteriorate.

Under the supervision and with the participation of management, including the Principal Executive Officer and Principal Financial Officer, the Company conducted an evaluation of the effectiveness of the Company's internal control over financial reporting as of December 31, 2019, using the criteria established in "*Internal Control - Integrated Framework*" issued by the Committee of Sponsoring Organizations of the Treadway Commission ("COSO").

A material weakness is a deficiency, or combination of deficiencies, in internal control over financial reporting, such that there is a reasonable possibility that a material misstatement of the Company's annual or interim financial statements will not be prevented or detected on a timely basis.

In its assessment of the effectiveness of internal control over financial reporting as of December 31, 2019, the Company determined that there were control deficiencies in the following areas that constituted material weaknesses, as described below:

- ① segregation of duties in some areas of Finance;
- ① oversight in the area of Information Technology ("IT"), where certain processes may affect the internal controls over financial reporting; and
- ① monitoring of review controls with respect to accounting for complex transactions.

Accordingly, the Company concluded that these control deficiencies resulted in a possibility that a material misstatement of the annual or interim financial statements will not be prevented or detected on a timely basis by the Company's internal controls.

As a result of the material weaknesses described above, management has concluded that the Company did not maintain effective internal control over financial reporting as of December 31, 2019, based on criteria established in Internal Control—Integrated Framework issued by COSO.

### ***Changes in Internal Control over Financial Reporting***

The Audit Committee of the Board of Directors meets regularly with our financial management, and with the independent registered public accounting firm engaged by us. Internal accounting controls and the quality of financial reporting are discussed during these meetings. The Audit Committee has discussed with the independent registered public accounting firm matters required to be discussed by the auditing standards adopted or established by the Public Company Accounting Oversight Board (“PCAOB”). In addition, the Audit Committee and the independent registered public accounting firm have discussed the independent registered public accounting firm’s independence from the Company and its management, including the matters in the written disclosures required by PCAOB Rule 3526 “*Communicating with Audit Committees Concerning Independence.*”

As of December 31, 2019, we did not maintain sufficient internal controls over financial reporting in the following areas:

- ① segregation of duties in some areas of Finance;
- ② oversight in the area of IT, where certain processes may affect the internal controls over financial reporting; and
- ③ monitoring of review controls with respect to accounting for complex transactions.

We have developed, and are currently implementing, a remediation plan for these material weaknesses. Specifically, we have identified and selected a system for financial reporting that will allow further automation of the reporting process, thereby strengthening the control environment over financial reporting.

As we continue to evaluate and work to enhance our internal controls over financial reporting, we may determine that additional measures should be taken to address these or other control deficiencies, and/or that we should modify our remediation plan.

There have been no changes in our internal control over financial reporting that occurred during the fiscal year ended December 31, 2019, other than those described above, that have materially affected, or are reasonably likely to materially affect, our internal control over financial reporting.

The Company is not required by current SEC rules to include, and does not include, an auditor’s attestation report. Consequently, the Company’s registered public accounting firm has not attested to management’s reports on the Company’s internal control over financial reporting.

### ***Continuing Remediation Efforts to address deficiencies in Company’s Internal Control over Financial Reporting***

Once the Company is engaged in stable business operations and has sufficient personnel and resources available, then our Board of Directors, in particular and in connection with the aforementioned deficiencies, will establish the following remediation measures:

- ① Additional Finance resources will be recruited to resolve the segregation of duties control weaknesses noted above;
- ② Internal audit resources will be contracted to review and advise on control weaknesses across the organization; and
- ③ Specialist resources in IT and Human Resources will be recruited to recommend and implement relevant policy and processes to strengthen IT and Human Resources internal controls associated with financial reporting.

## **ITEM                    OTHER INFORMATION**

### **9B.**

On February 17, 2020, Volition America, Inc., or Volition America, a wholly-owned subsidiary of the Company, entered into an amendment, or the Amendment, to that certain Clinical Study Agreement, or the CSA, by and between Volition America and the Regents of the University of Michigan, or the Regents, with regards to Volition America’s participation with the Regents and the National Cancer Institute, or NCI, Early Detection Research Network in a clinical study involving approximately 13,500 asymptomatic screening samples provided by the Regents and/or NCI (including more than 4,600 previously collected samples) from people aged 50 and over who had not previously undergone screening or diagnostic colonoscopy, referred to as the Original Study. Pursuant to the terms of the Amendment, the parties acknowledged that, although not fully-completed, the requirements of the Original Study had been satisfied, including any and all payment obligations by Volition America. Further, the Amendment provided that a new clinical study, referred to as the New Study, would be undertaken at no additional cost to Volition America that involves approximately 1,800 asymptomatic screening samples provided by the Regents and/or NCI (including approximately 500 previously collected samples) from people aged 18 and over (i) who are being seen preoperatively for colon adenocarcinoma or adenoma and who had not previously had any radiation or chemotherapy for the current diagnosis, or (ii) who are undergoing colonoscopy procedures for colonic neoplasia screening, surveillance or resection of known neoplastic lesions. The screening samples from the New Study will be tested by Volition America for blood-based, cell-free circulating biomarkers on Volition’s proprietary Nu.Q™ platform to validate Volition’s Nu.Q™ Colorectal Cancer Screening Test for U.S. regulatory purposes. The enrollment period and sample collection is anticipated to take up to 14 months to complete. The remaining terms of the CSA remain unchanged. The foregoing description of the Amendment does not purport to be complete and is qualified in its entirety by such Amendment, a copy of which is filed as Exhibit 10.22 to this Report

**PART III**

**ITEM DIRECTORS, EXECUTIVE OFFICERS AND CORPORATE GOVERNANCE**

**10.**

The information required under this item is incorporated by reference from our definitive proxy statement related to our 2020 Annual Meeting of Stockholders, or the Proxy Statement, to be filed pursuant to Regulation 14A, on or before April 29, 2020.

**ITEM 11. EXECUTIVE COMPENSATION**

The information required under this item is incorporated herein by reference from the Proxy Statement.

**ITEM 12. SECURITY OWNERSHIP OF CERTAIN BENEFICIAL OWNERS AND MANAGEMENT AND RELATED STOCKHOLDER MATTERS**

The information required under this item is incorporated herein by reference from the Proxy Statement

**ITEM 13. CERTAIN RELATIONSHIPS AND RELATED TRANSACTIONS, AND DIRECTOR INDEPENDENCE**

The information required under this item is incorporated herein by reference from the Proxy Statement.

**ITEM 14. PRINCIPAL ACCOUNTANT FEES AND SERVICES**

The information required under this item is incorporated herein by reference from the Proxy Statement.

PART IV

ITEM EXHIBITS, FINANCIAL STATEMENT SCHEDULES

15.

- (a) The following documents are filed as part of this Report:
1. *Financial Statements*. Included in Part II, *Item 8* of this Report and are incorporated by reference herein.
  2. *Financial Statement Schedules*. Financial statement schedules are omitted because they are not applicable or the required information is shown in the financial statements or notes thereto.
  3. *Exhibits*.

Exhibit Number	Exhibit Description	Incorporated by Reference			Filing Date	Filed Herewith
		Form	File No.	Exhibit		
<a href="#">2.1</a>	Share Purchase Agreement by and between Singapore Volition and ValiRX dated September 22, 2010.	8-K/A	000-30402	2.01	5/8/12	
<a href="#">2.2</a>	Supplementary Agreement to the Share Purchase Agreement by and between Singapore Volition and ValiRX dated June 9, 2011.	8-K/A	000-30402	10.15	1/11/12	
<a href="#">2.3</a>	Share Exchange Agreement by and among Standard Capital Corporation, the controlling shareholders of Standard Capital Corporation and Singapore Volition dated September 26, 2011.	8-K	000-30402	2.1	9/29/11	
<a href="#">2.4</a>	Agreement, Consent and Waiver by and between Standard Capital Corporation and its Shareholders dated September 27, 2011.	8-K/A	000-30402	10.28	4/5/12	
<a href="#">3.1</a>	Second Amended and Restated Certificate of Incorporation, as currently in effect.	8-K	001-36833	3.1	10/11/16	
<a href="#">3.2</a>	Amended and Restated Bylaws, as currently in effect.	S-8	333-208512	4.2	12/11/15	
<a href="#">4.1</a>	Description of Capital Stock.					X
<a href="#">10.1</a>	Non-Exploitation and Third-Party Patent License Agreement by and among ValiBio SA, ValiRX and The Walloon Region dated December 17, 2009.	8-K/A	000-30402	10.06	2/24/12	
<a href="#">10.2</a>	Common Stock Purchase Agreement, by and among VolitionRx and the purchasers thereto dated February 26, 2014.	8-K	000-30402	10.1	2/28/14	
<a href="#">10.3#</a>	Employment Agreement by and between VolitionRx and Jason Terrell MD, dated December 29, 2015.	10-K	001-36833	10.24	3/11/16	

Exhibit Number	Exhibit Description	Incorporated by Reference				Filed Herewith
		Form	File No	Exhibit	Filing Date	
<a href="#">10.4#</a>	2011 Equity Incentive Plan dated November 17, 2011.	8-K	000-30402	4.01	11/18/11	
<a href="#">10.6(b)#</a>	Form Stock Award Agreement for Restricted Stock under the 2011 Equity Incentive Plan.	8-K	000-30402	4.03	11/18/11	
<a href="#">10.7#</a>	2015 Stock Incentive Plan, as amended March 27, 2019.	8-K	001-36833	10.1	06/18/19	
<a href="#">10.8(a)#</a>	Form of Notice of Stock Option Grant and Stock Option Agreement under the 2015 Stock Incentive Plan.	S-8	333-214118	10.2	10/14/16	
<a href="#">10.9(b)#</a>	Form of Notice of Restricted Stock Award and Restricted Stock Agreement under the 2015 Stock Incentive Plan.	S-8	333-214118	10.3	10/14/16	
<a href="#">10.10(c)#</a>	Form of Notice of Stock Bonus Award and Stock Bonus Award Agreement under the 2015 Stock Incentive Plan	S-8	333-214118	10.4	10/14/16	
<a href="#">10.11(d)#</a>	Form of Notice of Stock Appreciation Right Award and Stock Appreciation Right Award Agreement under the 2015 Stock Incentive Plan.	S-8	333-214118	10.5	10/14/16	
<a href="#">10.11(e)#</a>	Form of Notice of Restricted Stock Unit Award and Restricted Stock Unit Agreement under the 2015 Stock Incentive Plan.	S-8	333-214118	10.6	10/14/16	
<a href="#">10.11(f)#</a>	Form of Notice of Performance Shares Award and Performance Shares Agreement under the 2015 Stock Incentive Plan.	S-8	333-214118	10.7	10/14/16	
<a href="#">10.12#</a>	Independent Director Agreement.	10-Q	001-36833	10.33	5/12/15	
<a href="#">10.13</a>	Real Estate Capital Lease Agreement by and between Belgian Volition and ING Asset Finance Belgium S.A., dated October 4, 2016 (English translation of French original).	8-K	001-36833	10.1	10/31/16	
<a href="#">10.14</a>	Deed of Sale to the Sale Agreement by and between and Gerard Dekoninck S.A., dated October 25, 2016 (English translation of French original).	8-K	001-36833	10.2	10/31/16	
<a href="#">10.15#</a>	Employment Agreement by and between Volition Diagnostics UK Limited and Cameron Reynolds, dated March 7, 2017.	10-K	001-36833	10.27	03/10/17	

Exhibit Number	Exhibit Description	Incorporated by Reference				Filed Herewith
		Form	File No.	Exhibit	Filing Date	
<a href="#">10.16#</a>	Employment Agreement by and between Volition Diagnostics UK Limited and Jacob Micallef, dated March 7, 2017.	10-K	001-36833	10.28	03/10/17	
<a href="#">10.18#</a>	Employment Agreement by and between Volition Diagnostics UK Limited and Martin Faulkes, dated March 7, 2017.	10-K	001-36833	10.30	03/10/17	
<a href="#">10.19#</a>	Employment Agreement by and between Volition Diagnostics UK Limited and David Vanston, dated April 10, 2017.	10-Q	001-36833	10.1	05/11/17	
<a href="#">10.20</a>	Unsecured Credit Agreement dated September 20, 2017, by and among VolitionRx Limited, Belgian Volition SPRL and SOFINEX (English translation of French original).	8-K	001-36833	10.1	09/21/17	
<a href="#">10.21</a>	Clinical Study Agreement dated July 17, 2017, by and between Volition America, Inc. and the Regents of the University of Michigan.	10-Q	001-36833	10.1	11/09/17	
<a href="#">10.22</a>	Amendment #1 to Clinical Study Agreement, dated February 17, 2020, by and between Volition America, Inc. and the Regents of the University of Michigan.					X
<a href="#">10.23</a>	Common Stock Purchase Agreement, dated August 8, 2018, by and between VolitionRx and Cotterford Company Limited, including the form of Warrant attached as Exhibit B thereto.	8-K	001-36833	10.1	8/9/18	
<a href="#">10.24</a>	Equity Distribution Agreement, dated September 7, 2018, by and between VolitionRx and Oppenheimer & Co. Inc.	S-3	333-227248	1.2	9/10/18	
<a href="#">10.25#</a>	Warrant to Purchase Common Stock by and between VolitionRx and Jason Terrell MD, dated March 20, 2013; First Amendment to Warrant Agreement dated February 14, 2017; and Second Amendment to Warrant Agreement dated July 1, 2019.	S-3	333-236335	4.3	2/7/20	
<a href="#">21.1</a>	List of Subsidiaries.					X
<a href="#">23.1</a>	Consent of independent registered public accounting firm.					X



Exhibit Number	Exhibit Description	Incorporated by Reference			Filed Herewith
		Form	File No.	Exhibit	
24.1	Power of Attorney (included on the signature page of this Report).				X
<a href="#">31.1</a>	Certification of Chief Executive Officer pursuant to Rule 13a-14(a) or Rule 15d-14(a) promulgated under the Securities Exchange Act of 1934, as amended.				X
<a href="#">31.2</a>	Certification of Chief Financial Officer pursuant to Rule 13a-14(a) or Rule 15d-14(a) promulgated under the Securities Exchange Act of 1934, as amended.				X
<a href="#">32.1</a> *	Certifications of Chief Executive Officer and Chief Financial Officer, pursuant to 18 U.S.C. Section 1350, as adopted pursuant to Section 906 of the Sarbanes-Oxley Act of 2002.				X
10.1.INS	XBRL Instance Document				X
101.SCH	XBRL Taxonomy Extension Schema Document.				X
101.CAL	XBRL Taxonomy Extension Calculation Linkbase Document.				X
101.DEF	XBRL Taxonomy Extension Definition Linkbase Document.				X
101.LAB	XBRL Taxonomy Extension Label Linkbase Document.				X
101.PRE	XBRL Taxonomy Extension Presentation Linkbase Document.				X
#	Indicates a management contract or compensatory plan or arrangement.				
*	The certifications attached as Exhibit 32.1 accompany this Report pursuant to 18 U.S.C. Section 1350, as adopted pursuant to Section 906 of the Sarbanes-Oxley Act of 2002, and shall not be deemed “filed” by the registrant for purposes of Section 18 of the Exchange Act and are not to be incorporated by reference into any of the registrant’s filings under the Securities Act or the Exchange Act, irrespective of any general incorporation language contained in any such filing.				

**ITEM 16. FORM 10-K SUMMARY**

None.

## SIGNATURES

Pursuant to the requirements of Section 13 or 15(d) of the Securities Exchange Act of 1934, the registrant caused this Report to be signed on its behalf by the undersigned, thereunto duly authorized.

### VOLITIONRX LIMITED

Dated: February 20, 2020

By: /s/ Cameron Reynolds  
Cameron Reynolds  
President, Chief Executive Officer and Director

### POWER OF ATTORNEY

KNOW ALL PERSONS BY THESE PRESENTS that each individual whose signature appears below constitutes and appoints Cameron Reynolds and Rodney Rootsart, and each or either of them, acting individually, his or her true and lawful attorney-in-fact and agent, with full power of substitution, for him or her and in his or her name, place and stead, in any and all capacities, to sign any and all amendments to this Report on Form 10-K, and to file the same, with all exhibits thereto and other documents in connection therewith, with the Securities and Exchange Commission, granting unto said attorneys-in-fact and agents, and each of them, full power and authority to do and perform each and every act and thing requisite and necessary to be done in connection therewith, as fully to all intents and purposes as he or she might or could do in person, hereby ratifying and confirming all that said attorneys-in-fact and agents, or either of them, or his, her or their substitute or substitutes, may lawfully do or cause to be done or by virtue hereof.

Pursuant to the requirements of the Securities Exchange Act of 1934, this Report on Form 10-K has been signed below by the following persons in the capacities and on the date indicated.

<u>Signature</u>	<u>Title</u>	<u>Date</u>
<u>/s/ Cameron Reynolds</u> Cameron Reynolds	President, Chief Executive Officer and Director (Principal Executive Officer)	February 20, 2020
<u>/s/ David Vanston</u> David Vanston	Chief Financial Officer and Treasurer (Principal Financial and Accounting Officer)	February 20, 2020
<u>/s/ Dr. Martin Faulkes</u> Dr. Martin Faulkes	Director	February 20, 2020
<u>/s/ Guy Innes</u> Guy Innes	Director	February 20, 2020
<u>/s/ Dr. Alan Colman</u> Dr. Alan Colman	Director	February 20, 2020
<u>/s/ Dr. Phillip Barnes</u> Dr. Phillip Barnes	Director	February 20, 2020
<u>/s/ Dr. Edward Futcher</u> Dr. Edward Futcher	Director	February 20, 2020

## DESCRIPTION OF CAPITAL STOCK

The following is a summary of all material characteristics of the capital stock of VolitionRx Limited, as set forth in our Second Amended and Restated Certificate of Incorporation, or our Charter, and our Amended and Restated Bylaws, or our Bylaws. References to “we,” “us,” and “our” refer to VolitionRx Limited. This summary does not purport to be complete and is qualified in its entirety by reference to our Charter and our Bylaws, copies of which have been filed as exhibits to our public filings with the Securities and Exchange Commission.

### Common Stock

*General.* We have authority under our Charter to issue up to 100,000,000 shares of our common stock, par value \$0.001 per share.

*Voting Rights.* Holders of shares of our common stock are entitled to one vote per share held of record on all matters submitted to a vote of stockholders, including the election of directors.

*Dividend Rights.* The holders are entitled to receive dividends when, as and if declared by our board of directors, in its discretion, out of funds legally available therefor.

*Right to Receive Liquidation Distributions.* In the event of our liquidation, dissolution or winding up, the holders of our common stock are entitled to share ratably in all of our assets remaining after payment of liabilities.

*No Preemptive or Similar Rights.* The holders of our common stock have no preemptive or other subscription rights, and there are no conversion rights or redemption or sinking fund provisions with respect to such shares.

### Anti-Takeover Effects of Delaware Law, Our Charter and Our Bylaws

Certain provisions of Delaware law, our Charter and our Bylaws could have the effect of delaying, deferring or discouraging another party from acquiring control of us. These provisions, which are summarized below, are expected to discourage certain types of coercive takeover practices and inadequate takeover bids. These provisions are also designed, in part, to encourage persons seeking to acquire control of us to first negotiate with our board of directors. We believe that the benefits of increased protection of our potential ability to negotiate with an unfriendly or unsolicited acquirer outweigh the disadvantages of discouraging such proposals, including proposals that are priced above the then-current market value of our common stock, because, among other reasons, the negotiation of such proposals could result in an improvement of their terms.

*Delaware Law.* We are subject to the provisions of Section 203 of the DGCL regulating corporate takeovers. In general, Section 203 prohibits a publicly-held Delaware corporation from engaging, under certain circumstances, in a business combination with an interested stockholder for a period of three years following the date the person became an interested stockholder unless:

- Ⓢ prior to the date of the transaction, our board of directors approved either the business combination or the transaction which resulted in the stockholder becoming an interested stockholder;
  - Ⓢ upon completion of the transaction that resulted in the stockholder becoming an interested stockholder, the interested stockholder owned at least 85% of the voting stock of the corporation outstanding at the time the transaction commenced, excluding for purposes of determining the voting stock outstanding, but not the outstanding voting stock owned by the interested stockholder, (1) shares owned by persons who are directors and also officers and (2) shares owned by employee stock plans in which employee participants do not have the right to determine confidentially whether shares held subject to the plan will be tendered in a tender or exchange offer; or
  - Ⓢ at or subsequent to the date of the transaction, the business combination is approved by the board of directors of the corporation and authorized at an annual or special meeting of stockholders, and not by written consent, by the affirmative vote of at least 66 2/3 % of the outstanding voting stock which is not owned by the interested stockholder.
-

Generally, a “business combination” includes a merger, asset or stock sale, or other transaction resulting in a financial benefit to the “interested stockholder” and an “interested stockholder” is a person who, together with affiliates and associates, owns or, within three years prior to the determination of interested stockholder status, did own 15% or more of a corporation’s outstanding voting stock. We expect the existence of this provision to have an anti-takeover effect with respect to transactions our board of directors does not approve in advance. We also anticipate that Section 203 may discourage business combinations or other attempts that might result in a premium over the market price for the shares of common stock held by our stockholders. The provisions of the DGCL, our Charter and our Bylaws could have the effect of discouraging others from attempting hostile takeovers and, as a consequence, they may also inhibit temporary fluctuations in the market price of our common stock that often result from actual or rumored hostile takeover attempts. These provisions may also have the effect of preventing changes in our management. It is possible that these provisions could make it more difficult to accomplish transactions that stockholders may otherwise deem to be in their best interests.

*Charter and Bylaw Provisions.* Our Charter and our Bylaws include provisions that:

- ① require that any action to be taken by our stockholders be effected at a duly-called annual or special meeting and not by written consent;
- ① specify that special meetings of our stockholders can be called only by the board of directors, the chairman of the board, or the chief executive officer (or the president if there is no chief executive officer);
- ① establish an advance notice procedure for stockholder approvals to be brought before an annual meeting of our stockholders, including proposed nominations of persons for election to the board of directors;
- ① provide that the number of directors on our board of directors is fixed exclusively by our board of directors;
- ① provide that vacancies on our board of directors may be filled only by a majority of directors then in office, even though less than a quorum;
- ① establish the Court of Chancery of the State of Delaware as the sole and exclusive forum for certain derivative actions or proceeding brought on our behalf, any action asserting a claim of breach of fiduciary duty, any action asserting a claim against us arising pursuant to the General Corporation Law of the State of Delaware, or the DGCL, or any action asserting a claim governed by the internal affairs doctrine; and
- ① provide that there is no right to cumulate votes with respect to any shares of capital stock.

**AMENDMENT #1 TO  
CLINICAL STUDY AGREEMENT**

The following is Amendment #1 to the Clinical Study Agreement dated July 17, 2017 between Volition America, Inc. ("Laboratory") and the Regents of the University of Michigan (the "University") for Dean Brenner, ORSP Reference # AWD005981, and incorporates all of the terms therein. This Amendment is effective as of September 1, 2019.

The purpose of this Amendment is to acknowledge that the requirements of the GLNE010 study have been satisfied. This Amendment serves to end the completed sections of the Study Protocol in Exhibit A (GLNE010) add Study Protocol Exhibit A-1 {GLNE007}, Exhibit B and Exhibit C. This Amendment shall also replace Section 3.1 in its entirety:

3.1 In consideration of its participation in the Clinical Study on the terms and conditions of this Agreement, Laboratory shall provide direct and indirect funding in the amount of up to One Million, Five Hundred Thousand United State Dollars (US\$1,500,000). Direct and indirect payments by the Laboratory for the Clinical Study has concluded, are paid in full, and no additional future funding will be provided.

All terms of the Clinical Study Agreement shall remain unchanged.

ACCEPTED AND AGREED TO:

**Volition America, Inc.**

<u>/s/ Jason Terrell</u>	<u>02/17/2020</u>
Signature of Responsible Officer for Laboratory	Date

Jason Terrell, MD, Chief Executive Officer  
Typed Name and Title

**Regents of the University of Michigan:**

<u>/s/ Julie Olivero</u>	<u>02/11/2020</u>
Signature of Responsible Officer for Laboratory	Date

Julie Olivero, Project Representative  
Typed Name and Title

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**Evaluation of Stool Based Markers for the Early Detection of Colorectal Cancers and Adenomas  
Great Lakes New England (GLNE) Clinical Validation Center  
NCI Early Detection Research Network**

**STUDY INVESTIGATORS:**

Clinical Validation Center Investigators (GLNE)

Henry Appelman, M.D.<sup>1</sup>

John A. Baron, M.D.<sup>2</sup>

Dean E. Brenner, M.D.<sup>1</sup>

Robert Bresalier, M.D.<sup>3</sup>

Tim Church Ph.D.<sup>4</sup>

Seth Crockett, M.D., MPH<sup>2</sup>

Marcia Cruz-Correa, M.D., Ph.D.<sup>9</sup>

William M. Grady, M.D.<sup>8</sup>

Mack Ruffin, M.D., M.P.H.<sup>5</sup>

Ananda Sen, Ph.D.<sup>1</sup>

Elena Stoffel, M.D., M.P.H.<sup>1</sup>

Sapna Syngal, M.D.<sup>6</sup>

Christopher Teshima M.D.<sup>7</sup>

<sup>1</sup>University of Michigan Medical Center, Ann Arbor, MI

<sup>2</sup>University of North Carolina, Chapel Hill, NC

<sup>3</sup>MD Anderson Cancer Center, Houston, TX

<sup>4</sup>University of Minnesota, Minneapolis, MN

<sup>5</sup>Hershey Medical Center-PSU, Hershey, PA6

<sup>6</sup>Dana-Farber Harvard Cancer Center, Boston, MA

<sup>7</sup>Saint Michael's Hospital, University of Toronto, Toronto, Ontario

<sup>8</sup>University of Washington Medical Center, Seattle, WA

<sup>9</sup>University of Puerto Rico Comprehensive Cancer Center, San Juan, Puerto Rico

Contact information for Great Lakes New England Clinical Validation Center

2150 Cancer Center and Geriatrics Center

University of Michigan Medical Center Ann Arbor, MI 48109-0930

Telephone: (734) 647-1417

Fax: (734) 647-9817

Email: [dbrenner@umich.edu](mailto:dbrenner@umich.edu) (PI)

[kherman@umich.edu](mailto:kherman@umich.edu) (Lead CRA)

EDRN Data Management and Coordinating Center (DMCC)

Fred Hutchinson Cancer Research Center

1100 Fairview Ave N Seattle, WA 98109-1024

206-667-6038

Ziding Feng, Ph.D. (PI)

[spage@fredhutch.org](mailto:spage@fredhutch.org) (DMCC GLNE 007 Project Manager)

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**GLNE 007**  
**EVALUATION OF STOOL BASED MARKERS FOR THE EARLY DETECTION OF**  
**COLORECTAL CANCERS AND ADENOMAS**

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## 1.0 SUMMARY OF STUDY

As part of the National Cancer Institute-funded Early Detection Research Network (EDRN), the Great Lakes-New England Clinical Epidemiological Center (GLNE CEC) proposes a research study that validates potential molecular markers (“biomarkers”) for the detection of precancerous and cancerous conditions and cancer risk assessment. Although examples of such biomarkers are currently in clinical use (i.e. CEA, CA-125), there are limitations to all of them. Our consortium focuses on gastrointestinal neoplasia.

The goals of this phase of the proposed research are:

1. Assessment of the utility of individual stool-based, and serum-based biomarkers for discriminating between patients with adenocarcinomas, patients with adenomas with high grade dysplasia, patients with advanced adenomas defined as adenoma histology of any combination including sessile serrated adenoma, tubulovillous adenoma, villous adenoma, sessile serrated polyp/adenoma, traditional serrated adenoma OR any adenoma  $\geq 1$  cm OR three or more adenomas, patients with adenomas that are not advanced, and normal colonoscopy subjects both at normal and high risk for developing colon cancer.
2. Construction of a panel of markers from those considered in Objective 1 to discriminate, under a number of assumptions concerning prevalence and cost of misclassification, between:
  - a. (Primary) Subjects with normal colons or non-advanced adenomas versus patients with cancers
  - b. (Secondary) Subjects with normal colons versus patients with cancers.
3. Comparison of the characteristics of individual markers and panels as discriminators to those of the established current standard, fecal immunochemical test (FIT).
4. Development of a bank of stool samples linked to serum, tissue, and clinical data from patients with colorectal cancer, adenomas and normal controls for validation of stool-based markers that may be developed in the future.

To build our collection, we propose to collect stool, FIT, serum, plasma, and tissue samples from 1,000 new subjects. EDRN Common Data Elements (CDEs) will be completed by the recruiting sites and provided for the laboratories developing the assay. Each biomarker will be analyzed individually and considered as a potential panel marker to be used for future large-scale screening longitudinal trials.

This protocol had previously recruited subjects from January 2006 to June 2010. The samples from this recruitment period are 9-13 years old as of the development of this protocol in April, 2019. Prior recruited subjects:

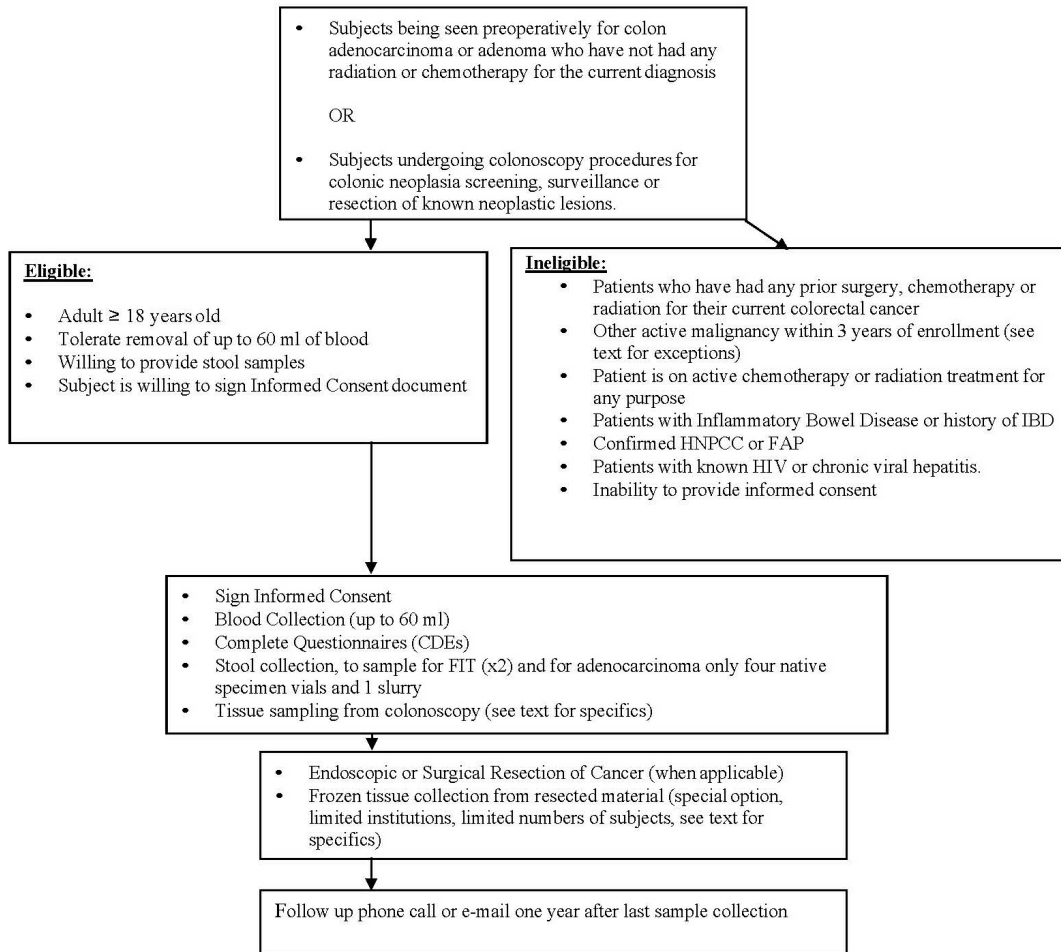
262 adenomas (54 of those advanced)  
191 cancers  
65 high risk, colonoscopic normal  
164 colonoscopic normal

From each subject, we collected 30 serum, 30 plasma, 5 stool, 20 5- ml urine aliquots  
Current Status of GLNE 007 repository:

Total circulating space samples collected: 16,900 serum, 15,700 plasma, 3,000 stool, and 7,000 urine aliquots (42,600 total)  
Total circulating space samples disbursed: 12,600 aliquots of various types.  
Remaining in the collection: (total 30,000)  
10,200 Serum aliquots  
11,500 Plasma aliquots  
2,100 stool aliquots (representing 585 unique subjects with at least 1 aliquot left)  
6,200 urine aliquots  
Total tissue samples: 2,100 tissue pieces snap frozen in liquid nitrogen  
Total tissue samples disbursed: 1,050  
Total tissue samples remaining: 1,050

This amended protocol (version 7) proposes to restart GLNE 007 to recruit 1,000 new subjects, (400 colorectal cancers, 200 adenomas, 200 higher risk but endoscopically normals and 200 endoscopically normal colons for controls). Thus, bringing our total from 682 to 1,682 total subjects.





**NOTES:**

- ① Nursing women who otherwise meet the eligibility criteria may participate.
- ② Subjects who had CRC that was successfully treated at least three years ago are eligible.
- ③ Recent screening colonoscopy (within 3 weeks of enrollment), poor preparation found at colonoscopy and returning for repeat colonoscopy are eligible.

### 3.0 OBJECTIVES

1. Assessment of the utility of individual stool-based, and serum-based biomarkers for discriminating between patients with adenocarcinomas, patients with adenomas with high grade dysplasia, patients with advanced adenomas defined as adenoma histology of any combination including sessile serrated adenoma, tubulovillous adenoma, villous adenoma, sessile serrated polyp/adenoma, traditional serrated adenoma OR any adenoma  $\geq 1$  cm OR three or more adenomas, patients with adenomas that are not advanced, and normal colonoscopy subjects both at normal and high risk for developing colon cancer.
2. Construction of a panel of markers from those considered in Objective 1 to discriminate, under a number of assumptions concerning prevalence and cost of misclassification, between:
  - a. (Primary) Subjects with normal colons or non-advanced adenomas versus patients with cancers;
  - b. (Secondary) Subjects with normal colons versus patients with cancers.
3. Comparison of the characteristics of individual markers and panels as discriminators to those of the established current standard, fecal immunochemical test (FIT).
4. Continued support of a renewal of a bank of stool samples linked to serum, tissue, and clinical data from patients with colorectal cancer, adenomas and normal controls for validation of stool- based markers that may be developed in the future.

### 4.0 BACKGROUND AND SIGNIFICANCE

#### 4.1 Biomarkers

##### Definitions, underlying assumptions, and rationale.

A biomarker is defined as a characteristic that is measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to therapeutic interventions (3). An NCI Working Group further characterized a biomarker as a Clinical Endpoint--a characteristic or variable that measures how a patient feels, functions or survives; as a Surrogate Endpoint--a biomarker intended to substitute for a clinical endpoint in a clinical trial-- and as a Global Assessment: an evaluation of risk and benefit balance for a patient or group of patients. However, the working group did not address biomarkers specific to the carcinogenesis process or for cancer detection.

The underlying assumption of a surrogate endpoint for cancer prevention is that a measured biological event will predict a cancer outcome, either immediately or at a later time (4) and, in the same circumstances, be affected by the intervention. The primary motivations for development of such surrogate endpoints concerns the ability to diagnose cancer at an early stage, to identify individuals at high risk for development of cancer and to enable reduction of sample size and trial duration for an interventional trial such that a rare or distal endpoint can be replaced by a more frequent and more proximate endpoint (5).

##### Specifications for a useful biomarker.

An "ideal" biomarker will have the following characteristics (6):

- (i) Variability of expression between phases of the carcinogenesis process (i.e. normal, pre- malignant, malignant).
- (ii) Detectable early in the carcinogenesis process.
- (iii) Associated with the risk of developing cancer or the occurrence of pre-cancer.
- (iv) Detected in body fluids (e.g. blood, urine, sputum) or tissues obtained via biopsy.
- (v) Capability for development of adequate quality control procedures.
- (vi) Potential for modification by a chemo preventive agent.

#### 4.2 The Early Detection Research Network (EDRN)

##### The mission of the Early Detection Research Network.

The Early Detection Research Network (EDRN) is a comprehensive effort supported by the NCI to develop highly sensitive, specific, and clinically reliable early detection tools. The Network is harnessing scientific expertise from national and international institutions to identify and validate molecular markers for the detection of precancerous and cancerous cells and to assess risk for developing cancer.

The GLNE CVC is a funded EDRN consortium dedicated to the characterization and validation of biomarkers for the early detection and risk assessment of colorectal adenocarcinoma. The consortium provides the EDRN with expertise in population epidemiology, biostatistics, pharmacology and medical oncology.

#### **4.3 Current State of the Art: Recommended Early Detection**

Randomized controlled trials have shown that annual or biennial fecal occult blood tests (FOBT) reduce colorectal cancer (CRC) mortality by 15% to 33% (7-9). The reduction is durable over 3 decades (10). Population based cohort studies of colonoscopic screening demonstrate reduced CRC mortality, primarily in distal but not in the proximal colon (11-13). This discrepancy has been attributed to endoscopic quality issues, the technical difficulties in detecting lesions in the right colon, and the more frequent occurrence of flat and depressed dysplastic lesions in the right colon (14-17). In tandem colonoscopy studies, a subset of large polyps may be missed by a single examiner. Shorter withdrawal is time-linked to a lower adenoma detection rate (18, 19). Flat and depressed lesions are more challenging to detect and have been described with a relatively high prevalence in a US colonoscopy cohort (20). While colonoscopic removal of adenomatous polyps reduces CRC mortality (21), prospective, randomized controlled trials of screening colonoscopy have been initiated by the VA and in Europe (21-23). Over-diagnosis (i.e. early detection of indolent invasive neoplasms that do not cause mortality) or lead-time bias in early detection of colorectal neoplasms do not degrade the efficacy of screening and early detection for colorectal cancers (24).

Current screening guidelines for average risk individuals vary world-wide. In the United States the American Gastroenterology Association recommends testing for early detection of adenomas and cancer (structural examination) or of cancer (non-invasive stool tests) beginning at age 50 (25). The United States Preventive Services Task Force (USPSTF) recommends fecal occult blood testing (FOBT) every two years with optional endoscopic screening with either flexible sigmoidoscopy or colonoscopy (26). The majority of developed countries recommend fecal occult blood testing every two years but do not support endoscopic screening (27); albeit with some exceptions (e.g. Germany (27, 28)). In 2012, 65.1% of the United States adults adhered to USPSTF colorectal screening guidelines with colonoscopy the commonly used screening method (61.7%) followed by FOBT (10.4%) (29) whereas colonoscopic screening adherence in Germany is 16% (28). Over 20 years of SEER data (1991 to 2011), United States CRC incidence (all races, males, females) has fallen from 59.5 cases in 1991 to 39.3 cases per 100,000 in 2011 (35% reduction) with a corresponding mortality reduction over the same time period from 24.0 to 15.1 deaths per 100,000 (37% reduction) (30). Widespread adherence to screening guidelines in the United States may be driven by the profound changes in the organization of medical care including enhanced access via the Affordable Care Act, rigid guideline enforcement by payers with physician performance incentives and disincentives, and the rapid adaptation of electronic medical record systems enabling ease referrals for screening, compliance reminders, and management tracking of compliance to care guidelines (31).

#### **4.4 Current State of the Art: Serum Based Biomarkers for Colorectal Neoplasia**

Rational for non-adherence with stool based or colonoscopic based CRC screening include the volume of bowel preparation, inadequate analgesia, no recommendation from primary physician, embarrassment (32) or cultural taboos surrounding collection or manipulation of stool provide rationale for discovery and validation of circulating biomarkers for early detection of colorectal neoplasia. Circulating signatures may be detected from neoplasm generated genetic products, antigens, antibodies, glycans, circulating tumor cells.

## Genetic Products

In a recent study of 24 CRC patients, mutant DNA fragments (circulating tumor DNA, ctDNA) are found in relatively high concentrations in the circulation of most patients with metastatic cancer and are detected in ~70% of patients with localized cancers (33). The direct detection of aberrant genes or genetic material specific to colorectal neoplasms (e.g. APC, b-catenin, K-ras, DCC, and p53) has been limited by the technical challenge of DNA recovery, the large number of potential underlying genetic mutations, and by the limited sensitivity of any single genetic alteration due to the extremely low abundance gene mutations in circulating plasma or serum (33- 38). DNA hypermethylation, in contrast, affects residues in regulatory portions of genes and provides major advantages in designing biomarker assays (37, 39-41). Digital based quantitative technologies improving upon bisulfite conversion while minimizing bisulfite associated DNA fragmentation and single molecule detection technologies (42) permit cost effective development of DNA hypermethylated gene biomarkers. Such technology detected circulating methylated vimentin with 59% sensitivity (42). Septin9, a methylated gene discovered in tissues with array technology (43, 44), detects CRC with 50% sensitivity and 92% specificity in a large (7941 participants) prospective colonoscopy verified screening trial (45). For early stage CRC, Septin9 sensitivity decreased to 35%. While circulating methylated CpG DNA promoter sites appear to have higher CRC detection performance than other genetic detection strategies, they substantially lag behind stool based detection of blood DNA markers or endoscopy. Nevertheless, for individuals refusing to use stool based screening, detection sensitivity of circulating methylated DNA markers appears equivalent to guaiac based stool screening and has the potential advantage of capturing the 40% of the population refusing stool screening. miRNAs are stable and detectable in serum and plasma. As in stool, numerous up and down regulated miRNA stool signatures discovered using unsupervised array technology may be useful as CRC detection biomarkers. A recent review identifies 19 miRNAs as individual or groups in panels as candidates for detection markers; but, insufficient clinical validation renders the data generated to date using small convenience sets confusing and not mechanism driven (46).

## Proteins

*Antigens:* Approximately 50% of all proteins are estimated to be glycosylated (47). Glycan abundance and their micro- and macro-heterogeneity can be changed in a disease-specific manner (48). Glycoprotein screening studies, many EDRN supported, have relied on immunoprecipitation or lectin affinity capture of whole glycoproteins and mass spectrometry identification of the de- glycosylated protein portion or probed with lectins in an array format containing up to a few hundred antibodies (49-53). Sialylated Lewis A and Lewis X moieties carrying proteins identify panels of potential markers. The Lampe EDRN laboratory has found seven such proteins (B3GNT5, CD44, HSPG2, IL6, INHBC, NOTCH4 and VWF) which when combined in discovery set plasma samples ROC AUC of 0.83 (54). GLNE discovered glycan ligand, galectin- 3 ligand is a circulating glycan biomarker in large population based prospective validation (55).

*Antibodies:* Serum antibodies recognizing multiple colon cancer antigens can be detected in colorectal adenocarcinoma patients' markers (56-58). Preliminary validation of single or small autoantibody panels have been disappointing (59). For example, antibodies to the Fas receptor have 17% sensitivity when 100% specific for CRC detection (60). Experience with p53, Hsp60, and nucleobindin 1 (Calnuc) autoantibodies has been better (~50% sensitivity/70 to 90% specific); but, they are not specific to CRC (59, 61, 62) and cannot be used as a colon specific screening tool. Discovery sets that include a miniarray of autoantibodies with other markers have reported improved detection accuracy (sensitivity 83%/specificity 90%) (63) but require clinical validation.

*Cytokines/growth factors:* High serum concentrations of insulin-like growth factors (IGF) and low levels of their binding proteins have been shown to correlate with CRC risk in large cohort studies (64-67) but have low sensitivities with high specificities for CRC detection. Other cytokines or angiogenesis factors such as TGF-b1 (68-74), VEGF (75, 76), angiogenin (77), endostatin (78), and endothelins (79, 80) also have low sensitivity in small convenience sets and have not proceeded to clinical validation.

*Other proteins:* Of the matrix metalloproteinases (81-83), plasma TIMP1 is elevated in CRC but has not had sufficient sensitivity in larger validation trials to merit development as a detection biomarker (84). Cell adhesion molecules (85) have low sensitivities for detection of early stage CRC.

Circulating tumor cells (CTCs) entering the vascular space from primary neoplasms have been considered to be initiators of metastases (86-88) and can be detected in early stage invasive neoplasms (89, 90). CTC isolation from epithelial cancers initially used antibody capture technology dependent upon epithelial adhesion (EpCAM) and cytokeratins (86). This technology limits CTC detection of early stage neoplasms because CTCs are thought to undergo epithelial to mesenchymal transition (EMT), epithelial traits are lost and epithelial marker such as EpCAM and cytokines are downregulated. CTCs present in as few as 1 cell in  $5 \times 10^9$  red cells, and up to  $5-10 \times 10^6$  white blood cells, are rare events (88). Newer microfluidic or centrifugation devices appear to more efficiently capture CTCs (89, 91). The inclusion of mesenchymal/EMT-specific antibodies, for example, vimentin, PLS3 may improve CTC capture and/or expansion (88). With the emergence of ex-vivo expansion protocols of CTCs and the increased ability to detect stem like or stem progenitor cells, CTCs are of future interest as an early cancer detection diagnostic (89, 91), but remain in the technology development phase.

Special consideration—EDRN discovered and preliminarily validated circulating biomarker: Galectin-3 Ligand ELISA as a Serum Biomarker for the Detection of Colorectal Neoplasia

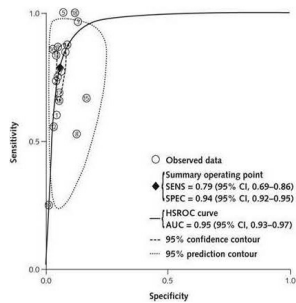
The galectins are widely distributed and evolutionarily conserved carbohydrate binding proteins characterized by their binding affinity for  $\beta$ -galactosides and by conserved sequence elements in the carbohydrate-binding region (92). Galectin-3 is the galectin that is of most interest in regard to colon cancer because of its demonstrated role in cancer progression, metastases, and interaction with mucins(93-97). Galectin-3 ligands include laminin, LAMP-1 and 2, LPS and colon cancer mucin. The major galectin-3 ligand detected in serum is a 40 kDa band distinct from MUC2 and other mucins CEA, and Mac-2-BP. We reported a true positive rate for the detection of CRC of 91% and false positive rate of 18% using preliminary data using quantitative Western blot technology on a convenience set of GLNE serum (55).

We developed a sensitive, reproducible ELISA assay for galectin-3 using a new antibody we created. This was used to assay the GLNE colorectal reference set (50 colorectal adenocarcinomas/50 adenomas/50 endoscopically normal controls). The ROC analyses for galectin-3 ligand combined with FOBT (fecal occult blood test-guaiac based) for detection of colorectal adenocarcinoma versus controls who had normal colonoscopy shows an area under the ROC curve of 0.91, while galectin-3 ligand detection of colorectal adenocarcinoma alone versus controls who had normal colonoscopy shows an area under the curve of 0.84. The true positive rate of galectin-3 ligand with FOBT for detection of CRC is 64% with a false positive rate of 5%. Without FOBT, true positive rate of galectin-2 ligand was 72% with a false positive rate of 20%.

**4.5 Rationale and Current State of the Art: Stool Based Biomarkers for Detection of Colorectal Neoplasia**

Occult blood tests

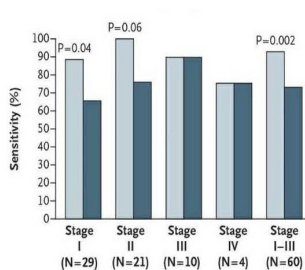
Stool testing as a screening approach offers the potential advantages of noninvasiveness, low cost, avoidance of cathartic preparation, and minimal impact on work time or daily activities. Guaiac based FOBT is not specific for human blood, and consequently, it has a high false positive rate for colorectal neoplasia. The fecal immunochemical test (FIT) detects human hemoglobin, thus eliminating the false positives caused by non-human hemoglobin in the diet (98, 99). FIT tests are more sensitive at detecting CRCs (sensitivity range 61% to 91%) and adenomas (sensitivity range 16% to 31%) than classical unrehydrated guaiac FOBT (Hemoccult II) (sensitivity range 25% to 38% for CRC; 16% to 31% for advanced adenomas) (100, 101). A recent meta-analysis that analyzed data from 19 prospective randomized trials or cohorts using 8 different commercially available FIT tests with colonoscopy or 2-year observation endpoints reported an overall sensitivity for detection of CRC of 79% (95% CI = 0.69-0.86), specificity of 94% (95% CI = 0.92-0.95) and overall accuracy (defined as hierarchical summary receiver operating characteristic (ROC) curve) of 95% (95% CI = 93% - 97%) (Figure 1). Differences in performance characteristics among FIT brands were small, particularly between the two major brands used OC-Light (Eiken Chemical) and OC-Micro/Sensor (Polymedco + Eiken Chemical). The Polymedco product is widely used in the USA. Quantitative FIT (Eiken OC-SENSOR)  $>177 \mu\text{g/gm}$  stool combined with age and sex predicts 11.46 fold risk of a large adenoma over lower risk groups (102).



**Fig 1 from Lee et al (1): Hierarchical ROC curve of the sensitivity versus specificity of FIT. The diamond = summary point of the curve to which the pooled sensitivity and specificity correspond. Dashed line = 95% CI for summary point; dotted line = 95% confidence area of FIT diagnostic accuracy. AUC = area under the curve; SENS = sensitivity;**

### Stool DNA tests

Since the neoplastic transformation process of the colonic epithelium results in cells shedding into the stool, collection of fecal material is likely to yield detectable molecular and biochemical events associated with cellular transformation (103, 104). First generation multi-marker stool DNA tests detected 52-73% of CRCs, 41-49% of CRCs plus adenomas with high grade dysplasia, and 15-46% of adenomas  $\geq 1$  cm, with specificities of 84- 95% (105, 106). Stool DNA test performance in both studies was compromised by failure to use stabilization buffer with stool collection, inefficient marker recovery from stool, and relatively insensitive analytical methods. Exact Sciences modified their previously published stool DNA panel (106) and now uses a panel consisting of methylated BMP3 and NDRG4 promoter regions, mutant K- ras (7 point mutations, Exon 2, codons 12,13), and a proprietary FIT test. In a recently published cross sectional validation study of 9,989 patients undergoing screening colonoscopy, the panel performed with a sensitivity of 92% for CRC; 84% for CRC + high grade dysplasia; and 42% for advanced adenomas (Figure 2) (2). The specificity was 87% for CRC, the ROC AUC for the Exact Sciences DNA stool panel for the detection of colorectal cancer is 0.94. FIT alone (Polymedco FIT) performed with sensitivity of 73.8% and specificity of 94.9% for detection of CRC and sensitivity of 23.8% for screen relevant neoplasia. Stool DNA component of the panels adds ~20% sensitivity to FIT. The USPSTF is currently assessing the role and contribution of fecal DNA panels such as the Exact Sciences panel to CRC screening (107).



**Fig 2 from Imperiale et al (2) sensitivity for detection of CRC by Exact Sciences stool DNA panel + FIT (light blue) vs Fit alone (dark blue) by stage.**

Aberrant methylation of vimentin exon 1 was initially described as a highly frequent biomarker of colorectal cancers and adenomas by Markowitz and co-workers (108). In reproducible studies, aberrant methylation of vimentin has been detected in 72%-83% of colon cancers and 70%-84% of colon adenomas (108, 109). The current assay for detection of vimentin exon 1 methylation is based on using methylation specific PCR (MSP). Adaptation of the vimentin MSP to testing fecal DNA is accomplished by recovery of vimentin DNA sequences from human stool using hybrid capture to vimentin specific oligonucleotides (108). Initial study showed that MSP assay of vimentin purified from feces (fecal vimentin DNA) detected methylated fecal vimentin DNA in 46% of cancer patients (N=94) at a specificity of 90% (N=198)(108). This initial study involved collaboration between the Markowitz laboratory who had discovered the methylated vimentin DNA marker, and Exact Sciences, who implemented detection of this marker in fecal DNA. This initial study was limited by use of samples that had suffered problems of DNA degradation during sample collection and shipping (106). A recently published two stage follow-up study lead by Itzkowitz et al in collaboration with Exact Sciences and the Markowitz laboratory showed markedly improved results with the use of a DNA stabilizing buffer added to stools at the time of collection (110). Detection of methylated fecal vimentin DNA was found in 77% of cancers (N=82) at 83% specificity (N=363). Six of 7 adenomas with high-grade dysplasia were also detected. This assay has successfully detected 55% (N=22) of adenomas that were greater or equal to 1cm in size (110). This is a published assay of capture of fecal vimentin DNA and then MSP detection of methylated vimentin exon 1 sequences (108, 110, 111).

## Other Stool Based Biomarkers Under Investigation

Considerable interest in fecal microbiome populations has triggered EDRN supported investigators into identifying unique bacterial species that are associated with colonic carcinogenesis and suggests that a microbiome signature may be a useful stool biomarker for CRC risk (112, 113). Metabolome signatures promise to identify amino acid or fatty acid profiles associated with colorectal cancer or high risk (114) have been preliminarily developed in EDRN supported research. Micro-RNAs (miRNA) have both oncogenic and suppressor properties, can be detected in stool, and have been explored as stool based early detection biomarkers (115, 116). Studies published to date have used small convenience samples and array technologies that have identified diverse and non-reproducible miRNAs as classifiers for colonic neoplasms.

### **4.6 Key Issues Driving Research Questions in CRC Early Detection Biomarkers**

Until therapeutic agents with much greater potency and minimal side effects are developed, the current best strategy for reducing cancer morbidity and mortality is early detection of neoplastic disease (117). Key opportunities in the current state of colorectal screening and early detection include:

1. *Enhancing adherence to current screening guidelines.* Screening and early detection reduce mortality from colorectal cancer; yet 35% of the population in the USA remain non-adherent.

Adherence is much lower in other countries (28). The barriers to these recommendations (cost, discomfort, cultural taboos) may be overcome with circulating biomarkers that provide individuals with persuasive evidence that undergoing invasive screening procedures, i.e. colonoscopy, will have important life-saving benefit that reduces mortality from CRC (11-13, 21). Developing, validating and bringing circulating biomarkers to population screening use remains a high priority that will likely increase adherence to endoscopic screening.

2. *Tailoring colonoscopic screening to individual risk:* Recently published data from the Clinical Outcomes Research Initiative found the prevalence of large polyps higher in blacks than whites among both men and women (118). Tailoring endoscopic screening to those at risk while limiting screening for those with minimal or no risk (119, 120) will enhance screening adherence and eliminate excess cost. Recommendations for tailoring were primarily population demographic based (119, 120); yet, the translation of carcinogenesis biology and genetics into biomarker panels with extremely high sensitivity (99%), i.e. no false negative tests, promises precise tailored endoscopic screening. The current state of art stool using based biomarker tools is coming close—92% sensitivity (2) but insufficient to permit tailored or individualized risk.

3. *Persistently positive stool DNA tests with negative colonoscopic screening* The stool methylated DNA panel's report 5% false positives (2, 111). A positive stool DNA test with a negative screening colonoscopy could potentially arise from neoplasia in the upper gastrointestinal tract or from occult and missed lesions in the colorectum. The latter is a particular concern in the right colon, where flat lesions and/or sessile serrated adenomas are more prevalent. Preliminary data from the Case Western EDRN BDL found near 100% vimentin methylation in gastric dysplasia while no methylation in adjacent gastric mucosa (S. Markowitz, Personal Communication). In Barrett's esophagus (BE), 7 of 7 high grade dysplasias (HGD), and 15 of 18 esophageal adenocarcinomas (EAC) and even in some squamous cancers (SCC) had methylated vimentin, whereas it was absent in all 9 normal squamous mucosa (121). A "false positive" stool DNA test may detect dysplasia or invasive neoplasms in the upper GI tract.

## **5.0 STUDY DESIGN**

### **5.1 Summary of Study Plan**

We propose a multi-center, prospective, cross-sectional cohort validation study of 1,682 subjects. We propose to increment the original GLNE 007 cohort with 400 subjects with diagnosed colorectal cancer, 200 subjects with colorectal adenomas, 200 subjects a prior history of adenomas, colorectal adenocarcinoma (>3 years previous), returning for surveillance or positive stool test (DNA or blood) but have a normal colonoscopy (higher risk normal), and 200 subjects who have a normal colon with NO prior history of adenomas, colorectal adenocarcinoma (not returning for surveillance) and who do not have a current (within 12 months) positive stool test (DNA or blood) (normal risk) and have a normal colonoscopy. Subjects will be recruited as described in Appendix B. The baseline visit should be done prior to a scheduled colonoscopy. If a subject is suspected of having a colon adenocarcinoma or an adenoma, the baseline samples should be collected before any procedure to remove the cancer or adenoma so the lesions are present when sample collection is done. Patients with cancer must have their baseline visit and all sample collection completed prior to endoscopic or surgical resection of CRC and chemotherapy and/or radiation therapy. Informed consent, demographic information and medical history via questionnaires, blood, and collection will be done at baseline. Stool collection, to sample for FIT (x2) and for adenocarcinoma four native stool specimen vials and 1 slurry will be done as described in the study calendar and Appendix D. All samples will be collected, handled, transported, processed, and stored according to detailed standard operating procedures and will be de-identified by random Specimen ID linked to the Participant ID in VSIMS. Selected subjects, based upon estimated future biomarker requirements, will have normal colonic epithelium collected during the colonoscopy procedure for future biomarker research. For those subjects with a large adenoma found on endoscopy, a frozen biopsy will be requested. Cancers, for the most part will be identified following endoscopic diagnosis.

### **5.2 Rationale for tissue collection**

A primary goal of GLNE 007 is to provide biosamples for training and testing of biomarkers that the EDRN believes have potential for future validation for regulatory review. A secondary goal of GLNE 007 meets the EDRN's discovery and early phase characterization of biomarkers. The EDRN is a vertically integrated organization that includes laboratories doing discovery research and early detection performance characterization research. The GLNE supports all of the EDRN's missions—discovery, characterization, training and testing in addition to large scale regulatory validation.

On occasion, investigators need fresh tissue to develop and test new biomarker technologies. The GLNE maintains a repository of frozen normal and adenomatous biopsy samples for this purpose. As with other biosamples proposed for GLNE 007, the frozen tissue samples need revitalization and updating.

GLNE collects fresh biopsies from adenomas as made available by local pathologists. If available, GLNE will also collect fresh biopsies from invasive cancers at endoscopy.

Biopsy tissue samples are not required from every subject entered into GLNE 007. The GLNE collects biopsies from normal colonic mucosa from subjects undergoing colonoscopy who are found to have a normal exam on an as needed basis (approximately 10% or 100 subjects will be asked to undergo biopsy of normal colonic mucosa) with a small repository, to be made available to EDRN investigators for discovery and early phase characterization. Because of the extra risk, time for participants and extra costs to the GLNE involved with performing these biopsies endoscopically, we do not require all patients with normal colonoscopies to undergo biopsy and tissue collection of normal colonic mucosa. The GLNE pays centers extra beyond the usual costs to procure frozen biopsy samples. Normal biopsies may be used as controls for EDRN laboratory biomarker discovery research controls for comparison with adenoma tissue and invasive neoplasm tissue. GLNE 007 has provided this resource to the EDRN over the last 15 years and continues to do so.

## **6.0 INCLUSION AND EXCLUSION CRITERIA**

### **6.1 Inclusion Criteria**

Willing to sign informed consent

Able to physically tolerate removal of up to 60 ml of blood

Adults at least 18 years old

Willing to collect 2 stool samples to prepare FIT test (x2) and for adenocarcinoma 4 native specimen vials and 1 slurry

Nursing women who otherwise meet the eligibility criteria may participate

Subjects undergoing colonoscopy for screening or surveillance (known prior neoplasms resected).



## Screening Colonoscopy

No known colonic neoplastic disease. Undergoing colonoscopic screening based upon current colon cancer screening guidelines.

Subjects whose screening colonoscopy shows any of these types of polyps may be included in the **normal** or the **higher risk normal** bin if they meet the other criteria noted above.

- ① Hyperplastic polyps
- ① Benign mucosal polyps
- ① Polypoid granulation tissue
- ① Prolapsed mucosal polyps
- ① Inflammatory polyp
- ① Transitional mucosal polyp
- ① Lipoma
- ① Gangleoneuroma
- ① Neuroma
- ① Hamartomatous polyp

Subjects who had colorectal adenocarcinoma that was successfully treated at least three years prior are eligible.

Recent screening colonoscopy (within 3 weeks of enrollment), poor preparation found at colonoscopy and returning for repeat colonoscopy.

Recent diagnostic colonoscopy (within 3 weeks of enrollment) with detection of adenocarcinoma or adenoma.

Known colorectal adenocarcinoma or adenoma remains in place after a diagnostic colonoscopy— adenocarcinoma or adenoma in colon at time of blood and stool collection.

Enrolled participants will be grouped into Bins according to one of the following

- ① **Colorectal Cancer**-pathologically confirmed colorectal cancer either present at time of stool collection or discovered during colonoscopy (Cancer Bin)
- ① **Adenoma**-pathologically confirmed adenoma (Adenoma Bin)
- ① **Higher Risk Normal (normal colonoscopy)**

Negative study colonoscopy and:

- Subjects with a personal history of adenomas (confirmed by pathology) with none present on qualifying colonoscopy
- Subjects with a personal history of CRC (longer than 3 years ago because of exclusion criteria of cancer within last 3 years) with none present at time of qualifying colonoscopy
- Any family history of CRC (1st degree relative)
- Current positive screening stool test for blood, for DNA or for both within 12 months.

- ① **Normal Control (normal colonoscopy)**

Negative colonoscopy and:

- No prior history of adenomas
- No prior history of CRC
- No family history of CRC
- Negative screening test (if performed) for blood, for DNA or for both within 12 months.

## 6.2 Exclusion Criteria

Cancer patients who have had any surgery, radiation, or chemotherapy for their current colorectal cancer prior to collecting the baseline samples

Other active malignancy within 3 years of enrollment except any of the following:

- a. Squamous cell carcinoma of the skin
- b. Basal cell carcinoma of the skin  
Carcinoma in situ of the cervix, Stages Ia or Ib invasive squamous cell carcinoma of the cervix treated by surgery only.
- c. (Excluded if had pelvic radiation)
- d. Stage Ia Grade 1 adenocarcinoma of the endometrium treated with surgery

Patient is on active chemotherapy or radiation treatment

Patients with a history of or clinically active Inflammatory Bowel Disease

Patients with known HNPCC or FAP

Subjects with known HIV or chronic viral hepatitis

Inability to provide informed consent

Women who are pregnant

## 7.0 STUDY PROCEDURES

### 7.1 Subject Recruitment

Patients diagnosed with colorectal cancer and adenomas and scheduled for surgical or endoscopic resection or subjects scheduled for a colonoscopy will be recruited from collaborating consortium centers.

The clinical research associate or study nurse (CRA) at each clinical site will identify subjects with appointments for colonoscopy, surgery, endoscopic polyp or cancer removal, or oncology. The study team will obtain permission to review the schedules from the physicians and the Institutional Review Boards. If the physician agrees that their patient can be contacted regarding participation, the research coordinator will meet with the patient in person or send a letter to the patient describing the study. Advertisements (e.g., newspapers, clinics) may also be used to recruit subjects from the surrounding communities.

The letter to the subject will include an opt-in response card. If we receive permission from the subject to contact them, the CRA will discuss the overall study with the potential subject, and arrange for a baseline visit to get consent, baseline samples, and provide stool kit for FIT and specimen vials.

### 7.2 Clinical Procedures

#### Enrollment and Registration Procedure

Eligible subjects will be enrolled into the study after providing informed consent to analyze stool samples and FIT, and blood samples for biomarkers, medical records review, and for completion of questionnaires. The subject will be assigned a Participant ID by the recruiting site and documented in VSIMS.

#### Timing of Sample Collection

##### *7.2.1.1 Sample collected prior to colonoscopy procedure*

Baseline samples, including stool, blood, and FIT must be collected prior to any colonoscopic preparation procedure.

### 7.2.1.2 Samples collected with known diagnosis of unresected, untreated colon adenocarcinomas or adenomas.

If any subjects are eligible to begin the study after their colonoscopy (e.g., a lesion remains in the colon), at least 7 days must elapse from the diagnostic colonoscopy, but no more than 3 weeks. Eligibility for the respective bins will be determined from the pathology and colonoscopy reports. Cancer patients must have a diagnosis of colon or rectal adenocarcinoma that has been previously untreated. Any stage is allowed. The baseline stool, FIT, and blood samples must be collected before any surgical resection or chemotherapy or radiation therapy is performed.

#### Baseline Visit

Informed consent will be obtained prior to any data or sample collection. Samples will be collected either prior to colonoscopic preparative procedure or 7 days or more after a diagnostic colonoscopy as outlined in Section 7.2.2.2. Detailed instructions will be provided to the subjects on the collection of the stool for the FIT tests and for adenocarcinoma four native stool specimen vials and 1 slurry. Samples will be collected as described below. Subjects will prepare two FIT tests and for adenocarcinoma four native specimen vials and 1 slurry from the stool sample for shipping to the University of Michigan.

#### Data Collection

The subject will be asked to complete EDNR demographic and medical history questionnaires (Appendix A) at baseline. Clarification or additional information may be obtained from the medical records. These data forms have gone through multiple stages of development and testing and are standardized across EDNR studies. Case report forms (CRFs) will also be used to collect information on concomitant medications, colonoscopy outcomes, resection information, cancer treatment, and diagnostics. The Follow up forms and medical record review will be completed at the follow up visit for the subjects in the adenoma and CRC bins if seen in clinic, otherwise done over the phone or e-mail. Long term data collection (medical records review and follow up CDE for all bins) will be done by a phone call or email once at one year post their last contact.

#### Sample Collection: Blood

Blood samples, up to 60 mLs, will be obtained according to standard operating procedures (Appendix C).

#### Sample Collection: Stool for FIT Testing

Adenocarcinoma subjects will be provided with a standard collection kit including detailed instructions on how to complete the FIT sampling. All other subjects will only obtain two FIT tests (Appendix D). The first FIT tube will be shipped inside the same shipping container with the stool sample. The second FIT tube will be mailed (pre-paid) to the University of Michigan at room temperature in the manufacturer's United States Department of Transportation-compliant envelope. The test will be analyzed at the Central Laboratory at the University of Michigan using analytic equipment provided by Eiken Chemical Company. (OC-SENSOR Diana).

#### Sample Collection: Stool for Biomarker Testing (ADENOCARCINOMA SUBJECTS ONLY)

Subjects with a known diagnosis of colorectal adenocarcinoma will be asked to collect their stool in the collection bucket (hat) provided. Subjects will be given detailed instructions and complete kits to collect the stool samples at home. They will prepare a FIT tube (FIT #1) from the stool sample. Subjects will also collect scoops of stool into a container with an EDTA-based buffer ("buffered stool") and additional scoops of stool into tubes provided to be sent on ice packs ("native stool").

The subjects will then package both the stool and the FIT for shipping per provided instructions. The US and Canadian subjects will ship the stool sample to the Central Laboratory at the University of Michigan using pre-paid DOT (Department of Transportation)-compliant packaging. Buffered stool samples will be homogenized and frozen in four 5 ml aliquots at  $-70^{\circ}\text{C}$  or colder for batch shipment to the analytical labs. The native stool will be placed at  $-70^{\circ}\text{C}$  or colder upon receipt.

#### Sample Labeling

All samples will be labeled or have an embedded barcode with a unique bar code and linked to the participant ID through VSIMS.

### 7.3 Biological Sample and Data Collection

#### Blood Collection and Storage

Subjects will provide up to 60 ml of blood in six 10 ml collection vials (2 red, 3 purple tops and 1 ACD-A, for serum and plasma respectively). Purple tops tubes must be filled to manufacturer's level to maintain blood: EDTA ratio. Additional blood draws, prior to prepping for the colonoscopy may be done to get to the necessary blood volume.

The serum samples (red top tubes) will sit at room temperature for a minimum of 30 minutes (maximum of 60 minutes) to allow the clot to form, and if not processed immediately, they can be held at 4° C for a maximum of 4 hours after collection. Plasma samples (purple {aka lavender}top tubes) and ACD-A (yellow top) will be held at 4° C for a maximum of 4 hours after collection. The red top collection tubes will be centrifuged at >1,300 x g at 4° C for 20 minutes (centrifuge brake off for first 10 minutes, then on for last 10 minutes). The serum will be removed, transferred to pre-labeled tubes, and frozen at -70° C or colder. The purple top and ACD-A collection tubes will be centrifuged at >1,300 x g at 4° C for 10 minutes without the centrifuge brake off for first 10 minutes and on for the last 10 minutes. The plasma will be transferred to a 15 ml conical tube for a second centrifugation step (>1,300 x g at 4° C for 10 minutes) prior to aliquoting in pre-labeled tubes, and frozen at -70° C or colder. All frozen samples will be stored at -70° C or colder at the collection site and shipped on dry ice monthly to the Central Laboratory at the University of Michigan and stored at -70° C or colder until assayed. Detailed Standard Operating Procedures including shipping and sample handling instructions are provided in Appendix C.

#### Stool Sample Collection and Handling (ADENOCARCINOMA ONLY)

Subjects with a known adenocarcinoma will be asked to collect a stool sample at baseline prior to any therapy or resection (when applicable). Subjects will be given a standard stool collection basin (hat) with detailed instructions, shipping container, pre-paid shipping labels, four native specimen collection vials, 1 stool slurry and cold packs, FIT vials, and all necessary supplies.

Subjects will be asked to collect a whole stool sample in the container provided, ensuring that no other materials (e.g. paper or urine) are collected in the hat. Subjects will collect scoops of stool into a container with an EDTA-based buffer ("buffered stool"), additional scoops of stool into tubes provided to be sent on ice packs ("native stool"), and a sample in a FIT vial. The subjects will then package both the stool and the FIT for shipping per provided instructions. Subjects will be asked to prepare four native specimen vials, 1 slurry and FIT tests (x2) (see appendix D) using the materials and instructions provided. The specimen vials will be shipped on the cold packs and frozen at -80° C at the Brenner laboratory at the University of Michigan.

#### Fecal immunochemical Test (FIT) (All enrolled subjects)

Subjects will be asked to prepare two FIT tests (see appendix D) using the materials and instructions provided. The OC-Sensor®, Eiken Chemical Company product, will be used according to manufacturer's instructions. The threshold for a positive test is 100 ng/ml. The Central Laboratory will process the samples using equipment provided by Eiken Inc. Technicians will undergo tutorial and quality assessment with Eiken support technicians prior to study launch. A quantitative result will be generated and recorded in the database.

#### Sample Collection: Tissue Samples

*NOTE: Tissue sample collection not required for protocol completion. A limited number of tissues per bin will be collected. Collection will be performed at specified designated institutions for incremental payment per accrual*

##### *7.3.1.1 Collection of Frozen Normal and Adenoma or Cancer Tissue*

For individuals with large adenomas who are undergoing endoscopic resection, the fresh surgical sample will be obtained by the endoscopist. Once the adenomas are located, a digital endoscopic picture will be obtained. Once the adenoma (s) is (are) removed, two biopsies will be done or two cuts will be made. The biopsies will then be frozen in liquid nitrogen after being placed in a pre-designated, labeled container. Normal sigmoid tissue will be collected as described below.

Bar coded vials will be sent to University of Michigan sample storage facility. The adenoma will then be sent to the institution's clinical pathology department according to standard clinical procedures.

For cancer or adenoma patients who are undergoing surgical resection, the Site CRA will notify the Pathology Service or the Institutional Tissue Procurement Service of a surgical sample needed for study purposes. Once the specimens are removed, two to four biopsies will be done or two- four cuts will be made. At least one of the biopsies should be from normal colon. The biopsies will be frozen in liquid nitrogen after being placed in a pre-designated, labeled container. Bar coded vials will be sent to University of Michigan sample storage facility. The specimen will be sent to the institution's clinical pathology department according to standard clinical procedures.

#### *7.3.1.2 Collection of Fixed and Frozen Normal Sigmoid Colon Biopsies on Qualifying Colonoscopy*

For all subjects who agree to the biopsy portion of the study and are undergoing colonoscopy, the endoscopist will take up to 6 biopsies from the **normal sigmoid colon**. Of those, at least 2 (and up to 4) will be snap frozen and at least 1 (and up to 2) will be fixed in 10% formalin and sent to the University of Michigan for paraffin embedding by the Histology Core. The fixed and frozen samples will be stored at the University of Michigan GLNE Core Laboratory.

Sample management procedures including storage, tracking, and shipping instructions are provided in Appendix E.

Tissue samples from pathology specimens may be requested for future biomarker studies from samples collected during routine clinical management of patients with adenomas and CRC. Medical records may be re-reviewed to extract data including, but not limited to size and location of tumor, histopathological features, patient treatment, and response to therapy. Patient permission will be obtained via the informed consent document. The University of Michigan core laboratory may request tissue blocks either to cut slides or to keep for future biomarker studies.

#### Medical Records Documentation

Medical records will be reviewed to collect information regarding the results of the procedures, pathology analysis, surgery, treatment, history, or outcomes and documented in the CRFs/CDEs. The medical records will serve as the source documents and will be maintained at the site enrolling the subject. Since these records necessarily contain subject identifiers, they will not be sent to the Data Coordinating Center at Dartmouth or to the University of Michigan. Medical records may be reviewed at the site during audits or monitoring visits.

#### Sample labels

All samples will be labeled with bar-coded labels or have embedded bar-codes. The labels will be provided by the DMCC and will link to the subject identification number in VSIMS. Labels will be placed on all tubes in the blood drawing kit, FIT, and frozen stool sample vials.

#### Sample tracking

All samples will be tracked by a bar code through a computerized program called VSIMS. Upon receipt of the specimen in the University of Michigan Laboratory Core, the bar code will be read and the date and time of arrival, documented. The Data Management Center will be notified at completion of each individual assay performed on a sample.

#### Long-term Follow up

The CRA will contact the subject via phone or email one year after their last sample collected for additional follow up data. Data will be collected on medical record review and follow up CDE (Appendix A), and include information related to their GI tract history or cancer history and related treatments, procedures, and outcomes. The consent form describes the long-term data collection.

#### **7.4 Circulating methylated genes BCAT1/IKZF1 (Clinical Genomics)**

A Good Laboratory Practice validated bisulfite PCR assay developed by Clinical Genomics will be used for this assay. Clinical Genomics will perform this assay on blinded samples at their laboratory facility in Rutherford, NJ. Clinical Genomics is not responsible for analysis of any other biomarkers other than their BCAT1/IKZF1 product. Sample distribution schedule is outlined in the Clinical Study Agreement.

### **7.5 Hypomethylated LINE1 from circulating cell free DNA (VolitionRx)**

A Good Laboratory Practice validated assay developed by VolitionRx will be used for this assay. VolitionRx will perform this assay on blinded samples at their laboratory facility in Namur, Belgium. Volition is not responsible for analysis of any other biomarkers other than their hypomethylated LINE1 assay. Sample distribution schedule is outlined in the Clinical Study Agreement.

### **7.6 Disclosure of results to subjects**

Subjects will be informed as part of the consent process that neither they nor their health care providers will receive any results from participation in this study.

### **7.7 Evaluable subjects**

A subject is considered evaluable and on-study if all samples are collected per protocol. Subjects without a full set of samples or data may need to be replaced on the study to get 400 evaluable cancers and 200 evaluable subjects the other three bins.

A subject will be asked to provide a replacement sample if:

- a. The stool specimens are received outside the time window required (i.e. greater than 36 hours after collection time and/or not kept cold) (Adenocarcinoma subjects only)
- b. No FIT test
- c. Blood cannot be obtained (must be obtained while target lesion is still present for adenomas and cancers)
- d. Blood is subject to some kind of handling error (no EDTA, too long at room temperature, etc.) and subject is still eligible to provide the blood again

### Protocol deviations

Subjects who do not provide one of the samples or all of the data, but are otherwise eligible to remain on study, will not be reported as deviations.

### **7.8 Completion of Study**

A subject has completed the study when the CRF data, blood samples, stool samples (adenocarcinoma only) and FIT have been obtained, properly processed and delivered to University of Michigan, and the one year follow up phone call has been done. A subject may be asked to provide a replacement sample if there is a problem with one collected, including an additional stool sample. The subject may decline, if they choose.

### **7.9 Subject Compensation**

To compensate for the inconvenience and cost of driving and parking, \$25 will be provided to each subject once blood samples, and stool samples are completed or \$50 for adenocarcinoma subjects who have provided all required samples. Recruiting sites will receive gift cards to distribute to subjects that complete the requirements to receive payment. Sites are required to account for distribution of gift cards to subjects. Sites outside the US will receive reimbursement by invoice, instead of gift cards.

## 8.0 STUDY CALENDAR

Procedures	Baseline <sup>1</sup>	Baseline stool collection <sup>2</sup>	Colonoscopy/ Resection	Long-Term Follow up
<b>Eligibility Checklist</b>	X			
<b>Consent Documentation</b>	X			
<b>General Information</b>	X			
<b>Medical History</b>	X			
<b>Concomitant Medications</b>	X			
<b>Colonoscopy</b>			X	
<b>Surgery</b>			X	
<b>Colon Cancer Treatment</b>				X
<b>Blood Collection</b>	X			
<b>FIT TESTS</b>		X <sup>2</sup>		
<b>Stool Sample</b>		A <sup>2,4</sup>		
<b>Frozen/Fixed Tissue collection</b>			S <sup>3</sup>	
<b>Follow up CDEs</b>				X

<sup>1</sup>Baseline clinic visit—Prior to treatment of any colon lesion, or prior to a colonoscopy (specifically, prep), OR at least 7 days post colonoscopy but no later than 3 weeks post colonoscopy.

<sup>2</sup>Stool collection any time after baseline visit and subject returns home with kits

<sup>3</sup>Frozen tissue is collected at the time of surgical or endoscopic resection of cancer or colonoscopy findings (fixed and frozen).

<sup>4</sup>Stool samples in EDTA buffer and 4 vials collected in adenocarcinoma subjects per stool collection and handling SOPs.

S = Special circumstance. Not a required component of protocol completion unless institution is registered as Special circumstance. Additional remuneration provided for a specific number of patients for frozen tissue collection in each of specified bins.

A = Stool collection required for subjects who have adenocarcinoma of the colon or rectum. The patient incentive is increased from \$25 to \$50 because of their diagnosis and additional effort compared to subjects who do not have cancer.

## 9.0 STATISTICAL CONSIDERATIONS

### 9.1 Study Population

This study is stratified: normal subjects (Stratum 1); subjects at high risk or previously with adenomas who currently are without adenomas (Stratum 2); subjects with adenomas (screen relevant neoplasia (SRN) and non-screen relevant neoplasia (Stratum 3) subjects with colorectal adenocarcinoma (Stratum 4). 200 subjects are to be accrued to each stratum except 400 subjects in Strata 4. Subjects in both Strata 2 and 3 are expected to be more likely to be positive for upstream markers of carcinogenesis than the normal subjects in Stratum 1 (who are both not at high risk and have never had adenomas), while subjects in Strata 3 and 4 are expected to be more likely to be positive for downstream markers indicating the presence of adenomas or adenocarcinomas than those in Strata 1 and 2. Stratum 2 may, therefore, be pooled with Stratum 1 or Stratum 3, depending on the context. From the screening perspective, Stratum 3 will be further divided into SRN or non-SRN. Non-SRN in stratum 3 could be combined with Strata 1&2 to form a non-SRN group and compared to Stratum 4, or compared to SRN in Stratum 3. Oversampling of subjects with adenocarcinoma of the colon or rectum is necessary to provide sufficient dedicated samples and data for validation trials aimed at regulatory approval. Samples and data from subjects recruited in this trial may be used to update and enhance reference sets used by the EDRN to further train, test and/or validate new biomarkers for future inclusion in validation trials aimed at regulatory approval. Strata 1-3 are necessary to ensure these comparison groups collected under the same protocol to Stratum 4 are available.

Training and validation: The prospective GLNE010 study has recruited many subjects in each stratum except adenocarcinoma. The samples from this protocol will need to be combined with GLNE010 samples to allow both panel building (training) and panel validation for each of above comparisons.

## 9.2 Data Analysis Plan

### Assessment of the utility of individual biomarkers for discriminating between patients with adenocarcinomas, patients with adenomas, patients without adenomas and normal subjects.

For markers measured on a continuous scale, the within-class distributions of the marker values will be assessed by graphical means (e.g., q-q plots). Maximum likelihood estimates of distribution parameters will be calculated. For markers measured on a dichotomous scale, the proportions of positive tests in each class will be determined. For each marker, non-parametric (via SAS PROC LOGISTIC) and fully parametric ROC curves will be constructed for: Stratum 4 versus all others except SRN-adenomas (define as adenoma  $\geq 1$  cm or adenoma with high grade dysplasia or sessile serrated polyp  $\geq 1$  cm) (**primary comparison**); Stratum 4 versus Strata 1 and 2 (**secondary comparison**); and other **exploratory comparisons**: Stratum 4 versus Stratum 1; Stratum 4 and SRN-adenomas in Strata 3 versus Strata 1 and 2 and non-SRN adenoma; Strata 2, 3 and 4 versus Stratum 1. While the non-parametric ROCs are generally preferred, decision rules for population screens may require very high specificity, which will require accurate estimation in the distribution tails; parametric ROC curves may be better for this application. The area under each ROC curve (AUC) for each comparison will be determined.

### Construction or testing of a panel of markers from those considered in Objective 1

**Construction of a panel of markers** from those considered in Objectives 1 and 2 to discriminate, under specific of assumptions concerning prevalence and cost of misclassification, for the primary, secondary, and exploratory comparisons described above. Candidate markers will be chosen according to both statistical (e.g., high patient or tissue sensitivity) and practical (less expensive assays, all markers assessed on blood) criteria. Forward stepwise logistic regression will be used to construct a panel to discriminate between the two classes of patients. The ROC curve will be constructed and AUC will be determined. Other panel building approach will also be used when appropriate, e.g. an “OR” rule will be used, that a test is positive if either one test is positive, if each of the biomarker is very specific but only for a subset of cancers.

**Validation of a panel of biomarkers:** If the cutoff has not been locked-down but the combination rule has been pre-determined, the optimal cutoff will depend on the intended clinical use. For example, for a blood based biomarker as a first step screening for those who do not want to do stool FIT tests, we might the cutoff that is corresponding to sensitivity of FIT test for colorectal cancers, then evaluate if the specificity of this cutoff is adequate. False positive is of less concern because it will lead to colonoscopy, a recommended screening in US. For a stool- based test (only for adenocarcinomas in this study when combined with the previous GLNE007 or GLNE010 set where stool samples were collected for all participants), if a test with much lower cost than that of the Exact Sciences multi-marker panel that includes a fecal immunochemical test and methylated DNA gene markers is brought for testing, the threshold sensitivity required to enter a large validation trial might be lower than that of the Exact Sciences panel (in the range of 85% to 92% for detection of adenocarcinoma) but better than FIT alone.

We would then compare the specificity to that of Exact Sciences multi-marker panel. If the cutoff has been pre-determined, then the evaluation will be a simple joint 1-sided 95% confidence region for sensitivity and specificity.

### Comparison of the characteristics of individual markers and panels as discriminators to those of the established current standard, Fecal Immunohistochemistry test (FIT).

For biomarker validation we assume at least the panel combination rule has been locked-down. The biomarkers or panels could be from outside of the consortium or from the one built in 9.2.2 using previous GLNE007 specimens collected between 2006-2010 and GLNE 010 samples collected between 2011 and 2019. As in Objective 3, the following analysis will be performed for each of the primary, secondary, and exploratory comparisons. For blood-based biomarkers, we will test whether it has a similar sensitivity as that of FIT and has a reasonable specificity (e.g.  $> 70\%$ ) if the cutoff is pre-determined, or whether at a cutoff corresponding to the same sensitivity of FIT the specificity is better than 50% (target specificity  $> 70\%$ ) if a cutoff is not pre-determined. This performance criterion is also used for training set panel building, i.e., a panel will need to have this performance before it is locked-down for validation. For stool-based biomarkers, we will test whether the sensitivity is better than that of FIT, with compatible specificity. With non-screening colonoscopies, we will collect information whether the colonoscopy was triggered by a positive FIT test or triggered by symptom. The performance of biomarker will be evaluated with each of these two groups separately and compared, to gauge the potential bias caused by FIT positive results triggering colonoscopy that will lead to over- estimate of sensitivity for FIT.



Markers available in the future will be developed in a similar fashion to Objectives 1-4. Every effort will be made to ensure that samples from and data concerning subjects in all four strata are collected, processed and stored according to the same procedures (Section 8.2 and Appendices), so that data and sample banking do not introduce bias into future studies.

### 9.3 Justification of Design and Sample Size

The primary goals of this protocol are to enhance the already available EDRN reference set and provide biosamples and data as required to fill in validation sets for the purposes of regulatory approval. The reference sets and other GLNE 007 samples will be used to assess the ability of different markers to discriminate between patients with adenocarcinoma, patients with adenomas and normal subjects (Objective 1) and to strategically use this information to construct panels of markers to discriminate cases (adenocarcinomas and/or screen relevant adenomas) from controls (Objective 2). An additional reference set might be set aside for the purposes of regulatory validation. Such samples may not be used for training or testing of a given marker that might be validated with samples from the GLNE 007 reference set or other validation sets previously collected by EDRN.

We justify the sample size for the primary comparison for training and validation separately:

**Panel training and testing:** Cancer (n=200) versus 560 normal (normal colonoscopies (200 average risk subjects, 200 high risk subjects) or non-screen relevant adenomas (estimated to be 80% of 200 adenomas, i.e. n=160)) for a blood-based test as the first line test for people who do not do any colorectal cancer screening. We assume the cutoff has not been locked-down (statistical power would be much larger if the cutoff is locked-down) so we will use cutoff corresponding to sensitivity of FIT (75%). We argue that with this sensitivity a test with at least 70% specificity would have great clinical utility. With the study sample size, we will have >90% power to reject a null hypothesis specificity of 58% if the true specificity is at least 70%.

For stratified analysis if there is evidence that specificities for normal colonoscopy high risk subjects and subjects with non-screen relevant adenoma are significantly lower than that for normal colonoscopy low risk group, suggesting they may have higher risk for screen relevant neoplasms in the future and should be analyzed separately. With 200 cancers and 200 normal colonoscopies in low risk group, we have at least 82% power to reject a null hypothesis specificity of 58% if the true specificity is at least 70%. With 200 cancers and 360 subjects in high risk or non-SRN adenoma groups, we have at least 89% power to reject a null hypothesis specificity of 58% if the true specificity is at least 70%.

For validation of stool-based test, we use the scenario that using a cutoff corresponding to 92% sensitivity (that of the Exact Sciences multi-marker panel) and test the adequacy of specificity, with 200 cancers and 747 normal or non-screen relevant neoplasms from GLNE010 training set, we have at least 86% power to reject a null hypothesis of specificity 76% if the true specificity is at least 85%. Such a test if it is substantially cheaper than that of the Exact Sciences multi-marker panel will have clinical value to increase the sensitivity of FIT.

**Panel validation:** Cancer (n=200) versus 560 normal (normal colonoscopies (200 average risk subjects, 200 high risk subjects) or non-screen relevant adenomas (estimated to be 80% of 200 adenomas, i.e. n=160)) for a blood-based test as the first line test for people who do not do any colorectal cancer screening. We assume the cutoff has not been locked-down (statistical power would be much larger if the cutoff is locked-down) so we will use cutoff corresponding to sensitivity of FIT (75%). We argue that with this sensitivity a test with at least 70% specificity would have great clinical utility. With the study sample size, we will have >90% power to reject a null hypothesis specificity of 58% if the true specificity is at least 70%. If the validation is done using all 3070 normal and non-screen relevant neoplasms from GLNE010 (the number as of April 2019), then we have >90% power for to reject a null hypothesis specificity of 60%.

For validation of stool-based test, we use the scenario that using a cutoff corresponding to 92% sensitivity (that of the Exact Sciences multi-marker panel) and test the adequacy of specificity, with 200 cancers from this protocol and 3070 normal and non-screen relevant neoplasms from GLNE010 as of April 2019, we have at least 90% power to reject a null hypothesis of specificity 76% if the true specificity is at least 85%. Such a test if it is substantially cheaper than that of the Exact Sciences multi-marker panel will have clinical value to increase the sensitivity of FIT.

## 10.0 DATA SAFETY AND MONITORING

### 10.1 Data Safety and Monitoring

#### Authority

The DSMC reviews, makes recommendations, and acts on the following:

- a. All protocols being run through the GLNE EDRN will be monitored by the DSMC.
- b. Progress towards completion of the trial—recruitment and retention of study participants.
- c. Insufficient accrual to warrant continuation of the trial.
- d. Evaluation of interim data analyses.
- e. Evaluation of interim new information.
- f. Evaluation of toxicity events including reporting of adverse events.
- g. Timeliness of data.
- h. Quality of data.
- i. Ethical conduct of research.

The DSMC is empowered with the authority to recommend a trial be suspended or terminated based upon concerns in any of the above areas of review. The DSMC reviews all serious adverse events and ensures that these events have been correctly reported to all institutional review boards, and that adverse events have been correctly classified as serious or not serious. The Board assesses the impact of these events upon the conduct of the clinical trial. The Board is empowered with the authority to suspend or terminate any trials for which there are concerns of toxicity that endanger human participants. Monitoring also considers factors external to the study, such as scientific or therapeutic developments that may have an impact on the safety of the participants or the ethics of the study. Recommendations that emanate from monitoring activities are reviewed by the principal investigator and addressed.

#### Composition

The principal investigator is present in an open session portion of the meeting and absent in a closed session. All DSMC official subjects in the review of confidential data and discussions regarding continuance or stoppage of a study have no conflict of interest and no financial stake in the research outcome. The current UM Prevention research base Data and Safety Monitoring Committee is Chaired by the Research Base Biostatistician and comprised of Faculty members from Gastroenterology, Family Medicine, Hematology/Oncology. At least 3 faculty members, not including the study PI, must be present along with the biostatistician as chair to have quorum. If the DSMC cannot meet face-to-face, a conference call is acceptable.

#### Meeting Frequency

The UM Prevention Research Base DSMC meets monthly by means of regularly scheduled meetings. Prior to each meeting, the UM Prevention Research Base clinical research associate distributes a standard summary report detailing accrual, biomarker modulations data, new publications or presentations relevant to the ongoing project, quality control audit information, any ethical concerns, patient-subject complaints and adverse events or serious adverse events of all prevention protocols.

#### Recommendations and Reporting

Recommendations for action are sent to the Principal Investigator. The Principal Investigator is responsible for implementing DSMC recommendations. In addition to the Principal Investigator, minutes from the monthly meetings are forwarded to the following as needed:

- a. DSMB members and the principal investigators at other sites
- b. The University of Michigan Comprehensive Cancer Center Prevention and Control Protocol Review Committee Chair, per PRC policies;
- c. IRBMED (University of Michigan Medical School IRB);
- d. NCI/DCP Program Staff;
- e. Any other trial sponsor.

Serious adverse events and adverse events are reported to the institutional review boards of all clinical sites, University of Michigan IRBMED per standard SAE reporting guidelines, and the sponsor as required by Federal regulations. A yearly summary report of trial activities is made to all trial investigators, supervisory committees and the sponsor. The UM prevention data management office and the DMCC have the responsibility of informing other trial investigators concerning the data and safety monitoring policy, procedures, and decisions.

## 11.0 ADVERSE EVENT REPORTING

### Definition

An adverse event (AE) is any condition, which appears or worsens after the participant is enrolled in an investigational study.

### AE Information

No adverse events are expected, as there is no intervention for this trial. Any adverse events related to the subject's participation in this study will be forwarded to the data coordinating center and reported to the UM IRBMed per Standard Adverse Event Guidelines.

### Serious Adverse Events

One-third of the participants will have colon cancer by study design, and deaths due to disease progression or serious adverse events due to cancer treatment are expected. The only procedures that are part of this study are blood, and stool collection, so it is unlikely that any deaths or hospitalizations will be related to the sample collection in this study. Only Serious Adverse Events that are deemed to be directly related to a study procedure (sample collection) by the DSMB will be reported to any regulatory body.

A serious adverse event is defined (by ICH Guideline E2A and Fed. Reg. 62, Oct. 7, 1997) as an event, occurring at any dose, which meets any of the following criteria:

- Ⓢ Results in death
- Ⓢ Is immediately life threatening
- Ⓢ Requires inpatient hospitalization or prolongation of existing hospitalization
- Ⓢ Results in persistent or significant disability/incapacity
- Ⓢ Is a congenital anomaly/birth defect

In addition, events that may not meet these criteria, but which the investigator finds very unusual and/or potentially serious, will be reported in the same manner.

## 12.0 DATA MANAGEMENT

### 12.1 Registration

Institutional collaborators will enter IRB information into the secure VSIMS database, including IRB approval date, expiration date, and document versions. Subject registrations will not be allowed without IRB approval. The DMCC will provide recruiting sites with Participant ID numbers to be assigned in VSIMS. No exceptions to eligibility requirements will be permitted without prior permission of the protocol PI.

### 12.2 Timeliness

Timeliness is monitored by the DMCC and UM through various reporting mechanisms within VSIMS.

### 12.3 Completeness and Accuracy

The DMCC will assure the completeness of the data by writing data entry programs that will not allow for empty fields whenever possible. The accuracy of the data will be checked by identifying appropriate parameters allowed to be entered in a given data field. Periodic reviews of the paper CDEs and the database data will be conducted by the lead CRA and DMCC site monitor.

### 12.4 Accuracy--Revisions and Corrections

All corrections to paper study documents will be initialed and dated. If computer-readable data is corrected by replacement of a data set, the replaced version of the data set will be retained in an archive. The collection of these auxiliary data sets represents an audit trail of corrections to the database.

## **12.5 On Site Data Audits**

All consortium sites are subject to periodic on-site audits. The objective of the on-site audit is to conduct a general review of a random sample of registered subjects from the selected protocol to assess overall protocol adherence with respect to subject eligibility, appropriate procedure for informed consent, registration process, general protocol adherence, sample shipment process, follow-up and off-study process.

An On-Site Audit checklist will be developed which will contain all of the essential elements of an On-Site audit. Each of the essential elements are reviewed and discussed with the clinical site. The Checklist is signed by the auditors and retained at the DMCC.

In preparation for a site audit, the study statistician will select the subjects for review using a randomized selection procedure. Other cases may also be selected at the discretion of the audit team. A minimum of 10% of the subjects accrued since the last audit will be reviewed. The on-site audit team will audit two to three unannounced cases. The consortium site investigator and research coordinator will be notified of the impending audit not more than 3 months in advance. Two to four weeks prior to the site visit, the list of selected subjects will be sent to the consortium clinical site. All data and material pertinent to the subject will be reviewed including eligibility criteria, informed consent, and sample shipment logs.

Subject data will be extracted at the DMCC prior to the visit. At the audit, the data from the DMCC will be compared to the original data (source documents and/or CDEs). On-site audit staff will review the documentation of IRB approvals, for each audited protocol, any amendments or adverse events, and consent forms.

Based on the findings of the audit, a follow-up schedule will be defined. A report of the audit is written and faxed to the DMCC and the NCI within 5 working days of the audit. A copy of the report is emailed or faxed to the consortium site investigator. The site PI has 30 days from receipt of the report to respond in writing to the DMCC directly. After the 30-day response period, the report is finalized and sent to NCI and the consortium site investigator.

The DMCC will maintain a file containing the latest version of the On-Site Audit guidelines, a listing of all consortium institutions reviewed to date, a copy of the On-Site Audit results and all correspondence for each audit conducted. These results will be reviewed by the Center's Executive Committee at a monthly telephone conference and will be made available to the NCI.

## **12.6 Sample Tracking**

Sites receiving shipments of samples are notified via e-mail, so if samples are delayed or lost, tracking may be initiated by the sending site. Sample shipment forms are included with shipments. These data forms describe the date of sample receipt, and availability of sample, along with tracking information. The receiving site will evaluate the sample condition on arrival, scan the bar-coded samples in the VSIMS database, verify samples shipped match samples sent, and store at appropriate conditions until shipment to analytical labs.

## **12.7 Confidentiality**

Subjects will be identified in the database by their unique EDRN subject identification numbers only. Information that could identify subjects, such as name, address, or social security number will be kept only by the enrolling site and will not be supplied to the DMCC at Fred Hutch. The Coordinating Center at UM will have a separate payment form with name, address, and social security number for payment purposes only as previously described. During an on-site audit or NCI site visit, staff may review medical records and other information that contains PHI, but this information will not be removed from the enrolling site. The Coordinating center at UM will not keep copies of signed informed consent documents. No information, including copies of the informed consent unless required by the institution, obtained during the study will be placed in a subject's medical record.

## **12.8 Security**

All subject files will be stored under lock and key at all times. All computer systems will be password-protected against intrusion; all network-based communications between sites of confidential information are encrypted.

An on-going computer-virus-protection program is available and used, maintained, and audited on all computers and pathways into the system, including good practice policies, screening of data files, executable software, diskettes, text macros, downloads, and other concerns as they arise. The DMCC will assist in maintaining appropriate levels of network security.

## **13.0 ETHICAL AND REGULATORY CONSIDERATIONS**

### **13.1 Institutional Review**

This study must be approved by an appropriate institutional review committee as defined by Federal Regulatory Guidelines (Ref. Federal Register Vol. 46, No. 17, January 27, 1981, part 56). The protocol and informed consent form for this study must be approved in writing by the appropriate Institutional Review Board (IRB). The IRB must be from an institution that has a valid Federal Wide Assurance, Multiple Project Assurance, Single Project Assurance or Cooperative Oncology Group Assurance on file with the Office for Human Research Protections, Department of Health and Human Services. The institution must comply with regulations of the Food and Drug Administration and the Department of Health and Human Services. Changes to the protocol, consent, as well as a changes to the investigator list at each site, must also be approved by the IRB and documentation of this approval provided to the Coordinating center. Records of the Institutional Review Board review and approval of all documents pertaining to this study must be kept on file by the investigator and are subject to OHRP or NCI inspection at any time during the study. Periodic status reports must be submitted to the Institutional Review Board at least yearly, as well as notification of completion of the study and a final report within 3 months of study completion or termination. The investigator must maintain an accurate and complete record of all submissions made to the Institutional Review Board, including a list of all reports and documents submitted.

#### **Inclusion of New Biomarkers Discovered by EDRN Investigators over the Next Two Years**

The design of this project including the collection of serum, DNA and tissue samples permit the inclusion of new EDRN discovered biomarkers into this panel. Should EDRN investigators provide sufficient preliminary data to justify inclusion in this panel; new biomarkers will be included in the validation program using the procedures described above.

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**EXHIBIT B**

**Specimen Volumes for Volition**

GLNE010 Old Samples (2012-2015)		
	Plasma <sup>1</sup>	Serum <sup>2</sup>
Cancer	10	10
Adenoma	1025	1025
Advanced Adenoma	749	749
High Grade Dysplasia	19	19
SRN (Hyperplastic)	506	506
Healthy Controls	2315	2315
<b>TOTAL</b>	<b>4624</b>	<b>4624</b>
Plasma <sup>1</sup> : 1.2ml (4x300ul)		
Serum <sup>2</sup> : 3.0ml (10x300ul)		

GLNE010 New Samples (2017-2019)		
	Plasma <sup>1</sup>	Serum <sup>2</sup>
Cancer	NA	3
Adenoma	NA	302
Advanced Adenoma	NA	429
High Grade Dysplasia	NA	4
SRN (Hyperplastic)	NA	176
Healthy Controls	NA	686
<b>TOTAL</b>	<b>NA</b>	<b>1600</b>
Plasma <sup>1</sup> : None Available		
Serum <sup>2</sup> : 4.0ml (2x2ml)		

GLNE007 Old Samples (2006-2010)		
	Plasma <sup>1</sup>	Serum <sup>2</sup>
Cancer	84	132
Adenoma	210	241
High Risk Normal	34	40
Healthy Controls	80	109
<b>TOTAL</b>	<b>408</b>	<b>522</b>
Plasma <sup>1</sup> : 1.0ml (2x500ul)		
Serum <sup>2</sup> : 2.0ml (4x500ul)		

GLNE007 New Samples (projected)		
	Plasma <sup>1</sup>	Serum <sup>2</sup>
Cancer	400	400
Adenoma	200	200
High Risk Normal	200	200
Healthy Controls	200	200
<b>TOTAL</b>	<b>1000</b>	<b>1000</b>
Plasma <sup>1</sup> : 1.8ml (6x300ul)		
Serum <sup>2</sup> : 5.0ml (2x2.5ul)		

## EXHIBIT C

### Statement of Work (for University of Michigan Grant Activity) Volition

#### Aims

Aim 1: Complete GLNE 007, a trial designed to train and test and circulating biomarkers for early detection of colorectal adenocarcinoma.

Aim 2: To perform phase 1 validation trials (training and test set designs) of promising biomarkers discovered by EDRN Biomarker Validation Laboratories, external academic collaborating institutions, and collaborating EDRN industrial partners for the early detection of colorectal cancer.

Aim 3: To enhance and curate an archive of appropriately preserved stool, serum, plasma, urine, tissue and DNA biospecimens to be used by EDRN investigators for future validation and biomarker discovery research.

#### Work Plan

As part of the overarching EDRN project, adults age 50 or older undergoing a screening or surveillance colonoscopy will be enrolled as Study Subjects for this Project. Samples obtained from these Study Subjects will be sent to the GLNE Central Laboratory for preparation for storage/shipment to Volition (Laboratory). There, the samples will be tested for blood-based, cell-free circulating biomarkers on their proprietary Nu.QTM platform.

Laboratory will provide a copy of all test results for the Clinical Study to Principal Investigator's designated Data Management Coordinating Center (DMCC) following the completion of their services. The DMCC will collect and store all such test results, and shall share test results with Institutions for collaborative analysis.

GLNE010  
VALIDATION AND COMPARISON OF BIOMARKERS FOR THE EARLY  
DETECTION OF COLORECTAL ADENOCARCINOMA

Great Lakes New England Clinical Validation Center  
NCI Early Detection Research Network  
2 UOI CA086400-16

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**Alliance for Clinical Trials in Oncology (Alliance)**

**Alliance- GLNE 010**

Validation and Comparison of biomarkers for the Early Detection of Colorectal Adenocarcinoma

*For any communications regarding this protocol, please call the protocol resource person on the following page.*

Study Chair: Jan Buckner, M.D.  
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**Alliance Protocol Resources**

<b>Questions:</b>	<b>Contact Name:</b>
Protocol document, consent form, Regulatory issues	Jacqueline M. Latky <i>Research Base</i> Research Protocol Specialist Phone: (507) 538-4633 Fax: (507) 284-5280 E-mail: <a href="mailto:lafky.jacqueline@mayo.edu">lafky.jacqueline@mayo.edu</a>

\* No waivers of eligibility per NCI

**CLINICAL TRIALS SUPPORT UNIT (CTSUS) ADDRESS AND CONTACT INFORMATION**

<b>To submit site registration documents:</b>	<b>For patient enrollments:</b>	<b>To Submit Study Data:</b>
CTSUS Regulatory Office 1818 Market Street, Suite 1100 Philadelphia, PA 19103 Phone - 1-866-651-CTSUS Fax - 215-569-0206	Refer to Appendix H for specific instructions.	All Groups must submit data via the EDNR's Validation Study Information Management System (VSIMS) . To obtain access for data entry, sites will be trained by webinar and given their own user name and password and access to the system.  For additional information about VSIMS, refer to the Manual of Operations, Appendix 13. For assistance with VSIMS or other data entry questions, call the VSIMS Helpline: 206-667-3438.  Do not submit study data or forms to CTSUS Data Operations. Do not copy the CTSUS on data submissions.



- ① **Patient enrollments** at all participating sites will use the GLNE's Validation Study Information Management System (VSIMS). Refer to Appendix H for specific enrollment details.
  - ① **Data management** will be performed as follows:
    - All participating institutions will enter their data directly into the Early Detection Research Network (EDRN) supported VSIMS system of the Data Management and Coordinating Center (DMCC) as discussed in Section 4 of the protocol.
    - ▶ Do not send study data or case report forms to the CTSU Data Operations. DO NOT copy the CTSU on data submissions.
  - ① **Data query and delinquency reports** will be sent directly to the enrolling site by the EDRN DMCC Operations Office. Please send query responses and delinquent data to the DMCC and do not copy the CTSU Data Operations.
  - ① CTSU sites should follow procedures outlined in Appendix H for Site Registration, Patient Enrollment, Adverse Event Reporting, and Data Submission.
  - ① **For patient eligibility or treatment-related questions:** Missy Tuck (734-763-1141 or [mtuck@umich.edu](mailto:mtuck@umich.edu))
  - ① **For questions unrelated to patient eligibility, treatment, or data submission** contact the CTSU Help Desk by phone or e-mail:
    - CTSU General Information Line- 1-888-823-5923, or [ctscontact@westat.com](mailto:ctscontact@westat.com). All calls and correspondence will be triaged to the appropriate CTSU representative.
  - ① **For detailed information on the regulatory and monitoring procedures for CTSU sites** please review the CTSU Regulatory and Monitoring Procedures policy located on the CTSU members' website <https://www.ctsu.org>
  - ① **CTSU Web site** is located at [http s://www.ctsu.org](http://www.ctsu.org)
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## Abbreviations and Definitions

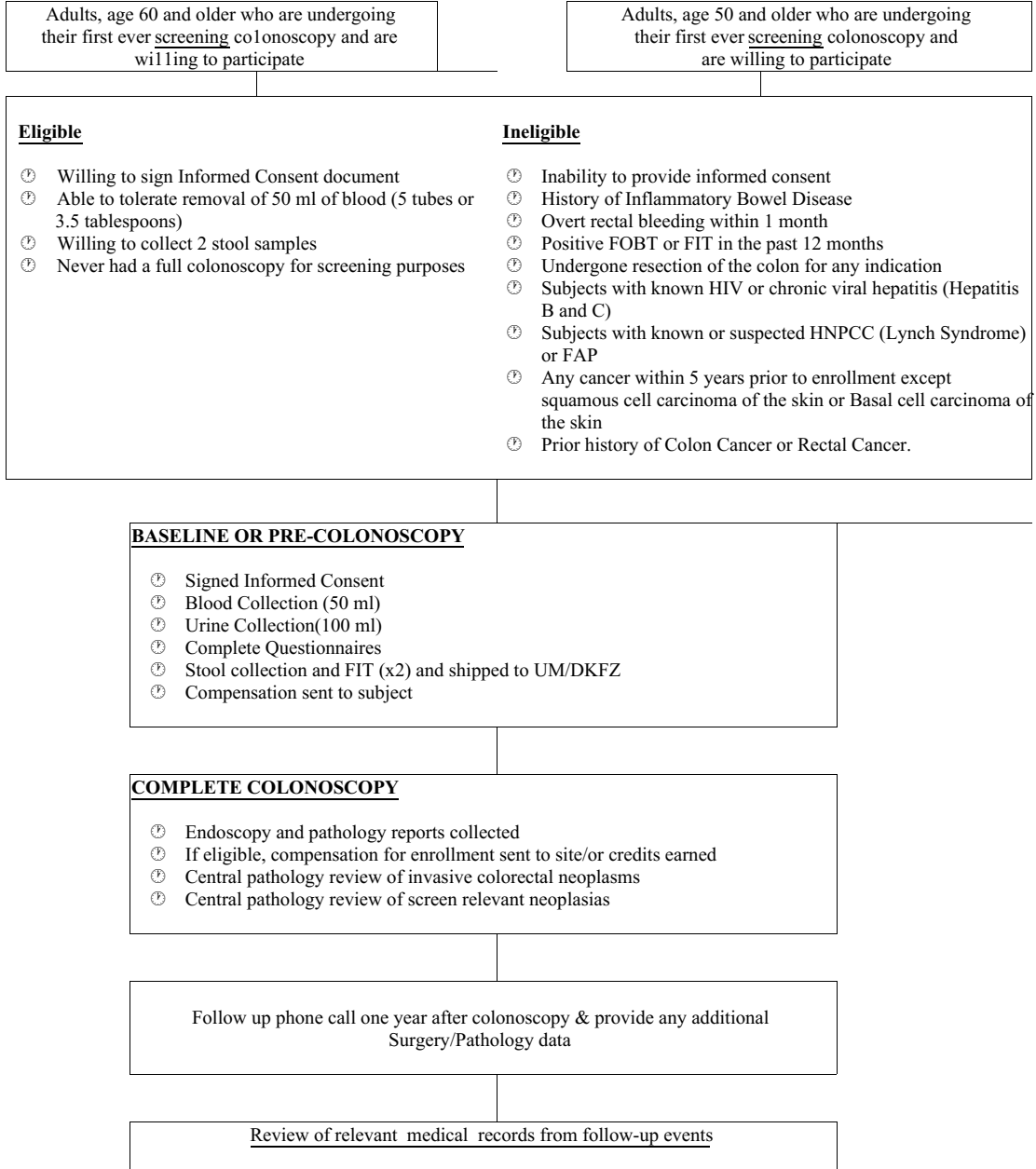
Early Detection Research Network (EDRN)  
Biomarker Reference Laboratory (BRL)  
Standard operating procedures (SOPs)  
University of Michigan (UM)  
German Cancer Research Center (Deutsches Krebsforschungszentrum, DKFZ), Heidelberg, Germany  
Deoxyribonucleic acid (DNA)  
FIT test (sampling bottle provided by Polymedco for testing two different stool samples)  
Data Management and Coordinating Center (DMCC)  
Participant Identification Number (PIO)  
Clinical Research Associate or study nurse (CRA)  
Inflammatory Bowel Disease (IBD)  
Hereditary non-polyposis colon cancer (HNPCC)  
Familial Adenomatous Polyposis (FAP)  
Validation Study Information Management System (VSIMS)  
Fecal Occult Blood Test (Guaiac-based) FOBT  
Immunoassay fecal occult blood test (FIT)  
Department of Transportation (DOT)  
Personal Health Information (PHI)  
Data Safety Monitoring Committee (DSMC)  
National Cancer Institute (NCI)  
National Institute of Health Diet History Questionnaire II (DHQII)  
Colorectal adenocarcinoma or adenomas with high grade dysplasia or adenomas greater than or equal to 1 cm (Screen Relevant Neoplasia- {SRN})  
Colorectal adenocarcinoma **OR** adenomas with high grade dysplasia (CRC/ HGD)  
Cancer Trials Support Unit (CTSU)  
Clinical Ligand Assay Satellite Services (CLASS)

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## 1.0 SUMMARY OF STUDY

The goal of this trial is to estimate the sensitivity and specificity of stool vimentin methylation, serum galectin-3 ligand, and fecal immunochemical testing for 1) colorectal adenocarcinoma, or 2) screen relevant neoplasms (high-grade dysplasia or adenoma with 2:25% villous histologic features or adenoma measuring 2:1 cm in the greatest dimension or sessile serrated polyps measuring 1 cm or more in diameter) as single markers and in combination. Four thousand asymptomatic subjects aged 60 and older undergoing a first ever routine colonoscopic screening for colorectal cancer from U.S. community and major medical center outpatient settings across multiple centers and consortia will be recruited. An additional five thousand subjects age 50 and older undergoing routine colonoscopic screening for colorectal cancer will be recruited in Germany and Canada (non-US sites). Up to 9,000 subjects will be recruited in this protocol, adding to the 4,677 confirmed and evaluable subjects already recruited. Subjects will meet with research staff prior to initiation of any colonoscopic preparative procedure. After completing informed consent, they will complete Early Detection Research Network (EDR.i<sup>®</sup>-J) data element forms. Blood and urine will be obtained following EDR.i<sup>®</sup>-J standard operating procedures (SOPs). Subjects will be provided with kits to collect stool samples for fecal immunochemical test (FIT) and stool tests. The collected samples will be shipped to the Central Laboratory at the University of Michigan or German Cancer Research Center (Deutsches Krebsforschungszentrum, DKFZ), Heidelberg, Germany where the stool will be homogenized, aliquoted, and stored at the University of Michigan CLASS laboratories. The FIT tests will be sent to the Central Laboratory at the University of Michigan or to DKFZ for quantitative analysis following standard operating procedures provided by Eiken Chemical Company. Data from the screening colonoscopy will be obtained. One year after colonoscopy, subjects will be contacted to determine if they have had a neoplastic colorectal diagnosis or other neoplastic events. Data management and protocol coordination will be performed by the Data Management and Coordinating Center (DMCC) of the EDNRN along with the GLNE Prevention Research Base at the University of Michigan and will include a Web-based front end and relational database backend, with biosample tracking (VSIMS). Biosamples will be managed in a high quality repository facility at the University of Michigan until shipment to the EDR.i<sup>®</sup>-J repository at NCI at Frederick Central Repository and to analytic partners.

We will estimate sensitivities and specificities and the corresponding confidence intervals of the stool DNA tests and serum/plasma tests for detection of invasive colorectal neoplasms and for screen relevant neoplasias (Aim 1). We will then test the primary hypothesis to confirm the clinical accuracy of a particular biomarker test or panel (Aim 2). The specific primary hypothesis will be defined prior to data analysis based on state of the art information available at that time about candidate biomarkers and tests. Several specific examples of potential primary hypotheses are given to justify study sample size. Finally, several alternative tests and multi-marker panels will be evaluated. (Aim 3). In secondary analysis, we will (a) provide measures of diagnostic accuracy standardized to the age and gender distribution of US population and (b) assess the effect of subject heterogeneity on the marker performance. A primary objective is to establish an archive of appropriately preserved stool, serum, plasma and DNA human biospecimens to be used by ED -approved investigators for future validation and biomarker discovery research (Aim 4).



### 3.0 OBJECTIVES

We propose a prospective cross-sectional PROBE-compliant validation trial of stool-based and serum-based tests for the detection of colorectal neoplasia (3). The trial is powered to evaluate tests for detecting early stage colorectal adenocarcinoma. This is the most stringent, conservative approach to the early diagnosis of colonic neoplasia and addresses the most important endpoint of identifying individuals with curable, early stage cancer.

**Aim 1:** To estimate the sensitivity and specificity for 1) colorectal adenocarcinoma or 2) screen relevant neoplasms (high-grade dysplasia or adenoma with  $\geq 25\%$  villous histologic features or adenoma measuring  $\geq 1$  cm in the greatest dimension or sessile serrated polyps measuring 1 cm or more in diameter) of the following individual colorectal neoplasia early detection biomarkers using colonoscopy as the gold standard:

- ① stool vimentin methylation
- ② serum galectin-3 ligand
- ③ fecal immunochemical tests (FIT)
- ④ Circulating methylated genes BCAT1 /IKZF1 (Clinical Genomics)
- ⑤ Hypomethylated LINE1 from circulating cell free DNA (VolitionRx)
- ⑥ Other currently unspecified biomarkers

**Aim 2 (primary objective):** To assess the accuracy and potential clinical value of a test for detection of colorectal adenocarcinoma. The specific test and relevant hypothesis are not defined now but will be chosen when all samples have been collected. This will allow the primary hypothesis to incorporate all information about markers and clinical practice that is available at the time of analysis and will ensure that the most compelling and timely hypothesis is tested. Statistical power calculations demonstrate that the study is well powered for these hypotheses (Section 8.0).

**(Secondary objective):** While the study will be powered for the primary objective, we shall also carry out a similar assessment of potential utility of a clinical test for SRN.

#### Aim 3

To validate, or to construct, a combined early detection biomarker panel using the above individual biomarkers (stool vimentin methylation, serum galectin-3 ligand, FIT, circulating methylated genes BCAT1 /IKZF1, hypomethylated LINE1 from circulating cell free DNA), and other unspecified future biomarkers, and describe its performance for 1) colorectal adenocarcinoma and for 2) screen relevant neoplasms.

#### Aim 4

To establish an archive of appropriately preserved stool, serum, plasma and DNA human biospecimens to be used by EDR: -approved investigators for future validation and biomarker discovery research.

### 4.0 BACKGROUND AND SIGNIFICANCE

#### 4.1 Current State of the Art: Recommended Early Detection

Randomized controlled trials have shown that annual or biennial fecal occult blood tests (FOBT) reduce colorectal cancer (CRC) mortality by 15% to 33% (4-6). The reduction is durable over 3 decades (7). Population based cohort studies of colonoscopic screening demonstrate reduced CRC mortality, primarily in distal but not in the proximal colon (8-10). This discrepancy has been attributed to endoscopic quality issues, the technical difficulties in detecting lesions in the right colon, and the more frequent occurrence of flat and depressed dysplastic lesions in the right colon (11-14). In tandem colonoscopy studies, a subset of large polyps may be missed by a single examiner. Shorter withdrawal is time linked to a lower adenoma detection rate (15, 16).

Flat and depressed lesions are more challenging to detect and have been described with a relatively high prevalence in a US colonoscopy cohort (17). While colonoscopic removal of adenomatous polyps reduces CRC mortality (18), prospective, randomized controlled trials of screening colonoscopy have been initiated by the VA and in Europe (18-20). Over-diagnosis (i.e. early detection of indolent invasive neoplasms that do not cause mortality) or lead-time bias in early detection of colorectal neoplasms do not degrade the efficacy of screening and early detection for colorectal cancers (21).

Current screening guidelines for average risk individuals vary world-wide. In the United States the American Gastroenterology Association recommends testing for early detection of adenomas and cancer (structural examination) or of cancer (non-invasive stool tests) beginning at age 50 (22). The United States Preventive Services Task Force (USPSTF) recommends fecal occult blood testing (FOBT) every two years with optional endoscopic screening with either flexible sigmoidoscopy or colonoscopy (23). The majority of developed countries recommend fecal occult blood testing every two years but do not support endoscopic screening (24); albeit with some exceptions (e.g. Germany (24, 25)). In 2012, 65.1% of the United States adults adhered to USPSTF colorectal screening guidelines with colonoscopy the commonly used screening method (61.7%) followed by FOBT (10.4%) (26) whereas colonoscopic screening adherence in Germany is 16% (25). Over 20 years of SEER data (1991 to 2011), United States CRC incidence (all races, males, females) has fallen from 59.5 cases in 1991 to 39.3 cases per 100,000 in 2011 (35% reduction) with a corresponding mortality reduction over the same time period from 24.0 to 15.1 deaths per 100,000 (37% reduction) (27). Widespread adherence to screening guidelines in the United States may be driven by the profound changes in the organization of medical care including enhanced access via the Affordable Care Act, rigid guideline enforcement by payers with physician performance incentives and disincentives, and the rapid adaptation of electronic medical record systems enabling ease referrals for screening, compliance reminders, and management tracking of compliance to care guidelines (28).

## 4.2 Current State of the Art: Serum Based Biomarkers for Colorectal Neoplasia

Reasons for non-adherence with stool based or colonoscopic based CRC screening include the volume of bowel preparation, inadequate analgesia, no recommendation from primary physician, embarrassment (29) or cultural taboos surrounding collection or manipulation of stool provide rationale for discovery and validation of circulating biomarkers for early detection of colorectal neoplasia. Circulating signatures may be detected from neoplasm generated genetic products, antigens, antibodies, glycans, circulating tumor cells.

### 4.2.1 Genetic Products

In a recent study of 24 CRC patients, mutant DNA fragments (circulating tumor DNA, ctDNA) are found in at relatively high concentrations in the circulation of most patients with metastatic cancer and at were detected in ~70% of patients with localized cancers (30). The direct detection of aberrant genes or genetic material specific to colorectal neoplasms (e.g. APC, -catenin, K-ras, DCC, and p53) has been limited by the technical challenge of DNA recovery, the large number of potential underlying genetic mutations, and by the limited sensitivity of any single genetic alteration due to the extremely low abundance gene mutations in circulating plasma or serum (30-35). DNA hypermethylation, in contrast, affects residues in regulatory portions of genes and provides major advantages in designing biomarker assays (34, 36-38). Digital based quantitative technologies improving upon bisulfite conversion while minimizing bisulfite associated DNA fragmentation and single molecule detection technologies (39) permit cost effective development of DNA hypermethylated gene biomarkers. Such technology detected circulating methylated vimentin with 59% sensitivity (39). Septin9, a methylated gene discovered in tissues with array technology (40, 41), detects CRC with 50% sensitivity and 92% specificity in a large (7941 participants) prospective colonoscopy verified screening trial (1). For early stage CRC, Septin9 sensitivity decreased to 35%. While circulating methylated CpG DNA promoter sites appear to have higher CRC detection performance than other genetic detection strategies, they substantially lag behind stool based detection of blood DNA markers or endoscopy. Nevertheless, for individuals refusing to use stool based screening, detection sensitivity of circulating methylated DNA markers appears equivalent to guaiac based stool screening and has the potential advantage of capturing the 35% of the population refusing stool screening. miRNAs are stable and detectable in serum and plasma. As in stool, numerous up and down regulated miRNA stool signatures discovered using unsupervised array technology may be useful as CRC detection biomarkers. A recent review identifies 19 miRNAs as individual or groups in panels as candidates for detection markers; but, insufficient clinical validation renders the data generated to date using small convenience sets confusing and not mechanism driven (42).

### 4.2.2 Proteins

*Antigens:* Approximately 50% of all proteins are estimated to be glycosylated (43). Glycan abundance and their micro- and macro-heterogeneity can be changed in a disease-specific manner (44). Glycoprotein screening studies, many EDRs supported, have relied on immunoprecipitation or lectin affinity capture of whole glycoproteins and mass spectrometry identification of the de-glycosylated protein portion or probed with lectins in an array format containing up to a few hundred antibodies (45-49). Sialylated Lewis A and Lewis X moieties carrying proteins identify panels of potential markers. The Lampe EDNRN laboratory has found seven such proteins (B3GNT5, CD44, HSPG2, IL6, INHBC, NOTCH4 and VWF) which, when combined in discovery set plasma samples ROC AUC of 0.83 (50). GLNE discovered glycan ligand, galectin-3 ligand is circulating glycan biomarker in large population based prospective validation (51).

*Antibodies:* Serum antibodies recognizing multiple colon cancer antigens can be detected in colorectal adenocarcinoma patients' markers (52-54). Preliminary validation of single or small autoantibody panels have been disappointing (55). For example, antibodies to the Fas receptor have 17% sensitivity when 100% specific for CRC detection (56). Experience with p53, Hsp60, and nucleobindin 1 (Calnuc) autoantibodies has been better (~50% sensitivity/70 to 90% specific); but, they are not specific to CRC (55, 57, 58) and cannot be used as a colon specific screening tool. Discovery sets that include a miniarray of autoantibodies with other markers have reported improved detection accuracy (sensitivity 83%/specificity 90%) (59) but require clinical validation.

*Cytokines/growthfactors:* High serum concentrations of insulin-like growth factors (IGF) and low levels of their binding proteins have been shown to correlate with CRC risk in large cohort studies (60-63) but have low sensitivities with high specificities for CRC detection. Other cytokines or angiogenesis factors such as TGF-P 1 (64-70), VEGF (71, 72), angiogenin (73), endostatin (74), and endothelins (75, 76) also have low sensitivity in small convenience sets and have not proceeded to clinical validation.

*Other proteins:* Of the matrix metalloproteinases (77-79), plasma TIMP1 is elevated in CRC but has not had sufficient sensitivity in larger validation trials to merit development as a detection biomarker (80). Cell adhesion molecules (81) have low sensitivities for detection of early stage CRC.

#### **4.2.3** Circulating Tumor Cells

Circulating tumor cells (CTCs) entering the vascular space from primary neoplasms have been considered to be initiators of metastases (82-84) and can be detected in early stage invasive neoplasms (85, 86). CTC isolation from epithelial cancers initially used antibody capture technology dependent upon epithelial adhesion (EpCAM) and cytokeratins (82).

This technology limits CTC detection of early stage neoplasms because CTCs are thought to undergo epithelial to mesenchymal transition (EMT), epithelial traits are lost and epithelial marker such as EpCAM and cytokines are downregulated. CTCs present in as few as 1 cell in  $5 \times 10^9$  red cells, and up to  $5-10 \times 10^6$  white blood cells, are rare events (84). Newer microfluidic or centrifugation devices appear to more efficiently capture CTCs (85, 87). The inclusion of mesenchymal/EMT-specific antibodies, for example, vimentin, PLS3 may improve CTC capture and/or expansion (84). With the emergence of ex-vivo expansion protocols of CTCs and the increased ability to detect stem like or stem progenitor cells, CTCs are of future interest as an early cancer detection diagnostic (85, 87), but remain in the technology development phase.

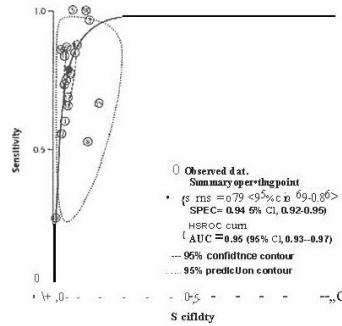
#### **4.2.4** Special consideration-EDRN discovered and preliminarily validated circulating biomarker: Galectin-3 Ligand ELISA as a Serum Biomarker for the Detection of Colorectal Neoplasia

The galectins are widely distributed and evolutionarily conserved carbohydrate binding proteins characterized by their binding affinity for p-galactosides and by conserved sequence elements in the carbohydrate-binding region (88). Galectin-3 is the galectin that is of most interest in regard to colon cancer because of its demonstrated role and cancer progression and metastases and interaction with mucins(89-93). Galectin-3 ligands include laminin, LAMP-1 and 2, LPS and colon cancer mucin. The major galectin-3 ligand detected in serum is a 40 kDa band distinct from MUC2 and other mucins CEA, and Mac- 2-BP. We reported a true positive rate for the detection ofCRC of 91% and false positive rate of 18% using preliminary data using quantitative Western blot technology on a convenience set of GLNE serum (51).

We developed a sensitive, reproducible ELISA assay for galectin-3 using a new antibody we created. This was used to assay the GLNE colorectal reference set (50 colorectal adenocarcinomas/50 adenomas/50 endoscopically normal controls). The ROC analyses for galectin-3 ligand combined with FOBT (fecal occult blood test-guaiaic based) for detection of colorectal adenocarcinoma versus controls who had normal colonoscopy shows an area under the ROC curve of 0.91, while galectin-3 ligand detection of colorectal adenocarcinoma alone versus controls who had normal colonoscopy shows an area under the curve of 0.84. The true positive rate of galectin-3 ligand with FOBT for detection of CRC is 64% with a false positive rate of 5%. Without FOBT, true positive rate of galectin- 2 ligand was 72% with a false positive rate of 20%.

## 4.3.1 Occult blood tests

Stool testing as a screening approach offers the potential advantages of noninvasiveness, low cost, avoidance of cathartic preparation, and minimal impact on work time or daily activities. guaiac based FOBT is not specific for human blood, and consequently it has a high false positive rate for colorectal neoplasia. The fecal immunochemical test (FIT) detects human hemoglobin, thus eliminating the false positives caused by non human hemoglobin in the diet (94, 95). FIT tests are more sensitive at detecting CRCs (sensitivity range 61% to 91%) and adenomas (sensitivity range 16% to 31%) than classical unhydrated guaiac FOBT (Hemoccult II) (sensitivity range 25% to 38% for CRC; 16% to 31% for advanced adenomas) (96, 97). A recent meta-analysis that analyzed data from 19 prospective randomized trials or cohorts using 8 different commercially available FIT tests with colonoscopy or 2 year observation endpoints reported an overall sensitivity for detection of CRC of 79% (95% CI = 0.69-0.86), specificity of 94% (95% CI = 0.92- 0.95) and overall accuracy (defined as hierarchical summary receiver operating characteristic (ROC) curve) of 95% (95% CI= 93% - 97%) (Figure 1). Differences in performance characteristics among FIT brands were small, particularly between the two major brands used OC-Light (Eiken Chemical) and QC-Micro/Sensor (Polymedco + Eiken Chemical). The Polymedco product is widely used in the USA. Quantitative FIT (Eiken OC-SENSOR) >177 µg/gm stool combined with age and sex predicts 11.46 fold risk of a large adenoma over lower risk groups (98).

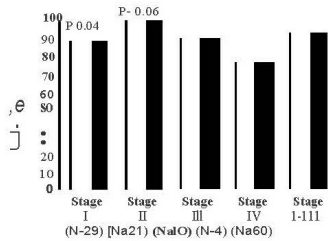


**Fig 1 from Lee et al (2): Hierarchical ROC curve of the sensitivity versus specificity of FIT. The diamond= summary point of the curve to which the pooled sensitivity and specificity correspond. Dashed line = 95% CI for summary point; dotted line = 95% confidence area of FIT diagnostic accuracy. AUC = area under the curve; SENS = sensitivity; SPEC = specificity.**

## 4.3.2 Stool DNA tests

Since the neoplastic transformation process of the colonic epithelium results in cells shedding into the stool, collection of fecal material is likely to yield detectable molecular and biochemical events associated with cellular transformation (99, 100). First generation multi-marker stool DNA tests detected 52-73% of CRCs, 41-49% of CRCs plus adenomas with high grade dysplasia, and 15-46% of adenomas 2:1 cm, with specificities of 84-95% (101, 102). Stool DNA test performance in both studies was compromised by failure to use stabilization buffer with stool collection, inefficient marker recovery from stool, and relatively insensitive analytical methods. Exact Sciences modified their previously published stool DNA panel (102) and now uses a panel consisting of methylated BMP3 and NDRG4 promoter regions, mutant K-ras (7 point mutations, Exon 2, codons 12,13), and a proprietary FIT test). In a recently published cross sectional validation study of 9,989 patients undergoing screening colonoscopy, the panel performed with a sensitivity of 92% for CRC; 84% for CRC + high grade dysplasia; and 42% for advanced adenomas (Figure 2) (103).

The specificity was 87% for CRC, the ROC AUC for the Exact Sciences DNA stool panel for the detection of colorectal cancer is 0.94. FIT alone (Polymedco FIT) performed with sensitivity of 73.8% and specificity of 94.9% for detection of CRC and sensitivity of 23.8% for screen relevant neoplasia. Stool DNA component of the panels adds ~20% sensitivity to FIT. The USPSTF is currently assessing the role and contribution of fecal DNA panels such as the Exact Sciences panel to CRC screening (104).



**Fig 2 from Imperiale et al [Imperiale 2014 #5977] sensitivity for detection of CRC by Exact Sciences stool DNA panel + FIT (light blue) vs Fit alone (dark)**

#### 4.3.3 Vimentin Methylation as a Stool DNA Test

Aberrant methylation of vimentin exon 1 was initially described as a highly frequent biomarker of colorectal cancers and adenomas by Markowitz and co-workers (105). In reproducible studies, aberrant methylation of vimentin has been detected in 72%-83% of colon cancers and 70%-84% of colon adenomas (105, 106). The current assay for detection of vimentin exon 1 methylation is based on using methylation specific PCR (MSP). Adaptation of the vimentin MSP to testing fecal DNA is accomplished by recovery of vimentin DNA sequences from human stool using hybrid capture to vimentin specific oligonucleotides(105). Initial study showed that MSP assay of vimentin purified from feces (fecal vimentin DNA) detected methylated fecal vimentin DNA in 46% of cancer patients (N=94) at a specificity of 90% (N=198)(105). This initial study involved collaboration between the Markowitz laboratory who had discovered the methylated vimentin DNA marker, and Exact Sciences, who implemented detection of this marker in fecal DNA. This initial study was limited by use of samples that had suffered problems of DNA degradation during sample collection and shipping (102). A recently published two stage followup study lead by Itzkowitz et al in collaboration with Exact Sciences and the Markowitz laboratory showed markedly improved results with the use of a DNA stabilizing buffer added to stools at the time of collection (107). Detection of methylated fecal vimentin

DNA was found in 77% of cancers (N=82) at 83% specificity (N=363). Six of 7 adenomas with high-grade dysplasia were also detected. This assay has successfully detected 55% (N=22) of adenomas that were greater or equal to 1cm in size (107). This is a published assay of capture of fecal vimentin DNA and then MSP detection of methylated vimentin exon 1 sequences (105, 107, 108).

#### 4.3.4 Other Stool Based Biomarkers Under Investigation

Considerable interest in fecal microbiome populations has triggered EDRN supported investigators into identifying unique bacterial species that are associated with colonic carcinogenesis and suggests that a microbiome signature may be a useful stool biomarker for CRC risk (109, 110). Metabolome signatures promise to identify amino acid or fatty acid profiles associated with colorectal cancer or high risk (111) have been preliminarily developed in EDRN supported research. Micro-RNAs (miR\_ NA) have both oncogenic and suppressor properties, can be detected in stool, and have been explored as stool based early detection biomarkers (112, 113). Studies published to date have used small convenience samples and array technologies that have identified diverse and non-reproducible miRNAs as classifiers for colonic neoplasms.



#### 4.3.5 Urine base Biomarkers

We demonstrated previously that human urine contains circulation-derived DNA [ $< 300$  base pairs (bp), designated as low molecular weight (LMV) DNA] and that LMV urine DNA can be used to detect colorectal cancer (CRC) associated k-ras mutations from patients with CRC. A quantitative MethylLight PCR-based assay targeting a 39-bp template of the hypermethylated vimentin gene (mVIM) was developed to detect circulation derived mVIM DNA. A blinded concordance study was performed using matching tissue and urine DNA samples from patients with CRC. The 20 CRC tissue samples and 20 urine samples from patients with CRC were provided with barcodes. LMW urine DNA and tissue DNA were isolated, bisulfite converted and assayed for mVIM. The mVIM was detectable in 85% (17/20) of the CRC tissue DNA samples and 75% of LMW urine DNA. As control, LMW urine DNA isolated from 20 subjects with no known neoplasm was also tested for the mVIM DNA. Two of 20 (10%) normal control LMW urine DNA contained detectable mVIM DNA. After all of the samples were tested, the urine and tissue ID numbers were unblinded and matched. The concordance value between the mVIM-positive CRC tissue and matched urine DNA samples was 71% (12/17). We thus conclude that CRC-associated mVIM DNA can be detected in the urine of patients with CRC with a concordance of 71% between marker-positive tissue and matched urine samples with a sensitivity of 75% (Su Y-H, personal communication). These results support further development of a urine test for CRC screening.

#### 4.4 Key Issues Driving Research Questions in CRC Early Detection Biomarkers

Until therapeutic agents with much greater potency and minimal side effects are developed, the current best strategy for reducing cancer morbidity and mortality is early detection of neoplastic disease (114). Key opportunities in the current state of colorectal screening and early detection include:

1. *Enhancing adherence to current screening guidelines:* Screening and early detection reduce mortality from colorectal cancer; yet 35% of the population in the USA remain non-adherent. Adherence is much lower in other countries (25). The barriers to these recommendations (cost, discomfort, cultural taboos) may be overcome with circulating biomarkers that provide individuals with persuasive evidence that undergoing invasive screening procedures, i.e. colonoscopy, will have important life-saving benefit that reduces mortality from CRC (8-10, 18). Developing, validating and bringing circulating biomarkers to population screening use remains a high priority that will likely increase adherence to endoscopic screening. *GLNE 010 addresses this priority by working closely with EDNR and industry groups to clinically assess and validate circulating biomarkers of CRC risk that might drive individuals who might decline to endoscopic screening.*

2. *Tailoring colonoscopic screening to individual risk:* Recently published data from the Clinical Outcomes Research Initiative found the prevalence of large polyps higher in blacks than whites among both men and women (115). Tailoring endoscopic screening to those at risk while limiting screening for those with minimal or no risk (116, 117) will enhance screening adherence and eliminate excess cost. Recommendations for tailoring were primarily population demographic based (116, 117); yet, the translation of carcinogenesis biology and genetics into biomarker panels with extremely high sensitivity (99%), i.e. no false negative tests, promises precise tailored endoscopic screening. The current state of art stool using based biomarker tools is coming close-92% sensitivity (103) but insufficient to permit tailored or individualized risk. *GLNE 010 addresses the priority of biomarker driven tailored risk by completing the ongoing phase 3 validation trial of stool and circulating biomarkers and using the extensive repository created by this and other GLNE protocols to rapidly identify new markers that may enhance sensitivity of the current biomarker panels.*

3. *Persistently positive stool DNA tests with negative colonoscopic screening:* The stool methylated DNA panels report 5% false positives (103, 108). A positive stool DNA test with a negative screening colonoscopy could potentially arise from neoplasia in the upper gastrointestinal tract or from occult and missed lesions in the colorectum. The latter is a particular concern in the right colon, where flat lesions and/or sessile serrated adenomas are more prevalent. Preliminary data from the Case Western EDNR BDL found near 100% vimentin methylation in gastric dysplasia while no methylation in adjacent gastric mucosa (S. Markowitz, Personal Communication). In Barrett's esophagus (BE), 7 of 7 high grade dysplasias (HOD), and 15 of 18 esophageal adenocarcinomas (EAC) and even in some squamous cancers (SCC) had methylated vimentin, whereas it was absent in all 9 normal squamous mucosa (118). A "false positive" stool DNA test may detect dysplasia or invasive neoplasms in the upper GI tract. The GLNE will propose to address this priority in the future in a future project. This project, to be submitted as a separate proposal will propose a longitudinal study of participants registered in an ongoing cross sectional Phase 2 colon biomarker validation trial with a positive stool test and negative colonoscopy registered in the current

#### 4.5 Rationale for Food Frequency Assessment and the Use of the NIH DHQ II Food Frequency Questionnaire

Numerous epidemiologic studies have controversially implicated high total fat and saturated fat, alcohol, inadequate calcium, vitamin D, dietary fiber, vitamin B6, folate, methionine, antioxidant vitamins such as C, and E, and lack of fruits and vegetables as dietary risk factors for colon carcinogenesis (119-127). The causal contribution of these dietary factors to risk of colon cancer has been difficult to assess and compare in meta analyses due to different instruments used to assess diet, including diet records, 24 hr. dietary recalls and food frequency questionnaires (128, 129). However, because there is significant epidemiologic support for dietary variables affecting cancer risk in populations (130-132), it is important to collect dietary information along with human biosamples to allow future study of the relationship of selected dietary variables and their impact upon biomarkers of cancer risk, for early detection, or post diagnosis prognosis. For example, vimentin methylation is a key stool DNA marker we propose to validate as an early detection tool in this trial. Since the methylation reaction requires methyl tetrahydrofolate (133-135), it is conceivable that dietary folate may impact the methylation status of this and other future methylated biomarkers for cancer risk and detection.

In a large validation biosample and annotated data set such as the one in this trial, diet intake among different subjects is likely to be an important source of bias, thus an adjustable variable in the analysis of validated biomarkers. We have chosen to administer a food frequency questionnaire to the subjects enrolled in this trial because it assesses dietary exposures over time (typically 6 months). We recognize the weaknesses of a food frequency instrument, including recall bias, but the instrument has value when used as a semi-quantitative measure to rank order individuals according to their intake of a given nutrient rather than a continuous variable (136, 137). We propose to use the computerized self-administered user-friendly National Institute of Health Diet History Questionnaire II (DHQII). The DHQ I was developed specifically to study dietary risk factors for cancer and has been validated to adequately assess dietary intake over time against established FFQs such as the Block and Willett FFQ (136, 138). The questionnaire assesses supplement use in addition to dietary intake data and has been used in multiple cancer studies to date. The DHQ II is a refinement of the validated DHQ I with improved separation of some food sub categories to enhance detail of data intake.

### 5.0 STUDY DESIGN

#### 5.1 Subject Recruitment

The clinical research associate or study nurse (hereafter “CRA”) at each clinical site will identify subjects with appointments for colonoscopy via IRB-approved HIPAA-compliant site-specific methods (Appendix B-tailored to each site). Recruitment methods could include letters from the primary care physicians and gastroenterologists, direct referrals to the study team by physicians, in-clinic recruitment advertisements, use of navigator programs, county or statewide screening programs, and other IRB-approved means of identifying and contacting subjects. Interested subjects will be asked to participate in a baseline visit prior to initiation of colonoscopy preparative procedures, either at the local Center or during a visit to the subject’s home by a CRA. Advertisements (e.g., newspapers, AARP Magazine, Clinicaltrials.gov) may also be used to recruit subjects from the surrounding communities.

#### 5.2 Eligibility

##### 5.2.1 Inclusion Criteria (at time of consent)

###### Subjects at US Sites

- ① Adults 60 and older
- ① Never had a full colonoscopy for screening purposes
- ① Willing to sign informed consent
- ① Able to physically tolerate removal of about 50 ml of blood
- ① Willing to collect 2 stool samples

###### Subjects at Sites in Germany/Canada

- ① Adults 50 and older
- ① Never had a full colonoscopy for screening purposes
- ① Willing to sign informed consent
- ① Able to physically tolerate removal of about 50 ml of blood
- ① Willing to collect 2 stool samples

### 5.2.2 Exclusion Criteria (at time of consent)-All subjects

- ① Inability to provide informed consent
- ① History of Inflammatory Bowel Disease
- ① Overt rectal bleeding within 1 month (30 days) (including due to suspected hemorrhoids)
- ① Positive guaiac-based occult blood or fecal immunochemical test (e.g. FOBT, FIT) in the past 12 months (365 days)
- ① Undergone resection of the colon for any indication
- ① Subjects with known HIV or chronic viral hepatitis (Hepatitis Band C)
- ① Subjects with known or suspected HNPCC (Lynch Syndrome) or FAP
- ① Any cancer within 5 years prior to enrollment except squamous cell carcinoma of the skin or Basal cell carcinoma of the skin.
- ① Prior history of Colon Cancer or Rectal Cancer.

## 5.3 **Study Procedures**

### 5.3.1 Enrollment and Registration Procedure

Subjects who meet the eligibility criteria will be scheduled for a baseline visit. The baseline visit must occur prior to any preparative regimen for colonoscopy (e.g. PEG {Golytely, Halflytely}, Miralax/Gatorade, Suprep, etc.) and within 16 weeks of the scheduled colonoscopy procedure. At this baseline visit, subjects will provide informed consent (see model consent, appendix F) for analysis of stool, urine and blood samples for biomarkers; medical record review, including colonoscopy and pathology reports; and for completion of questionnaires.

The subject will be enrolled and given a unique participant identification number (PID) generated randomly by the DMCC. The sites will subsequently link the PID to the specimen collection kits once specimens are collected.

### 5.3.2 Demographic and Other Data Collection

The subject will be asked to provide data to complete EDRN demographic and medical history questionnaires. Clarification or additional information may be obtained from the medical records. Case report forms (CRFs) will also be used to collect information on concomitant medications, colonoscopy outcomes, resection information, any new cancer treatment, and new diagnostic tests. Long term data collection (medical records review and follow up data) will be prompted by information gathered at a phone call with the subject at one-year post colonoscopy. Data may be collected via face-to-face interviews, via phone or email interviews, or returned by mail dependent on subject preference. Subjects (U.S and Canada only) will be asked to complete a NCI DHQ II food frequency questionnaire (Appendix A) at home after the baseline visit as defined in Section 5.3.1. The NCI DHQII can be done online through a secure web-based system (<http://riskfactor.cancer.gov/dhq2>). If subjects chose to report diet data on paper forms, sites will be responsible for entering the data into the web-based system.

### 5.3.3 Sample Collection: Blood

Baseline blood samples will be obtained according to standard operating procedures (Appendix C). The blood will be collected during or after the baseline visit but prior to any preparative regimen or procedure as detailed in section 5.3.1 and the Operations Manual. Samples must be collected within 16 weeks prior to the qualifying colonoscopy (detailed in the operations manual). Blood may not be collected at or after the colonoscopy.

### 5.3.4 Sample Collection: Urine Sample

A baseline urine sample will be obtained according to standard operating procedures (Appendix G). The urine will be collected during or after the baseline visit, but prior to any preparative regimen or procedure as detailed in section 5.3.1 and the Operations Manual. The urine specimen must be collected within 16 weeks prior to the qualifying colonoscopy. Urine may not be collected at or after colonoscopy.

### **5.3.5** Sample Collection: Stool Sample and FIT #1

Subjects will be required to collect stool samples prior to any preparative regimen or procedure within 16 weeks prior to colonoscopy. Women will be asked to avoid collection during heavy menses if applicable. Subjects will be asked to collect their stool in the collection bucket (hat) provided. Subjects will be given detailed instructions and complete kits to collect the stool samples at home. They will prepare an OC\_SENSOR FIT (Eiken Chemical Company) (FIT #1) from the stool sample. Subjects will also collect scoops of stool into a container with an EDTA-based buffer (“buffered stool”) and additional scoops of stool into tubes provided to be sent on ice packs (“native stool”) The subjects will then package both the stool and the FIT for shipping per provided instructions. The US and Canadian subjects will ship the stool sample to the Central Laboratory at the University of Michigan using pre-paid DOT (Department of Transportation)-compliant packaging.

German subjects will send their stool samples to the German Cancer Center (DKFZ) (Dr. H. Brenner).

### **5.3.6** Sample Collection: FIT #2

Subjects will be asked to collect another bowel movement (ideally the next one) for a second FIT only (FIT #2). The subject will use the 2nd FIT to collect another sample from the stool collected on paper provided. The subject will mail the FIT using provided self addressed postage-paid envelopes. The US and Canadian subjects will ship the FIT #2 sample to the Central Laboratory at the University of Michigan and the German subjects will send their stool samples to the German Cancer Center (DKFZ) (Dr. H. Brenner).

### **5.3.7** Subject Compensation

To compensate for the inconvenience and cost of driving and parking, \$25 will be provided to each subject once blood samples, urine samples, stool samples and questionnaires are completed. If the research coordinator visits the subject at home, no payment will be offered at the site’s discretion. U.S. and Canadian recruiting sites will receive gift cards to distribute to subjects that complete the requirements to receive payment. Gift cards will be to places like Target, Walmart, or other similar stores in the specific region, purchased by UM Prevention Research Base staff and distributed to sites. Sites are required to account for distribution of gift cards to subjects. German subjects will be paid according to local policies.

### **5.3.8** Colonoscopy Standards

Colonoscopy standards for inclusion of the data into GLNE 010 will include verification of insertion to cecum, photos of all lesions (available at the site), size, histology and location in colon of all suspected colorectal cancers, adenomas, or other polyps. Case report forms will capture some of this information; the rest will be reviewed directly at site monitoring visits or by review of redacted reports.

### **5.3.9** Sample Collection: Tissue Samples

One H & E slide from clinical tissue blocks of all detected colorectal adenocarcinomas , high grade dysplasia and advanced adenomas will be obtained (given, shared digitally or borrowed), sent to the lead site and reviewed by a reference GI pathologist at the University of Michigan. Up to ten slides (10um thick sections) from clinical tissue blocks of all detected colorectal adenocarcinomas, high grade dysplasia and advanced adenomas will be obtained whenever possible and sent to the lead site for storage. Specific details will be worked out with each site depending on costs and standard practices.

### **5.3.10** Sample Labeling and Tracking

All samples will be labeled with a unique specimen ID (embedded barcodes or other labels) managed by the DMCC. The site will subsequently associate the specimen IDs to the PID. The bar codes will be scanned at each step of the procedure (collection, on-site processing, shipment, receipt, and storage in a repository). All biosamples are property of the EDRN.

## 5.4 Study Definitions

### 5.4.1 Assessing Inclusion Criteria-Definition of “Full Colonoscopy”

Prior colonoscopy eligibility requirements for screening (versus surveillance) indications are defined per AGA guidelines (22, 139) and are used to define the study group. A subject who has had a flexible sigmoidoscopy is eligible. An incomplete colonoscopy is one where the prep was considered “poor” or more than 15% obscured (see SOP, Appendix E) or the entire colon could not be visualized or the scope did not reach the cecum (unless an obstructing mass was the reason the scope didn’t reach the cecum). Subjects having a repeat colonoscopy due to a previous “incomplete” colonoscopy are eligible if they otherwise meet inclusion/exclusion criteria because incomplete colonoscopies are not a “full colonoscopy”.

### 5.4.2 Minimum Requirements for Subject Enrollment

- a. Two FIT tests shipped properly per SOPs (within tolerance)
- b. Stool samples shipped properly per SOPs (within tolerance)
- c. Blood: minimum 18 aliquots of serum, 18 aliquots of plasma, and 2 huffy coats processed per SOPs
- d. Complete colonoscopy to cecum with good or better bowel preparation (per colonoscopy SOPs) or an obstructing mass prohibiting insertion to cecum
- e. All data forms
- f. Four 5 ml vials of urine

### 5.4.3 Enrolled Subject

An enrolled subject is one that has signed the informed consent, is eligible based on inclusion and exclusion criteria at the time of consent (section 5.2) **AND** has the minimum specimens required (5.4.2). Replacement samples or additional visits before the screening colonoscopy are options to meet the minimum requirements to enroll a subject. Once a subject meets the inclusion and exclusion criteria and provides specimens within the 16- week window, the qualifying colonoscopy for study purposes is the first one that is complete (to cecum) with a good or better preparation (defined as less than 15% of mucosa obscured). An otherwise eligible subject may need a repeat colonoscopy due to poor prep, poor sedation, or some other technical or logistical issue. These subjects would be considered enrolled as long as the colonoscopy is done within 16 weeks of original specimen collection. Enrolled subjects are listed as “pending” in VSIMS until confirmed. (See 5.4.5) or deemed ineligible (see 5.4.6).

### 5.4.4 Protocol Deviations

Subjects who do not meet the minimum requirements (5.4.2), do not have a complete colonoscopy or have to provide replacement samples will not be reported as protocol deviations.

### 5.4.5 Evaluable Subjects

Once an enrolled subject has completed their colonoscopy, and the recruiting site has pathology and colonoscopy reports, the site should run “Confirm Eligibility” in VSIMS. The “Confirm Eligibility” function will verify that the subject met the inclusion/exclusion criteria, provided the required samples and data (5.4.2), and count the subject in a final group or bin based on the colonoscopy results (including no colonoscopy). Evaluable subject’s samples and data will be used for analysis or building a reference set.

Bin #1 - Colorectal Cancer  
Bin #2 - Carcinoma in Situ  
Bin #3 - Adenoma with High-Grade Dysplasia  
Bin #4 - Advanced Adenoma  
Bin #5 - Adenoma  
Bin #6 - Hyperplastic Polyps  
Bin #7 - Polyp of Other & Unknown Types  
Bin #11 - Normal Colon

#### **5.4.6** Screen Failures, Not Eligible and Unevaluable Subjects

Subjects who are approached to participate via a face-to-face visit and do not meet the eligibility criteria in section 5.2 are “screen failures”. These subjects will not be entered in VSIMS, should not be issued a PID, and will not receive payment for samples (\$25).

Screen Failures will not count as accruals.

Subjects who sign the informed consent, but end up not meeting the eligibility criteria in section 5.2 (with samples already collected) will be “ineligible”. Subjects who meet the inclusion/exclusion criteria but do not provide stool and blood will be labeled ineligible. An “ineligible” CRF will be completed. Ineligible subjects will be entered in VSIMS and may receive payment for stool samples if provided before determined ineligible. Ineligible subjects will not count as accruals toward the total 9000 subjects.

Subjects who are eligible, sign the consent form, and then do not meet the minimum requirements for subject enrollment (section 5.4.2) are unevaluable. Subjects that provide specimens but do not have a colonoscopy or subjects that have a colonoscopy with poor prep, poor sedation or an otherwise incomplete colonoscopy are considered unevaluable. The site should run “Confirm Eligibility”. Unevaluable subjects will be entered in VSIMS and may receive payment for stool samples if provided. Unevaluable subjects will count as accruals toward the total 9000 subjects.

Bin #8 - Uncategorized Colonoscopy

Bin #9 - Incomplete Colonoscopy

Bin #10 - No Colonoscopy

#### **5.4.7** Off-Study

A subject is off-study when the data, food frequency questionnaire, blood, urine and stool samples (including both FIT tests) have been obtained, properly processed and delivered to, the Central Laboratory at the University of Michigan/DKFZ, a colonoscopy has been completed, eligibility confirmed and the one year follow up contact has been conducted.

Data collection will continue on subjects that have findings of cancer or require surgical excision of lesions (i.e. adenomas) in order to obtain staging, treatment, and outcomes relevant to the use of the biomarkers, and these subjects will not be off study until that data collection is complete. Adverse events or serious adverse events will not be reported for subjects remaining on study between the completion of their baseline visit and going “off study” as this is a minimal risk, non-interventional study (section 9.6).

### **5.5** **Biological Sample and Data Collection**

#### **5.5.1** Blood Collection, Processing and Storage

Subjects will provide 50 ml of blood as defined above. Blood samples will be drawn in a specific order: 2 x 10 ml red top tubes and then 3 x 10 ml purple top tubes. Purple top tubes must be filled to manufacturer’s level to maintain blood:EDTA ratio. Sufficient blood is needed to ensure that a minimum of 18 aliquots of serum, 18 aliquots of plasma and 2 huffy coats are collected. Additional red and purple top tubes may be collected to get the full 50 mls needed. Additional blood draws, prior to prepping for the colonoscopy may be done to get to the necessary blood volume.

The serum samples (red top tubes) will sit at room temperature for a minimum of 30 minutes (maximum of 60 minutes) to allow the clot to form, and if not processed immediately, they can be held at 4° C for a maximum of 4 hours after collection. Plasma samples (purple {aka lavender} top tubes) will be held at 4° C for a maximum of 4 hours after collection. The red top collection tubes will be centrifuged at >1,300 x g at 4° C for 20 minutes. The serum will be removed, transferred to pre-labeled polypropylene capped tubes, and frozen at - 70° C or colder. The purple top collection tubes will be centrifuged at >1,300 x g at 4° C for 10 minutes without the brake on the centrifuge. The plasma will be transferred to a 15 ml conical tube for a second centrifugation step (>1,300 x g at 4° C for 10 minutes) prior to aliquoting in pre-labeled polypropylene capped tubes, and frozen at - 70° C or colder. The huffy coat, remaining in the purple top tubes above the red blood cells, will be removed and placed into 2 pre-labeled vials, up to 1.2 mls of RNeasy Lysis Buffer (Qiagen, Crawfordsville, IN) will be added and stored at -70° C or colder. All frozen samples will be stored at - 70° C or colder at the collection site and shipped on dry ice to the CLASS labs at the University of Michigan and stored at - 70° C or colder until assayed. Detailed Standard Operating Procedures including shipping and sample handling instructions are provided in Appendix C.

#### **5.5.2** Urine sample collection, processing, storage

At the baseline visit, subjects will be asked to provide a urine sample of at least 25 mls. The urine specimen will be stabilized with IM EDTA, and held at 4°C for up to 4 hours until aliquoted. The urine will be aliquoted and stored frozen at -70° C or colder. All frozen samples will be stored at -70° C or colder at the collection site and shipped on dry ice to the CLASS labs at the University of Michigan and stored at -70° C or colder until assayed. Detailed Standard Operating Procedures including shipping and sample handling instructions are provided in Appendix G.

#### **5.5.3** FIT Analysis

Subjects will be provided with a standard collection kit including detailed instructions on how to complete the FIT sampling (Appendix D). The first FIT tube will be shipped inside the same shipping container with the stool sample (see 5.3.5). The second FIT tube will be mailed (pre-paid) to the University of Michigan or DKFZ at room temperature in the manufacturer's DOT-compliant envelope. The test will be analyzed at the Central Laboratory at the UM or DKFZ using analytic equipment provided by Eiken Chemical Company. (OC-SENSOR Diana).

#### **5.5.4** Stool Sample Collection and Handling

Subjects will be asked to collect their stool in the collection bucket (hat) provided. Subjects will be given detailed instructions and complete kits to collect the stool samples at home.

They will prepare a FIT tube (FIT #1) from the stool sample. Subjects will also collect scoops of stool into a container with an EDTA-based buffer ("buffered stool") and additional scoops of stool into tubes provided to be sent on ice packs ("native stool") The subjects will then package both the stool and the FIT for shipping per provided instructions. The US and Canadian subjects will ship the stool sample to the Central Laboratory at the University of Michigan using pre-paid DOT (Department of Transportation)-compliant packaging. German subjects will send their stool samples to the German Cancer Center (DKFZ) (Dr. H. Brenner). Buffered stool samples will be homogenized and frozen in four 5 ml aliquots at -70° C or colder for batch shipment to the analytical labs. The native stool will be placed at -70° C or colder upon receipt.

#### **5.5.5** Follow up

The CRA will contact the subject via phone or letter or email about one year (window 11- 14 months) after their qualifying colonoscopy for additional follow up data including changes in family history of cancers, significant personal medical events such as hospitalizations or new medical diagnoses, and any diagnosis or treatments for cancer or dysplastic lesions (e.g. adenomas). Data will be collected on medical record review forms and follow up data forms (Appendix A).

#### **5.5.6** Medical Records Documentation

Medical records will be reviewed to collect information regarding the results of the procedures, pathology analysis, surgery, treatment, history, or outcomes and documented in the CRFs. The medical records will serve as the source documents and will be maintained at the site enrolling the subject. Redacted copies (identifiers blocked out) of colonoscopy reports and pathology reports may need to be sent to the University of Michigan for review. Medical records and/or source documents may be reviewed at the site during audits or monitoring visits.

### **5.6** **Disclosure of results to subjects**

Subjects will be informed as part of the consent process that neither they nor their health care providers will receive any subject-specific results from participation in this study including results of FIT, stool or blood sample assay results. Subjects and their health care providers will be furnished with published data (abstracts, published manuscripts) upon request.

### **5.7** **Biomarker Analytical Approach**

#### **5.7.1** Sample Shipment to Analytical Sites

Samples will be shipped in batches to analytical sites. Shipment date and time will be recorded in shipping logs in VSIMS. Date and time of receipt will be recorded at the analytical laboratory. Laboratory staff will be blinded to all subject data except specimen ID and relevant handling or processing information. No diagnostic or additional demographic data will be provided. The analytical laboratories will not have access to the database, and will not be able to link the bar code to a specific PID.

5.7.2 Analytical Performance and Reporting Standard

The laboratory will have 12 weeks (3 months) from the date of sample receipt to complete the analytical task and report the data to the DMCC. The data will be reviewed for quality control by the biostatistician (DMCC). If there are concerns regarding variance of the data, an on-site visit will occur to review the methods of assay quality control and data manipulation.

5.8 Data Collection, Management and Monitoring

Data will be collected, managed and monitored through the EDRN supported VSIMS system of the Data Management and Coordinating Center (DMCC). This system is a fully featured, Good Computing Practice compliant (secure, audit trail, daily backup) database system with biosample tracking capability. Data will be entered via a Web-fronted interface at each collaborating clinical site. The data will be subject to internal and external audits. The DMCC and GLNE Prevention Research Base at the UM will organize and implement on site audit procedures. Biosample tracking will be accomplished using a bar code reader and the VSIMS system in real time for each step in sample management.

6.0 STUDY CALENDAR (Table 1)

Procedures	Baseline <sup>1</sup>	Stool collection <sup>2</sup>	Colonoscopy	Follow up <sup>5</sup>
Informed Consent	X			
Study Documentation CRF <sup>4</sup>	X			
General Information CDE <sup>4</sup>	X			
Medical History CDE <sup>4</sup>	X			
Concomitant meds CRF	X			
Hormone Replacement TherapyCRF	X*			
Colonoscopy CRF			X	
Surgery Report CRF			X*	X*
CRC treatment CRF				X*
Ineligible CRF	X*			
Food Frequency Data Collection	X			
Blood Collection	X			
Urine Collection	X			
FITxl		X		
Protocol deviation CRF		X*	X*	X*
Stool Collection		X		
Fixed Tissue <sup>3</sup>				X <sup>3</sup>
One Year Follow up				X <sup>5</sup>
Subject Expense Payment		X		
Protocol deviation CRF		X	X	X
Study Termination CRF				X

<sup>1</sup>Visit prior to colonoscopy procedure; subject should not have started the colon preparation procedures.

<sup>2</sup>Stool collection any time after first visit and subject returns home with kits. All samples collected prior to beginning colon preparation procedures

<sup>3</sup>Fixed slides are obtained for pathology review.

<sup>4</sup>CDE=Common Data Elements; CRF=Case Report Form Note: CDEs and CRFs are in Appendix A

<sup>5</sup>To be completed 12 months after the completion of the colonoscopy

\* Only if needed/applicable



## 7.0 ANALYTICAL PROCEDURES

### 7.1 Vimentin methylation

This assay will be performed at an EDRN-designated BRL according to previously published methods described in the background. The assay will be run both qualitatively and quantitatively for presence of and quantity of methylated vimentin gene by the University of Maryland BRL (PI Sandy Stass). The vimentin methylation assay will be performed blinded without knowledge of clinical source or results of other assays.

### 7.2 Fecal immunochemical Test (FIT)

The QC-SENSOR product will be used according to manufacturer's instructions. The threshold for a positive test is 100 ng/ml. The Central Laboratory at UM and DKFZ will process the samples using equipment provided by Eiken Chemical Company. Technicians will undergo tutorial and quality assessment with Eiken Chemical Company support technicians prior to study launch. A quantitative result will be generated and recorded and uploaded into VSIMS by the DMCC. If either stool result is above the recommended cut off, that subject will be called positive.

### 7.3 Galectin-3 Ligand

The analytically validated ELISA method described in the preliminary data will be transferred to an EDRN Biomarker Reference Laboratory. Serum aliquots will be provided to the analytical sites in a blinded fashion. The Bresalier laboratory will assay 20% of the samples to ensure quality control. All of the samples will be assayed by the UCLA Biomarker Reference Laboratory. Galectin-3 Ligand assay result is a continuous variable. To facilitate comparison, a threshold corresponding to the specificity of vimentin methylation, estimated from controls in this study that do not have SR,N<sup>+</sup> from colonoscopy, will be used.

### 7.4 Circulating methylated genes BCAT1/IKZF1 (Clinical Genomics)

A Good Laboratory Practice validated bisulfite PCR assay developed by Clinical Genomics will be used for this assay. Clinical Genomics will perform this assay on blinded samples at their laboratory facility in Rutherford, NJ. Clinical Genomics is not responsible for analysis of any other biomarkers other than their BCAT1/IKZF1 product.

### 7.5 Hypomethylated LINE1 from circulating cell free DNA (VolitionRx)

A Good Laboratory Practice validated assay developed by VolitionRx will be used for this assay. VolitionRx will perform this assay on blinded samples at their laboratory facility in Namur, Belgium. Volition is not responsible for analysis of any other biomarkers other than their hypomethylated LINE I assay.

## 8.0 DATA ANALYSIS PLAN, SAMPLE SIZE JUSTIFICATION, AND STATISTICAL POWER

Aim 1: This aim proposes to estimate the sensitivity and specificity for 1) colorectal adenocarcinoma or 2) screen relevant neoplasms (high grade dysplasia or adenoma with ≥25% villous histologic features or adenoma measuring ≥1 cm in the greatest dimension or sessile serrated polyps measuring ≥1 cm in diameter) of the following individual colorectal neoplasia early detection biomarkers using colonoscopy as the gold standard:

- ① stool vimentin methylation
- ② serum galectin-3 ligand
- ③ fecal immunochemical tests (FIT)
- ④ Circulating methylated genes BCAT1/IKZF1 (Clinical Genomics)
- ⑤ Hypomethylated LINE1 from circulating cell free DNA (Volition)
- ⑥ Other currently unspecified biomarkers

For each of the individual biomarkers, we will first verify through a receiver operating curve (ROC) analysis the previously established thresholds for an optimum sensitivity and specificity. For ties in area of ROC, we shall choose the cut-off based on the highest percent agreement.

From this point on, we will treat the performance of the markers as a dichotomy. We will calculate accuracy summaries such as sensitivity, specificity, predictive values, as well as the likelihood ratios for each individual marker along with the associated confidence intervals.

Aim 2 (primary objective): Three potential scenarios of possible primary hypotheses and corresponding plans for data analysis and statistical power are presented. The choice of primary hypothesis will be finalized prior to data analysis based on state of the art information about candidate biomarkers and clinical practice at that time.

a. To determine if a blood based panel (for example, serum galectin-3 ligand, CEA, methylated genes BCAT1/IKZF1, Hypomethylated LINE1 from circulating cell free DNA), at the same sensitivity of that for fecal immunochemical testing (FIT) for the detection of colorectal adenocarcinoma, has a specificity greater than 0.55 with an anticipated specificity  $2^{\cdot}$ : 0.70.

b. To determine if stool vimentin methylation and the blood based panel (serum galectin-3 ligand, CEA, methylated genes BCAT1/IKZF1, Hypomethylated LINE1 from circulating cell free DNA) when combined with fecal immunochemical testing (FIT) will significantly improve the sensitivity of FIT for the detection of colorectal adenocarcinoma, and maintain specificity greater than 0.80.

c. To determine if stool vimentin methylation, the blood based panel (serum galectin-3 ligand, CEA, methylated genes BCAT1/IKZF1, Hypomethylated LINE1 from circulating cell free DNA), when combined will improve the detection of colorectal adenocarcinoma: at sensitivity  $2^{\cdot}$ :0.98 it will have a specificity significantly greater than 0.55.

The statistical power analysis assumes 71 CRC cases (63 from the additional 9,000 subjects plus 10 CRC cases already recruited less 2 CRC cases in the surveillance sub-cohort), and >13,000 non-CRC controls (assuming ~12,000 non-SRN controls and ~1,300 SRNs).

Example a: A blood based panel (serum galectin-3 ligand, or combined with other blood based biomarker if necessary) will be defined and locked-down prior to data analysis. At a cutoff with the same sensitivity as FIT (assumed here to be 0.75 but the value will be estimated from the study data) the specificity for non-SRN controls and its 95% C.I. will be calculated. The 1-sided hypothesis that this specificity is significantly higher than 0.55 ( $H_0$ ) will be tested with an anticipated specificity  $2^{\cdot}$ : 0.70 ( $H_1$ ), using the kernel method described by Bantis and Feng (141). The kernel ROC estimate has been proven to have smaller mean square error than that of the empirical ROC estimate (142).

Statistical power will be >85% or >90% with an anticipated specificity  $2^{\cdot}$ : 0.70 or  $2^{\cdot}$ : 0.75 respectively. Preliminary data from GLNE investigator Robert Bresalier supports the performance assumptions made. The blood based panel used Galectin 3 ligand, methylated genes BCAT1/IKZF1, Hypomethylated LINE1 from circulating cell free DNA, CEA with four different modeling approaches using GLNE data (94 normals, 50 small adenomas, 100 CRCs, and 51 advanced adenomas). The specificity at 75-80% sensitivity is >70% for both negative colonoscopy group and for negative colonoscopy plus small adenoma group.

Example b: To determine if stool vimentin methylation and the blood based panel (serum galectin-3 ligand, CEA, methylated genes BCAT1/IKZF1, Hypomethylated LINE1 from circulating cell free DNA) will significantly improve the sensitivity of fecal immunochemical testing (FIT) for the detection of colorectal adenocarcinoma, and maintain specificity greater than 0.80. From other training samples, the cutoff points and the combination rules of vimentin methylation and blood based panel will be determined based on their ability to detect FIT negative colon cancer and maintain high specificity, then combined with FIT either by an "OR" rule or a linear combination. This decision rule will be locked-down prior to GLNE10 protocol data analysis. The difference of the sensitivities of this decision rule and FIT and the 1-sided 95% confidence interval of this difference will be calculated from 10,000 bootstrap samples to accommodate the dependence between two tests. The null hypothesis will be rejected if the lower bound of this confidence interval is above zero.

The statistical power will be >0.83 if the true difference in sensitivity is  $2^{\cdot}$ :0.16 (e.g. 0.75 for FIT, 0.91 for the new test) under the conservative assumption of independence of the two tests. The actual power will be larger as these two tests are expected to be positively correlated.

Example c: To determine if stool vimentin methylation, the blood based panel (serum galectin-3 ligand, CEA, methylated genes BCAT1/IKZF1, Hypomethylated LINE1 from circulating cell free DNA), when combined will increase the detection of colorectal adenocarcinoma: at sensitivity  $2^{\cdot}$ : 0.98 it will have a specificity significantly greater than 0.55, with an anticipated specificity  $2^{\cdot}$ : 0.79. This has significant clinical value as it has potential to spare more than 55% people in US from colonoscopy screening, and improve screening rate for those who do not want to have colonoscopy as first-line screening modality. From other training samples, a panel will be built to achieve  $2^{\cdot}$ : 0.98 in sensitivity in detecting colorectal adenocarcinoma while maintaining specificity  $2^{\cdot}$ : 0.79 for subjects without SRN lesions. This is feasible if other markers can pick up majority of FIT negative CRCs without reducing specificity by more than 15%. The combination rule will be defined and locked-down prior to GLNE10 protocol data analysis. To test the study hypothesis, at cutoff corresponding to 0.98 sensitivity, the specificity of this decision rule, its 1-sided 95% C.I., and the 1-sided hypothesis that this specificity is significantly higher than 0.55 ( $H_0$ ) will be tested using the method using the kernel method described by Bantis and Feng (141). Statistical power is > 0.90 if the true specificity  $2^{\cdot}$ : 0.79. The 1-sided 95% C.I. for sensitivity will also be reported.

**Secondary objective:** While the study will be powered for the primary objective, we shall also carry out a similar assessment of potential utility of a clinical test for SRN.

Aim 3 To construct a combined early detection biomarker panel using the above individual biomarkers (stool vimentin methylation, serum galectin-3 ligand, FIT, the Exact Sciences stool DNA panel, circulating methylated genes BCAT1 /IKZF1 , hypomethylated LINE1 from circulating cell free DNA), and describe its performance for 1) colorectal adenocarcinoma and for 2) screen relevant neoplasms.

After the primary hypothesis (Aim 2) has been finalized, the examples described in Aim 2 and are not chosen as the primary hypotheses will be tested in Aim 3 as they are all clinically relevant hypotheses. The data analyses and their statistical power for these hypotheses have been described under Aim 2 above and so are not repeated here.

In addition, new panels could be constructed using the trial data as training set but these panels will need to be validated on other independent cohorts.

#### Aim 4

To establish an archive of appropriately preserved stool, serum, plasma and DNA human biospecimens to be used by EDRN-approved investigators for future validation and biomarker discovery research.

### **8.1 Secondary Analyses**

#### **8.1.1 Secondary Analyses for Screen Relevant Neoplasias**

Similar analyses as described above will be performed for the secondary endpoint (SRN). The minimally acceptable performance used for setting null hypotheses will differ: sensitivity=0.25 for secondary specific aim 2, and sensitivity=0.45 for secondary specific aim 3.

#### **8.1.2 Secondary Analyses for projecting biomarker performance in US population**

Since our enrollment is enriched with older subjects, in Aim 1a we will also report age and gender-standardized accuracy summaries that is calculated with an inverse probability weighted method in order to adequately reflect the performance of biomarkers in the US screening population which may have a different age distribution from that of our study population. The ratio of observed proportion within each age by gender stratum versus the corresponding proportion in the US population as reported by the Census data will be used as the weights for subjects in the stratum.

Note that the weighted analyses may provide reasonable projections for biomarkers' performance in the US population, however we will treat this as a secondary analysis and therefore our power calculation is based on unweighted analyses from the observed sample.

#### **8.1.3 Additional Secondary Analyses**

The cohort will be characterized in terms of demographics and epidemiological variables such as gender, education status, intake of dietary micro and macronutrients, hormone replacement therapy, alcohol intake and smoking. We will assess the relationship of each of these factors individually to each marker under evaluation and risk of colorectal cancer in multiple regression models. For factors that modulate the relationship between biomarkers and clinical endpoints, we will further evaluate their effects on the accuracy parameters of the marker, by evaluating for example the covariate-specific ROC curves. We will also test directly covariate effects on the accuracy of markers in order to identify subpopulations for which markers are mostly effective.

#### **8.1.4 Inclusion of New Biomarkers Discovered by EDRN Investigators over the Next Two Years**

The design of this project including the collection of serum, DNA and tissue samples permit the inclusion of new EDRN-discovered biomarkers into this panel. Should EDRN investigators provide sufficient preliminary data to justify inclusion in this panel; new biomarkers will be included in the validation program using the procedures described above.

**9.0 PROJECT MANAGEMENT PLAN**

**9.1 Strategies to Ensure Completion of Milestones**

Milestones set up and regularly reviewed: Milestones are set on a quarterly schedule and managed by the principal investigator.

Conference calls: a) *Investigator calls:* All GLNE investigators communicate every other week by scheduled telephone conference call organized and chaired by the PI, Dr. Brenner (11 AM to 12 Noon, Thursdays). b) *Coordinator calls:* All GLNE research coordinators and support staff communicate once per month by scheduled telephone conference call organized and chaired by the Project Manager, Mr. Kirk Herman and the DMCC.

Data and Safety Monitoring: The University of Michigan Cancer Center Prevention Program’s Data Safety and Monitoring Committee meets monthly and reviews progress towards milestones. Accrual, endpoints, toxicity, and strategies to ensure goals are met reviewed by this committee. Minutes are forwarded to the supervising IRB (IRBMED) and to relevant regulatory agencies.

**9.2 Timeline for Completing GLNE 010**

Table 2, a Gantt diagram, outlines our milestones for the proposed CRC early detection biomarker trial.

Month		-12	-6	0	6	12	18	24	30	36	42	48-54
Pre-Study	Document Completion											
	Regulatory											
	MTA/legal											
	Supplies/ Equipment											
	Kit Assembly											
	Database preparation											
Implementation	Accrual (Number of Participants)			1500	1500	1500	1500	1500	1500	0		
	Sample Analysis (Number of samples analyzed) • Galactin-3 ligand • Vimentin methylation											
Analysis	Data Cleaning											
	Data Analysis											
	EDRN Steering Cte Present					X		X		X		X
	Publication Submission										X	X

**Table 2: GLNE 010 (Colon Biomarker Validation Trial) Milestones**

We have broken down this trial into the re-organizational phase (Months -6-0) that began on April 6, 2016 and will be completed on April 1, 2017. Accrual phase beginning April 1, 2017, will last 3.5 years with data assay and data analysis being complete in Years 5. Milestones are outlined below and add detail to the events depicted in Table 2. We estimate 250 participants/month accrual at all sites (EDRN, Alliance/NCORP, and Germany, see Table 3).

Center	Accrue/mo	PI	%Minority
Case Western	2	Cooer	40%
Columbia	2	Kastrinos	80%
Dana Farber	7	Syngal	10%
MD Anderson	8	Bresalier	20%
Minnesota	30	Allen/Church	3%
St Michaels/Toronto	15	Marcon	3%
Univ IL Chicago	1	Carroll	55%
Univ Michigan	12	Turgeon	3%
Univ North Carolina	13	Crockett	1%
Univ Washington	11	Gradv	1%
<b>Alliance/NCORP-Mavo</b>	61	<b>Marshall</b>	3%
Total USA	162		
Heidelbera, Germanv	95	H Brenner	0%
Total USA-Germany	257		

**Table 3 (Left): Prior accrual experience, GLNE USA, limited to age >=65 yrs. DKFZ Germany documented accrual, all age groups over 7 years, BliTz (personal communication, H. Brenner).**

### 9.3 Endpoint Event Justification and Milestones

#### 9.3.1 Required Endpoints

In order to successfully complete this project a minimum of 70 invasive colorectal neoplasms must be detected. To ensure we reach this endpoint, we revised the protocol to 1. Expand the trial to include Germany; 2. Limit recruitment in USA sites to participants 60 years or older undergoing first colonoscopy.

#### 9.3.2 Justification for Requiring only Invasive Colorectal Neoplasms

First, recent as well as older large validation trials have found that dysplastic adenomas as endpoints degrade biomarker detection accuracy (102, 103). For example, in the recently published Exact Sciences stool DNA panel, sensitivity for invasive colorectal neoplasms only was 92% which reduced to 84% when high grade dysplastic adenomas were included with invasive colorectal neoplasms (103). Second, a strategy which allows high grade dysplasia in adenomas as primary endpoints reduces the likelihood of successful identification of early detection biomarkers for colorectal neoplastic disease. We run the risk of losing biomarkers to failure requiring classifier performance of single markers or panels that might be useful for the detection of curable invasive colorectal neoplasms despite poorer performance for detection of high grade but non-invasive neoplasms. We increase barriers for success to the very biomarkers that the EDNRN exists to discover and validate. Third, inclusion of high grade dysplasia in adenomas with invasive colorectal neoplasms degrades the usefulness of the repository samples generated by GLNE 010.

There will be insufficient invasive colorectal neoplasm events to use for large cross sectional validation of future biomarkers. Our only other resource for invasive colorectal neoplasms is a cross sectional reference set that is not PROBE compliant.

#### 9.3.3 Strategies to Ensure Sufficient Invasive Colorectal Cancer Events

Expand GLNE to Germany: The rapid expansion of colonoscopic screening in the USA to younger age groups (ages 50-59) (Table 4) with high adherence to colonoscopic screening guidelines (61%), while reducing incidence and presumably mortality from invasive colorectal neoplasms may be reducing the numbers of screen detected invasive colorectal neoplasms in USA screening trials such as GLNE 010. In GLNE 010, the detected rate of screen relevant (“advanced”) adenomas of 13% exceeds the expected rates in recently published biomarker trials using screening populations [Lieberman, 2014 #5685; Lieberman, 2014 #5684; Imperiale, 2014 #5977; Church, 2014 #4704]. In Germany, colonoscopic screening is provided as a benefit to the population, but with much lower colonoscopic screening adherence rates (16%) (25) as opposed to 61% adherence in the USA (19). The higher invasive colorectal neoplasm case proportion of 0.7% in a large ongoing prospective screening trial (Table 4) makes a primary colorectal neoplasm endpoint feasible.

Age	Exact	PRESEPT	Blitz	GLNE
50-59	29%	35%	36%	42%
60-64	8%	27%	23%	18%
65-69	37%	21%	25%	21%
70-74	17%	11%	13%	21%*
≥75	9%	6%	4%	---
CRC Event	0.7%	0.8%	0.7%	0.2%
HGD Event	0.4%	0.7%	0.7%	0.4%

**Table 4: Enrollment Ages, Recent Large Cross Section CRC Screening Trials.**  
Exact Sciences (2014, (1)), PRESEPT (2014, (2)), Blitz  
(ongoing, Heidelberg, H. Brenner, Personal Communication.)

Enrich North America Risk to Bring Endpoint Event Percentages to the Level of Other Trials: The incidence of invasive colorectal neoplasm increases with age, particularly at ≥60 yrs. The recent published PRESEPT and Exact Sciences trial enriched their screening populations to increase invasive colorectal neoplasm event rates. PRESEPT restricted enrollment to first colonoscopic procedures. PRESEPT did not limit age of enrollment, two thirds of participants were ≥60 yrs. Exact Sciences required two thirds of enrolled participants be ≥65 yrs, but permitted prior colonoscopic screens ≥9 yrs. Blitz has no age limits or prior endoscopy limits, but the low screening adherence rate in Germany suggests that the population represents first colonoscopic screening procedures.

## 9.4 Endpoint Event Monitoring

### 9.4.1 Endpoint Monitoring

As part of weekly accrual monitoring, endpoints events (invasive cancers, high grade dysplasia, screen relevant neoplasia, and adenoma) will be reported.

### 9.4.2 Monitoring Primary Endpoint Events

The primary endpoint, invasive colorectal cancer, will be monitored weekly.

### 9.4.3 Primary Endpoint Expectations

At an expected accrual rate of 1,500 per 6 months, we expect 10 invasive cancers to be detected every 6 months. Because events do not occur at regularly spaced intervals, but rather as randomly distributed events, to identify potential problems in meeting our primary endpoint goal we need a sufficient number of evaluable accruals to determine whether the current enrollment strategy will meet the accrual goal of at least 70 invasive neoplasms.

For these reasons, we will wait until 1,000 subjects have been enrolled to assess whether our case event rates will be sufficient to meet the current primary study endpoint.

### 9.4.4 Monitoring to Ensure Sufficient Cancer Endpoints

The following procedure will be followed in collaboration with the DMCC: Review every 1,000 evaluable subjects through the course of the project with the expectation that  $7 \pm 1$  new invasive neoplasm cases will be detected for every 1,000 evaluable subjects. If  $< 7$  new invasive neoplasm cases are enrolled for every 1,000 evaluable subjects, the project will be reviewed by statistical consultants and coinvestigators and revision of study goals and design by allowing FIT positive screens.

## 9.5 Data Safety and Monitoring

### 9.5.1 Authority

The University of Michigan Prevention Research Base Data Safety and Monitoring Committee (DSMC) reviews, makes recommendations, and acts on the following:

- a. All protocols being run through the GLNE EDRN will be monitored by the DSMC
- b. Progress towards completion of the study-recruitment and retention of study participants
- c. Evaluation of interim new information
- d. Evaluation of toxicity events including reporting of adverse events, if applicable
- e. Timeliness of data
- f. Quality of data
- g. Ethical conduct of research

The DSMC is empowered with the authority to recommend a study be suspended or terminated based upon concerns in any of the above areas of review. Monitoring also considers factors external to the study, such as scientific or therapeutic developments that may have an impact on the safety of the participants or the ethics of the study.

Recommendations that emanate from monitoring activities are reviewed by the principal investigator and addressed.

### 9.5.2 Composition

The current UM Prevention Research Base Data and Safety Monitoring Committee is Chaired by one of the faculty members present at the meeting, usually the most senior member who is not a principal investigator on studies being discussed. Membership includes faculty members from Gastroenterology, Family Medicine, and Hematology/Oncology. At least 3 faculty members must be present to have quorum. If the DSMC cannot meet face-to-face, a conference call is acceptable.

### 9.5.3 Meeting Frequency

The UM Prevention Research Base DSMC meets monthly by means of regularly scheduled meetings. Prior to each meeting, the UM Prevention Research Base Clinical Research Associate distributes a standard summary report detailing accrual, biomarker modulations data, new publications or presentations relevant to the ongoing project, quality control audit information, any ethical concerns, patient-subject complaints and adverse events or serious adverse events of all prevention protocols.

### 9.5.4 Recommendations and Reporting

Recommendations for action are sent to the Principal Investigator. The Principal Investigator is responsible for reviewing and if necessary, implementing DSMC recommendations.

## 9.6 **Adverse Event Reporting**

### 9.6.1 Definition

An adverse event (AE) is any condition, which appears or worsens after the participant is enrolled in an investigational study. For this minimal risk, sample collection study, we provide a definition of what would be considered related to the study participation.

### 9.6.2 AE Information

No adverse events are expected, as there is no intervention for this study. Any adverse events related (as judged by the site PI, overall PI or DSMC) to the subject's participation (sample or data collection) in this study will be forwarded to the data coordinating center and reported to regulatory bodies per study-specific guidelines. Adverse events or serious adverse events will not be reported for subjects remaining on study between the completion of their baseline visit and going off-study as this is a minimal risk, non-interventional study. Subjects could be considered on study for over a year and have adverse events completely unrelated to study participation. Examples of related adverse events that could be reported could include problems with the blood draw (bruising, fainting), loss of confidentiality of data, or lost samples. Examples of events that would not be reported would include any complications from the colonoscopy or events related to other medical conditions like colon cancer or diabetes.

### 9.6.3 Serious Adverse Events

Some percentage of participants will have colorectal cancer identified during colonoscopy by study design, and deaths due to disease progression or serious adverse events due to cancer treatment are expected. The only procedures that are part of this study are blood, urine and stool collection, so it is unlikely that any deaths or hospitalizations will be related to the sample collection in this study. Only Serious Adverse Events that are deemed to be directly related to a study procedure (sample collection) by the DSMC will be reported to any regulatory body.

A serious adverse event is defined (by ICH Guideline E2A and Fed. Reg. 62, Oct. 7, 1997) as an event, occurring at any dose, which meets any of the following criteria:

- Ⓢ Results in death
- Ⓢ Is immediately life threatening
- Ⓢ Requires inpatient hospitalization or prolongation of existing hospitalization
- Ⓢ Results in persistent or significant disability/incapacity
- Ⓢ Is a congenital anomaly/birth defect

In addition, events that may not meet these criteria, but which the investigator finds very unusual and/or potentially serious, will be reported in the same manner.

## **10.0 DATA MANAGEMENT**

### **10.1 Registration**

Institutional collaborators will enter IRB information into the secure database, including IRB approval date, expiration date, and document versions. Subject registrations will not be allowed without IRB approval. The DMCC will assign the participant ID number (PID).

No exceptions to eligibility requirements will be permitted.

### **10.2 Timeliness**

In collaboration with the DMCC, a data expectation system will be developed. Detailed instructions are provided in the operations manual. For sample shipments, data from the shipping and receiving laboratory describing the date of sample shipping and sample receipt are captured in the database.

### **10.3 Completeness and Accuracy**

The DMCC will assure the completeness of the data by writing data entry programs that will not allow for empty fields whenever possible. The accuracy of the data will be checked by identifying appropriate parameters allowed to be entered in a given data field. Periodic reviews of the paper forms and the database data will be conducted by the lead CRA and a Site Monitor from the Coordinating Center.

### **10.4 Accuracy--Revisions and Corrections**

All corrections to paper study documents will be initialed and dated. If computer-readable data is corrected by replacement of a data set, the replaced version of the data set will be retained in an archive. The collection of these auxiliary data sets represents an audit trail of corrections to the database.

### **10.5 On Site Data Audits**

All consortium sites will be subject to periodic on-site audits. The objective of the on-site audit will be to conduct a general review of a random sample of registered subjects from the selected protocol to assess overall protocol adherence with respect to subject eligibility, appropriate procedure for informed consent, registration process, general protocol adherence, sample shipment process, follow-up and off-study process.

An On-Site Audit checklist will be developed which will contain all of the essential elements of an On-Site audit. Each of the essential elements will be reviewed and discussed with the clinical site. The Checklist will be signed by the auditors and retained at the DMCC.

In preparation for a site audit, the study statistician will select the subjects for review using a randomized selection procedure. Other cases may also be selected at the discretion of the audit team. A minimum of 10% of the subjects accrued since the last audit will be reviewed for the first year. The number of subjects to be audited for the subsequent year will be determined based on findings of the audits from prior years in order to have sufficient power to identify important issues. The on-site audit team will audit additional unannounced cases. The consortium site investigator and research coordinator will be notified of the impending audit at least 3 weeks in advance. All data and material pertinent to the subject will be reviewed including eligibility criteria, informed consent, and sample shipment logs. All informed consent documents for all subjects may be reviewed at the on-site audit. At the audit, the data from the DMCC will be compared to the original data (source documents and/or CRFs). On-site audit staff will review the documentation of IRB approvals, for each audited protocol, any amendments or adverse events, and consent forms.

Based on the findings of the audit, a follow-up schedule will be defined. A report of the audit will be written and emailed or faxed to the consortium site investigator. The site PI will have 30 days from receipt of the report to respond in writing to the DMCC directly.

The DMCC will maintain a file containing the latest version of the On-Site Audit guidelines, a listing of all consortium institutions reviewed to date, a copy of the On-Site Audit results and all correspondence for each audit conducted. These results will be reviewed by the DSMC, the DMCC, and others as needed and will be made available to the NCI.



## **10.6 Sample Tracking**

CLASS labs must be notified via e-mail of a shipment due to arrive so if samples are delayed or lost, tracking may be initiated by the sending site. Sample shipment packing lists generated by the database are included with shipments. The receiving site will evaluate the sample condition on arrival, scan the bar-coded samples into database, verify samples shipped match samples sent and store at appropriate conditions until shipment to analytical labs or repositories.

## **10.7 Confidentiality**

Subjects will be identified in the database by their unique PID only. Information that could identify subjects, such as name, address, or medical record number will be kept only by the enrolling site and will not be supplied to the DMCC or GLNE Research Base. During an on-site audit or NCI site visit, audit staff may review medical records and other information that contains PHI, but this information will not be removed from the enrolling site. Neither the DMCC nor the research base at UM will keep copies of signed informed consent documents. No information, including copies of the informed consent unless required by the institution, obtained during the study will be placed in a subject's medical record.

## **10.8 Security**

All subject files will be stored under lock and key at all times. All computer systems will be password-protected against intrusion; all network-based communications between sites of confidential information are encrypted. An on-going computer-virus-protection program is available and used, maintained, and audited on all computers and pathways into the system, including good practice policies, screening of data files, executable software, diskettes, text macros, downloads, and other concerns as they arise. The DMCC will assist in maintaining appropriate levels of network security.

## **11.0 ETHICAL & REGULATORY CONSIDERATIONS**

### **11.1 Institutional Review**

This study must be approved by an appropriate institutional review committee as defined by Federal Regulatory Guidelines (Ref. Federal Register Vol. 46, No. 17, January 27, 1981, part 56). The protocol and informed consent form for this study must be approved in writing by the appropriate Institutional Review Board (IRB). The IRB must be from an institution that has a valid Federal Wide Assurance on file with the Office for Human Research Protections, Department of Health and Human Services. The institution must comply with regulations of the Food and Drug Administration and the Department of Health and Human Services. Changes to the protocol, consent, as well as a changes to the investigator list at each site, must also be approved by the IRB and documentation of this approval provided to the Coordinating center.

Records of the Institutional Review Board review and approval of all documents pertaining to this study must be kept on file by the investigator and are subject to OHRP, FDA or NCI inspection at any time during the study. Periodic status reports must be submitted to the Institutional Review Board at least yearly, as well as notification of completion of the study and a final report within 3 months of study completion or termination. The investigator must maintain an accurate and complete record of all submissions made to the Institutional Review Board, including a list of all reports and documents submitted.

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## SUBSIDIARIES OF VOLITIONRX LIMITED

<u>Name of Subsidiary</u>	<u>State or other Jurisdiction of Incorporation or Organization</u>
Singapore Volition Pte. Limited <i>(100% subsidiary of VolitionRx Limited)</i>	Singapore
Belgian Volition SPRL <i>(99.9% subsidiary of Singapore Volition Pte. Limited)</i>	Belgium
Volition Diagnostics UK Limited <i>(100% subsidiary of Belgian Volition SPRL)</i>	United Kingdom
Volition America, Inc. <i>(100% subsidiary of Belgian Volition SPRL)</i>	Delaware
Volition Veterinary Diagnostics Development LLC. <i>(87.5% subsidiary of Belgian Volition SPRL)</i>	Texas





**CONSENT OF INDEPENDENT REGISTERED PUBLIC ACCOUNTING FIRM**

To the Audit Committee  
VolitionRx Limited

As independent registered public accountants, we hereby consent to the incorporation by reference of our report dated February 20, 2020, contained in this annual report on Form 10-K with respect to the consolidated financial statements of VolitionRx Limited, in its registration statements on Form S-3 (Registration Statement Nos. 333-195213, 333-227248, 333-227731 and 333-236335) and its registration statements on Form S-8 (Registration Statement Nos. 333-208512, 333-214118, 333-221054, 333-227565 and 333-236336).

*/s/ Sadler Gibb & Assoc.*

Sadler, Gibb & Associates, LLC  
Salt Lake City, UT  
February 20, 2020

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**CERTIFICATION OF CHIEF EXECUTIVE OFFICER  
PURSUANT TO SECTION 302 OF THE SARBANES-OXLEY ACT OF 2002**

I, Cameron Reynolds, certify that:

1. I have reviewed this annual report on Form 10-K of VolitionRx Limited;
2. Based on my knowledge, this report does not contain any untrue statement of a material fact or omit to state a material fact necessary to make the statements made, in light of the circumstances under which such statements were made, not misleading with respect to the period covered by this report;
3. Based on my knowledge, the financial statements, and other financial information included in this report, fairly present in all material respects the financial condition, results of operations and cash flows of the registrant as of, and for, the periods presented in this report;
4. The registrant's other certifying officer and I are responsible for establishing and maintaining disclosure controls and procedures (as defined in Exchange Act Rules 13a-15(e) and 15d-15(e)) and internal control over financial reporting (as defined in Exchange Act Rules 13a-15(f) and 15d-15(f)) for the registrant and have:
  - (a) Designed such disclosure controls and procedures, or caused such disclosure controls and procedures to be designed under my supervision, to ensure that material information relating to the registrant, including its consolidated subsidiaries, is made known to us by others within those entities, particularly during the period in which this report is being prepared;
  - (b) Designed such internal control over financial reporting, or caused such internal control over financial reporting to be designed under our supervision, to provide reasonable assurance regarding the reliability of financial reporting and the preparation of financial statements for external purposes in accordance with generally accepted accounting principles;
  - (c) Evaluated the effectiveness of the registrant's disclosure controls and procedures and presented in this report our conclusions about the effectiveness of the disclosure controls and procedures, as of the end of the period covered by this report based on such evaluation; and
  - (d) Disclosed in this report any change in the registrant's internal control over financial reporting that occurred during the registrant's most recent fiscal quarter (the registrant's fourth fiscal quarter in the case of an annual report) that has materially affected, or is reasonably likely to materially affect, the registrant's internal control over financial reporting; and
5. The registrant's other certifying officer and I have disclosed, based on our most recent evaluation of internal control over financial reporting, to the registrant's auditors and the audit committee of the registrant's board of directors (or persons performing the equivalent functions):
  - (a) All significant deficiencies and material weaknesses in the design or operation of internal control over financial reporting which are reasonably likely to adversely affect the registrant's ability to record, process, summarize and report financial information; and
  - (b) Any fraud, whether or not material, that involves management or other employees who have a significant role in the registrant's internal control over financial reporting.

Date: February 20,  
2020

/s/ Cameron Reynolds  
Cameron Reynolds  
President and Chief Executive Officer

**CERTIFICATION OF CHIEF FINANCIAL OFFICER  
PURSUANT TO SECTION 302 OF THE SARBANES-OXLEY ACT OF 2002**

I, David Vanston, certify that:

1. I have reviewed this annual report on Form 10-K of VolitionRx Limited;
2. Based on my knowledge, this report does not contain any untrue statement of a material fact or omit to state a material fact necessary to make the statements made, in light of the circumstances under which such statements were made, not misleading with respect to the period covered by this report;
3. Based on my knowledge, the financial statements, and other financial information included in this report, fairly present in all material respects the financial condition, results of operations and cash flows of the registrant as of, and for, the periods presented in this report;
4. The registrant's other certifying officer and I are responsible for establishing and maintaining disclosure controls and procedures (as defined in Exchange Act Rules 13a-15(e) and 15d-15(e)) and internal control over financial reporting (as defined in Exchange Act Rules 13a-15(f) and 15d-15(f)) for the registrant and have:
  - (a) Designed such disclosure controls and procedures, or caused such disclosure controls and procedures to be designed under my supervision, to ensure that material information relating to the registrant, including its consolidated subsidiaries, is made known to us by others within those entities, particularly during the period in which this report is being prepared;
  - (b) Designed such internal control over financial reporting, or caused such internal control over financial reporting to be designed under our supervision, to provide reasonable assurance regarding the reliability of financial reporting and the preparation of financial statements for external purposes in accordance with generally accepted accounting principles;
  - (c) Evaluated the effectiveness of the registrant's disclosure controls and procedures and presented in this report our conclusions about the effectiveness of the disclosure controls and procedures, as of the end of the period covered by this report based on such evaluation; and
  - (d) Disclosed in this report any change in the registrant's internal control over financial reporting that occurred during the registrant's most recent fiscal quarter (the registrant's fourth fiscal quarter in the case of an annual report) that has materially affected, or is reasonably likely to materially affect, the registrant's internal control over financial reporting; and
5. The registrant's other certifying officer and I have disclosed, based on our most recent evaluation of internal control over financial reporting, to the registrant's auditors and the audit committee of the registrant's board of directors (or persons performing the equivalent functions):
  - (a) All significant deficiencies and material weaknesses in the design or operation of internal control over financial reporting which are reasonably likely to adversely affect the registrant's ability to record, process, summarize and report financial information; and
  - (b) Any fraud, whether or not material, that involves management or other employees who have a significant role in the registrant's internal control over financial reporting.

Date: February 20,  
2020

/s/ David Vanston  
David Vanston  
Chief Financial Officer and Treasurer

**CERTIFICATIONS OF CHIEF EXECUTIVE OFFICER AND CHIEF FINANCIAL OFFICER  
PURSUANT TO 18 U.S.C. SECTION 1350,  
AS ADOPTED PURSUANT TO SECTION 906 OF THE SARBANES-OXLEY ACT OF 2002**

The following certifications are hereby made in connection with the Annual Report on Form 10-K of VolitionRx Limited (the "Company") for the period ended December 31, 2019, as filed with the Securities and Exchange Commission on the date hereof (the "Report"):

I, Cameron Reynolds, President and Chief Executive Officer of the Company, hereby certify, pursuant to 18 U.S.C. Section 1350, as adopted pursuant to Section 906 of the Sarbanes-Oxley Act of 2002, that, to my knowledge, (i) the Report fully complies with the requirements of Section 13(a) or 15(d) of the Securities Exchange Act of 1934, as amended, and (ii) the information contained in the Report fairly presents, in all material respects, the financial condition and results of operations of the Company as of the dates and for the periods presented.

Date: February 20,  
2020

By: /s/ Cameron Reynolds  
Cameron Reynolds  
President and Chief Executive Officer

I, David Vanston, Chief Financial Officer of the Company, hereby certify, pursuant to 18 U.S.C. Section 1350, as adopted pursuant to Section 906 of the Sarbanes-Oxley Act of 2002, that, to my knowledge, (i) the Report fully complies with the requirements of Section 13(a) or 15(d) of the Securities Exchange Act of 1934, as amended, and (ii) the information contained in the Report fairly presents, in all material respects, the financial condition and results of operations of the Company as of the dates and for the periods presented.

Date: February 20,  
2020

By: /s/ David Vanston  
David Vanston  
Chief Financial Officer and Treasurer