FLOWING WITH THE TRANSLATIONAL TREND:
HOW FLOW CYTOMETRY CATAPULTS CLINICAL RESEARCH

THE PRINCIPLE OF MODERN FLOW CYTOMETRY

Cytometry is the process of measuring the properties of individual cells. These properties may include gene or protein expression, chemical properties, deoxyribonucleic acid (DNA) content, and various cellular functions. The earliest methods of cytometry relied upon light microscopy for the classification and observation of cells for the first time, leading to the classification of cells by morphology and insight into cellular functions. However, the time required for microscopic analysis constrains the number of samples or number of cells in each sample that can be examined. Therefore, the utility of microscopy for analysis of rare cells is limited by hardware, logistical, spatial, temporal and human factors. Among all these limitations, not being able to assess the protein expression and activation (e.g. phosphorylation) on live cells represent the main drawback of microscopy.

Fortunately, flow cytometry addresses these limitations.

APPLICATIONS FOR FLOW CYTOMETRY IN CLINICAL TRIALS

- Immunogenicity assays for peptide or protein-based therapeutics
- Identification of reactive antibodies predictive of graft rejection
- Drug-screening for a novel therapy with unmet medical need
- CAR-T cell PK/PD efficacy
- Monitoring malignancies and mosaicism in circulation due to genetics or transplantation
- Real-time quantification of minimal residue disease
- Transforming the management of cancer by characterizing the tumor microenvironment
- Elucidating mechanisms-of-action for therapeutics that modulate cellular function

Flow cytometry depends upon the ability to pass single cells (in most the cases – live cells) one by one in a single cell suspension through the optical path of multiple laser beams surrounded by sheath fluid. Flowing cells through the path of the laser permits the analysis of tens of thousands of cells per second. As a prerequisite, flow cytometry requires that the cells from the samples are in a single-cell, liquid suspension. Therefore, solid tissue samples (e.g. tumor tissues, biopsies) require dissociation through mechanical and/or enzymatic processing before analysis, a key rate-limiting process called “single cell suspension” generation.

A wide variety of information about the cell can be determined depending upon how the cell interacts with the light from the laser. Detectors that measure the way the cell scatters light can provide information about the size of the cell as well as its cellular complexity due to presence of intracellular granules and irregularities in the shape of the cell membrane. Based on light scatter properties alone, three distinct populations of leukocytes can be resolved in the peripheral blood: lymphocytes, monocytes and granulocytes.

Clinical applications for flow cytometry began in the mid-1980s with the monitoring of CD4+ lymphocytes in patients with HIV. Since then the FDA has granted IVDapproval for flow cytometric assays that enumerate T cells/B cells/NK cells as a single panel, enumerate CD34+ stem cells, identify of HLA-B27 expression, enumerate reticulocytes, and identify deficiency in glycophosphatidylinositol-linked proteins for the diagnosis of paroxysmal nocturnal hemoglobinuria (PNH).

Over the last three decades, flow cytometry has become well-established as a research tool due to the wide range of cellular parameters that can be measured with fluorescent reagents and the ability to measure these parameters simultaneously. In certain disciplines in which cellular phenotyping is necessary, the utility of flow cytometry is indispensable, as no other currently available techniques are able to achieve the robust and ultra-fast polychromatic multi-dimensional capability coupled with the relatively fast turnaround time.

THE MATCH OF ANTIBODY & FLUOROPHORE

The power and comprehensive utility of modern flow cytometry fundamentally rely on laser-excitable fluorescent compounds (termed "fluorophores") that are chemically conjugated to specific proteins of interest. The specific antigenicity determinant of a given protein recognizable by an antibody is called the "epitope". Likewise, in the case of a therapeutic humanized antibody (biologics), its antigenic determinant is called an "idiotope". Accordingly, the antibody recognizing the biologic antibody (for tracking and PK purpose) is termed "anti-idiotype (anti-ID) antibody".

Monoclonal antibodies which bind to cellular proteins can be conjugated to a large variety of fluorescent molecules (hence termed "fluorophores") and can be used to detect expression of specific proteins present on the cell surface or in the cytosols. Conceivably, this labelling system can be used to identify and characterize specific cell types defined by the expression profiles of a group of proteins. As needed, the fluorescent antibody can even penetrate deeply into the nuclear membrane and quantify the nuclear protein expression (e.g. FOXP3 for regulatory T cells), a process actively controlled by a process called "permeabilization" following prior fixation.

In the common cases of immune cells or cells of hematopoietic origin, e.g. pan WBC, lymphocytes etc., this process is frequently referred to as "immunotyping". Once a population of interest is identified through protein expression, flow cytometry can then provide both relative (percentage) and absolute counts (enumeration) with accuracy and precision. Or it can be coupled with other fluorescent reagents to characterize protein expression (e.g. intracellular cytokines and chemokines), function (e.g. receptor phosphorylation), or chemical properties of the cells (e.g. pH, calcium influx) at the individual level.

AT A GLANCE: MEDPACE'S FLOW CYTOMETRY LABS

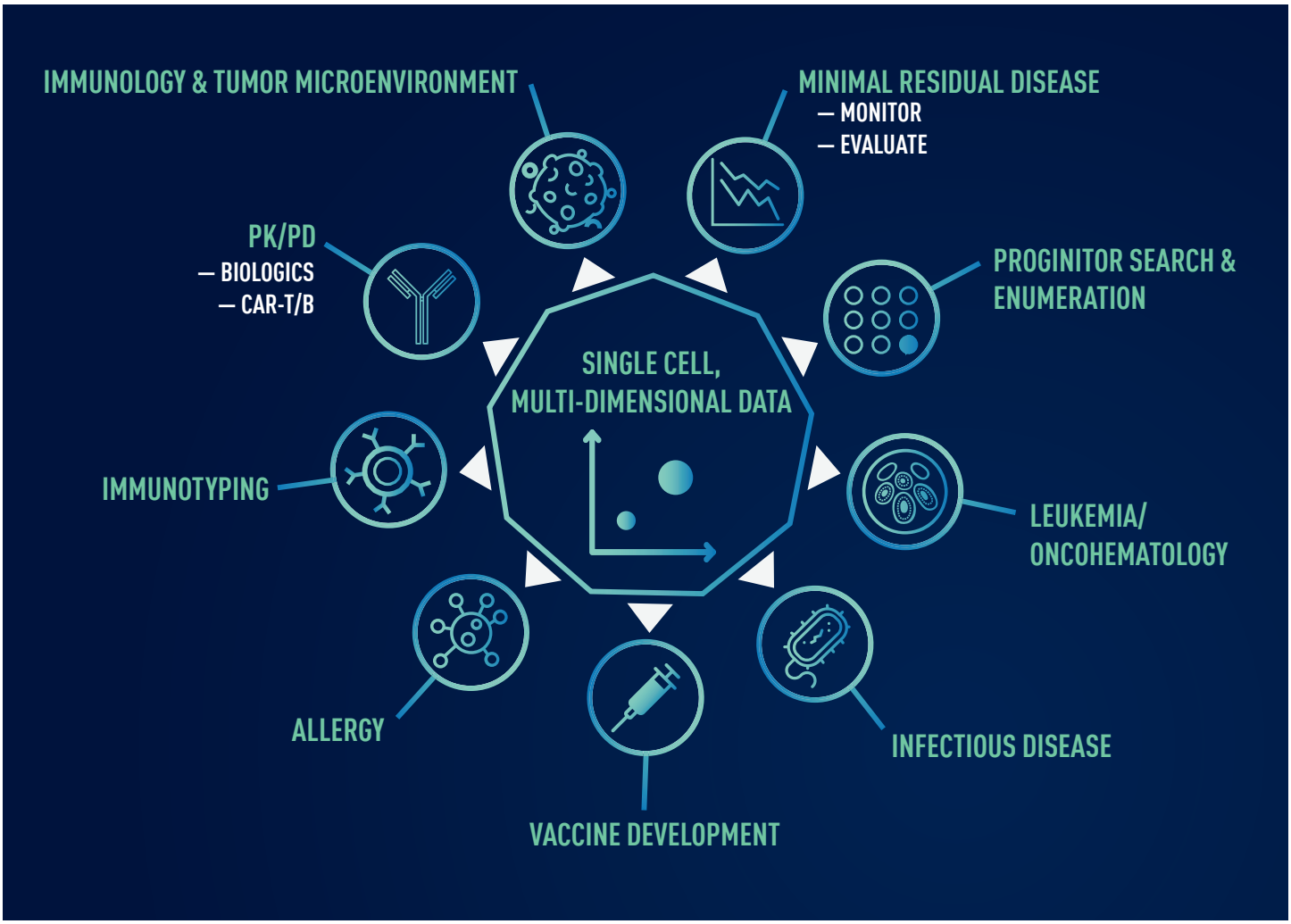
- Medpace supports global flow cytometry services (US, Europe, and Asia) in wholly owned, purpose-built central laboratories
- All labs follow global operating procedures, and utilize a single laboratory information system to ensure harmonization of global data
- PhD/MD flow scientists with extensive experience designing, analyzing, and interpreting multicolor flow cytometry assays
- Support custom panels and methods transferred to our lab
- Broad assay support including:
  - Immune cell phenotyping and Immune cell function assays
  - Immune cell enumeration
  - Intracellular cytokine assays
  - Receptor occupancy assays
  - CAR T-cell assays
  - TBNK assay
  - Epigenetic profiling assays
  - Stem cell enumeration
- Ability to measure two light scatter parameters (with three sub parameters each) and up to 10 fluorescent parameters for each cell at rate of more than 10,000 cells per second
- Medpace utilizes the BD FACSCanto or BD FACSLyric Systems (the only BD analyzers successfully cleared by the Food and Drug Administration (FDA) 510(k) for in vitro diagnostic (IVD) use offering the precision and confidence needed for clinical assay in a CAP accredited lab)
- "Same-day" diagnosis with expedient 24-hour turnaround time compared to traditional methods for some assays (e.g. TBNK panel)
- Readily address expression of more than 10 proteins of interest simultaneously at translation/protein levels
- Subsequent analysis is available to substantiate the biological/cellular mechanism, including Boolean gate analysis, expression overlapping, and developmental tree analyses
- Single cell analysis if needed, specifically tailored for the sponsor's request

LEARN MORE

Although flow cytometry is typically paired with fluorescent conjugated antibodies, especially in immunology, this pairing does not represent the full capacities of modern flow cytometry. Fluorescent dyes that are activated or quenched based on pH or redox status can penetrate the cell and provide real-time information regarding the chemical properties within individual cells. Fluorescent dyes that bind DNA can be used to determine the amount of DNA present in the cell, indicating the cell cycle phase and proliferation status. Positive ions (extracellular or intracellularly stored) are known to orchestrate a myriad of cellular response and functions, such as T cell cytokine release. In this sense, calcium sensitive fluorescent dyes can be used to identify cellular signaling events that result in calcium flux, a method readily extended to other metallic ions critical for proper cellular function, such as magnesium. Of note, in some trials where the therapeutic target is present in a solid organ hard to access, intracellular chemical properties from circulating cells (such as T, B cells) may be used as a surrogate biomarker to assess and monitor similar reactions occurring in other organ systems, underlining the practical utility of flow cytometry.

A ROBUST & IRREPLACEABLE METHOD WITH MANY CLINICAL UTILITIES

The flexibility of flow cytometry lends itself to a wide variety of therapeutic areas include hematology, oncology, allergy and immunology, infectious diseases, host-vs-graft disorders (GVHDs), autoimmunity, regenerative medicine, and even urgent unmet pulmonary condition of acute respiration distress syndrome (ARDS) directly contributing to the lethality of the COVID-19 pandemic.



Cancer-Associated Immunology & Tumor Microenvironment

While there exist multiple lines of therapies of varying clinical efficacy, the remission of tumor is best solved by our own immune system. Therefore, defining and profiling the capacity and strength of our own, or exogenously introduced (e.g. CAR-T/B therapy, humanized antibody/biologics) immune system is the key for anti-cancer therapies.

Flow cytometry is particularly useful in clinical trials in the area of oncology and tumor-associate immunology. A variety of primary and secondary endpoints can be measured using flow cytometry. Monitoring of leukemia and lymphoma in the peripheral blood is less invasive than sampling bone marrow and can provide longitudinal assessment of treatment effects on disease burden.

There are several lines of recent evidences showing that tumor microenvironment is paramount in regulating tumor tissue propagation, survival and metastasis. This is a complicated topic typically involving infiltrating/surrounding immune cells, extracellular matrix properties, cytokine milieu, and microbiota. With proper tissue digestion technique, flow cytometry represents the best tool to study this intriguing question at cellular levels, when combined with molecular techniques, such as RNA sequencing and tumor variant load. The combined platform may provide insight on key pathognesis for a particular type of solid tumor in a study. This platform also offers the opportunities to further interpret the possible difference in constitutional vs. somatic genetic information/variant/mutation, aiming to explain the genetic difference at cellular levels.

CAR-T/B Cells

CAR-T/B cell therapy is a popular cutting-edge technology shown to be 80% effective for treating tumors. CAR-T therapy has become indispensable in some areas of oncology such as leukemia management. Monitoring the CAR-T cells in the blood and different target organs can virtually only be performed by flow cytometry with the corresponding fluorescent conjugated anti-idiotype antibodies (a unique and efficient PK study). As to the effectiveness of the CAR-T therapy (PD), flow cytometry can also assess the target cell absolute count, immunocytes' activation marker, cytokine production within the CAR-T and endogenous T cells, and even the proliferation status of therapeutic T cells and target cells. Therefore, flow cytometry can be a useful tool to evaluate and monitor the clinical efficacy of CAR-T/B therapy.

Minimal Residual Disease

Since modern flow cytometry is super sensitive and highly proficient at rare event detection and enumeration, minimal residual disease can also be identified by flow cytometry particularly when it comes to monitoring leukemia and lymphoma. In terms of prognosis, flow cytometry data is an important predictor of relapse after treatment in some clinical scenarios. Molecular technique can also provide insights, but it is usually subjected to PCR amplification introducing potential bias. Flow cytometry can directly and rapidly label disease associated markers, surface or intracellular, at protein levels, and the findings can be collaboratively analyzed in the context of other pertinent surface markers.

Leukemia/Oncohematology

For other therapeutic strategies, particularly regarding the blood tumor leukemia, it may be necessary to ascertain the effectiveness of CD34+ stem cell mobilization by enumerating circulating CD34+ stem cells in peripheral blood for subsequent apheresis and autologous transplantation after chemotherapy or radiation therapy. The same story also holds true for CD117+ stem cells. Flow cytometry can identify the expression of specific biomarkers, such as ZAP-70, CD138 or CD38, which may predict response to anti-leukemia treatment. There are already multiple lines of ALL/CLL blast-evaluation panels clinically available and have been successfully applied to evaluate the progress/prognosis/treatment efficacy of leukemia. Therefore, flow cytometry can provide valuable information about how the treatment affects the function of individual cells and identify subsets of patients that respond to treatment.

Malignant Progenitor Search & Enumeration

When positioned in an exploratory arm, flow cytometry can also aid in identifying new malignant progenitor markers, surface or intracellular, that are specifically linked to the pathogenesis for any given morbidity of investigation. Rates of immune cell reconstitution after chemotherapy, radiation therapy, and bone marrow transplantation may be readily measured by flow cytometry.

Circulating tumor cells and cancer stem cells may be evidence of invasive carcinoma and metastatic potential. Flow cytometry can be used to monitor the level of circulating tumor cells when therapeutic interventions aim to reduce metastasis, since modern flow cytometry is capable of detecting rare cellular events as low as 10-100 out of 1 million cells depending on the low linearity of the test. Taken together, flow cytometry can provide key measurements of critical endpoints in trials of oncology therapies.

Cardiovascular & Pulmonary Disorders

Cardiovascular diseases greatly impact global health with increasing mortality and prevalence. Flow cytometry can contribute to our better understanding of this disease category. Recent research indicates that immune cell phenotyping capacity of flow cytometry can be leveraged to assess cardiovascular disease risk and monitor disease progression, an unmet need in cardiovascular field. A flow cytometry panel could be designed and validated per a sponsor's need to precisely quantify the differences in granulocytes, monocytes, and lymphocytes in the context of an increased risk for cardiovascular diseases. There is a number of published studies indicating the promising utility in predicative medicine. Furthermore, the macrophage subtyping classification (mainly M1/M2 tilt) and platelet-mediated coagulation are key factors determining cardiovascular diseases' pathogenesis and clinical outcome. Accordingly, sub-populations of macrophage subtype balance and platelet aggregation can be readily examined by flow cytometry with several panel prototypes already available at Medpace, which may serve as an innovative and effective tool to evaluate the development and progression of cardiovascular disease.

Flow cytometry also has broad application investigating the mechanisms for pulmonary diseases. Considering the COVID-19 pandemic, quantifiable phosphorylation status of a specific drug receptor may alter pulmonary capillary permeability and cytokine storm during a lethal lung condition termed acute respiration distress syndrome (ARDS). ARDS was shown to be the main cause for mortality observed in COVID-19, SARS, combat/ER trauma, and COPD. Herein, flow cytometry not only optimizes the dose transitioning from phase I to phase II, but also readily assesses the circulating biomarkers that are reflecting what is going on in the targeted organs, such as lung and brain, where a biopsy would not be practically available.

Key player in Pharmacokinetics (PK) & Pharmacodynamics (PD) studies

PK and PD typically represent the first step in new drug studies/clinical trials, as they are critical in evaluating the safety and efficacy of a new therapeutic. Flow cytometry can facilitate studies in each arm.

For PK, regarding what "the body does to the drug", flow cytometry typically represents a unique tool to track cellular drugs infused into the circulation, (e.g. a CAR-T/B cell present in the subject's blood.) Conventional PK methods (such as HPLC-MS/MS) are not the ideal way to track a live therapeutic cell in the patient body. Moreover, these engineered T cells are expected to engraft and clonally proliferate. In this sense, flow cytometry is a useful tool to assess their preferred anatomical niche and quantify surface markers associated with these therapeutic parameters.

Flow cytometry can also measure pharmacodynamics endpoints and biomarkers that might be predictive of therapeutic success in oncology trials. Based on the ability of flow cytometry to measure a variety of cellular processes, the effects of treatment on the cells of interest can be specifically and precisely defined at a single cell level. Depending on the mechanism-of-action, flow cytometry can monitor treatment effects on the viability of leukemic blasts, cell cycle status, and intracellular signaling events. In some refractory leukemia situations, when the targeted B cell downregulate their target marker, (e.g. CD19, during a process called "B cell escape" in ALL/CLL) a simple flow cytometry panel could readily detect this phenomenon and suggest an alternative management plan. Many side effects of highly effective CAR-T therapy result from cytokine storms from these

T cells. An intracellular T cell cytokine panel would help track these CAR-T cells (typically labeled and traced with an anti-idiotype antibody) to know the CAR-T's Th cytokine producing potentials.

Immunophenotyping

Flow cytometry has numerous applications in the monitoring of immune system status, cell-based classification and function. There are at least 300+ classical "CD markers" and 50+ cytokine/interleukins with commercial conjugated antibodies readily available for a surface or intracellular staining panel development. These applications can provide important measurements in clinical trials for therapies of autoimmune disease, infectious disease, and allergy. As an example, using T cells in Phase I and II trials, investigators would want to gain multi-dimensional information regarding surface activation marker expression, exhaustion marker expression, intracellular cytokines, proliferation markers, and many other molecules of interest per specific study. Immunotyping also means different immunocytes can be addressed/analyzed simultaneously with multiple dimension data obtained per cell population. A well-designed polychromatic flow panel (10-15 colors) is highly capable of addressing surface/intracellular expression of these markers of interest in more than 6 populations (e.g. Tc, Th, Treg, B, DC, macrophage, neutrophils etc.). Combined with advanced single cell analysis (e.g. by Flowjo, FACSDiva etc.) by qualified scientists, this vast amount of data would be valuable to guide studies focusing on drug mechanism, circulating markers, phase II dose probing, receptor occupancy, etc. Secondary hypothesis could be readily generated from these multi-dimensional data.

Acquired Immunodeficiency Syndrome (AIDS)

Immunotoxicological effects can also be ascertained by monitoring the numbers of specific leukocyte populations following a treatment regimen. HIV progression can lead to immunodeficiency due to an insufficiency in the population of CD4+ T lymphocytes. Flow cytometry can monitor the progression of disease and the efficacy of treatment by providing absolute counts of the number of CD4+ lymphocytes in circulation, a task routinely performed by a 6-color panel called "TBNK" to enumerate the major component of adaptive immunity. Coupled with the technique of HIV-specific tetramers, flow cytometry is also proficient in enumerating HIV-specific T cell clones. This flow capacity is largely irreplaceable and can often times be extended into other areas where certain antigen-reactive T cell clones need to be detected at cellular levels. Molecular technique such as NGS can infer this information from sequence-based analysis but it is not at cellular level and hence is not associated with other surface markers for a co-analysis.

Vaccines Development

In vaccination studies, the efficiency of the vaccination to induce a cell-mediated response can be measured by identifying effector and memory T cell subsets based on cell surface markers. To directly assess the antigen-specific T cells and B cells, a tetramer staining can identify individual T cells that specifically recognize the immunogen and further characterize their expression of pertinent inflammatory mediators. This test needs to be carried out in HLA context, which is also an expertise of modern flow cytometry.

Allergies and Hypersensitivity

With the same tool, allergists now use flow cytometry to identify and characterize the exact clones of T cells that are specifically reactive to the allergen (e.g. pollen) via epitope-TCR interaction, which can be assessed by MHC tetramers by flow cytometry with relative ease and certainty (as compared to TCR-CDR3 based molecular/bioinformatic methods). Activation or lineage markers on specific immune cell subsets can be used as biomarkers to track the effectiveness of therapeutic interventions in allergy and autoimmune disorders that aim to limit immune responses (e.g. the quantification and immunotyping of the inhibitory T regulatory cells.) In some types of hypersensitivity, such as bee venom allergy, a flow-based basophil activation test (BAT) is employed to assess the basophil's responsiveness to a suspected allergen. This is critical in preventing a more lethal condition such as anaphylaxis.

Collectively, flow cytometry is a powerful tool to provide cellular insights into various ways that therapeutic intervention can modulate immune function aiming to obtain an innovative treatment strategy.

FLOWING INTO THE FUTURE WITH MEDPACE

Medpace has extensive experience in the planning and execution of global clinical trials across a variety of therapeutic disciplines including oncology, cardiovascular and metabolic disease, infectious disease, neurology, and regenerative medicine.

Medpace's CAP accredited central laboratories have flow cytometry capabilities in the United States (CLIA-certified). Belgium, and Singapore all working together to support global clinical trials. We routinely participate in CAP Proficiency Tests (PT) survey, to maintain our CAP accreditation and ensure our accuracy and precision comparing to our peer laboratories.

The flow cytometry leadership team represents a state-of-the-art scientific cabinet. All holding PhD degrees, the team of flow cytometry scientists at Medpace have decades of experience in both clinical and research laboratory testing in support of clinical trials and translational science. Each member of the team possesses their own unique expertise obtained during their distinct career trajectories. They are the intellectual leaders of the flow cytometry division and can provide insightful and detailed guidance in the best application of flow cytometry for a study initiated by the sponsor, from flow panel design to validation parameter/matrix suggestions.

The flow cytometry field is enriched with many exciting technical advancements and complex clinical trial designs that are critical to improve human health. With robust capabilities, a well-trained technical team/scientific cabinet, and knowledge/experience obtained over years of research, Medpace has the expertise, experience and global resources to optimize the use of flow cytometry in support of your studies.

FULL-SERVICE CLINICAL DEVELOPMENT

Medpace is a scientifically-driven, global, full-service clinical contract research organization (CRO) providing Phase I-IV clinical development services to the biotechnology, pharmaceutical and medical device industries. Medpace's mission is to accelerate the global development of safe and effective medical therapeutics through its high-science and disciplined operating approach that leverages local regulatory and deep therapeutic expertise across all major areas including oncology, cardiology, metabolic disease, endocrinology, central nervous system and anti-viral and anti-infective.