**Understanding gene functions and disease mechanisms: Phenotyping pipelines in the German Mouse Clinic**

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**Abstract**

Since decades, model organisms have provided an important approach for understanding the mechanistic basis of human diseases. The German Mouse Clinic (GMC) was the first phenotyping facility that established a collaboration-based platform for phenotype characterization of mouse lines. In order to address individual projects by a tailor-made phenotyping strategy, the GMC advanced in developing a series of pipelines with tests for the analysis of specific disease areas. For a general broad analysis, there is a screening pipeline that covers the key parameters for the most relevant disease areas. For hypothesis-driven phenotypic analyses, there are thirteen additional pipelines with focus on neurological and behavioral disorders, metabolic dysfunction, respiratory system malfunctions, immune-system disorders and imaging techniques. In this article, we give an overview of the pipelines and describe the scientific rationale behind the different test combinations.

Keywords: mouse phenotyping, phenotyping pipeline, mouse model, gene function analysis

**1. Introduction**

For the use of mutant mice as models for human diseases and the understanding of mammalian gene function, the phenotypic characterization of mouse lines is an important prerequisite. The German Mouse Clinic (GMC) established a collaboration-based platform for phenotype characterization of mouse lines. In the GMC, experts from various fields of mouse genetics and physiology work in close collaboration with clinicians and cover the areas of allergy, behavior, cardiology, clinical chemistry, dysmorphology, bone and cartilage, diabetes and energy metabolism, steroids, eye and vision, immunology, lung function, gene expression profiling, neurology, nociception and pathology. The GMC has expertise and experience in mouse phenotyping for more than 15 years [1-5], and as a member of international consortia like EUMORPHIA or EUMODIC [6-9], the GMC contributed to the standardization of mouse phenotyping technologies and advancements in the use of mice for functional gene analysis and modelling of human diseases. As a member of the International Mouse Phenotyping Consortium (IMPC, [www.mousephenotype.org](http://www.mousephenotype.org), [10-12]), a consortium currently composed of 17 research institutions and 5 national funders that is recognized by the G7 as a global infrastructure, the German Mouse Clinic contributes to the endeavor to generate an encyclopedia of mammalian gene function by the generation and phenotypic characterization of 20,000 knockout mouse strains on a single background strain. In addition to activities in the IMPC, the GMC is also open for scientific collaborations. The special logistics that have been developed to import live mice from collaborating institutions worldwide are a unique feature of the GMC. The standard phenotyping procedure for mouse lines without or with incomplete knowledge of phenotypic alterations in the mouse line is a systemic screening of phenotypes that covers essentially all organs. In many of cases, the results of this initial screening pipeline are of immediate significance and high importance for the scientific community, and data can be published right on. In other cases, the screening results create new working hypotheses that provide a basis for in-depth mechanistic studies. For this purpose, the GMC developed specialized pipelines that enable further hypothesis-driven characterization of mouse lines. These hypothesis-driven pipelines cover the following disease areas:

* Emotionality
* Learning and memory
* Motoric deficits
* Sensory system
* General neuro-behavioral assessment
* Renal function
* Glucose metabolism
* Energy metabolism
* Imaging technologies
* Immunity
* Allergy
* Lung function
* Progression analysis

A further option for specialized, hypothesis-driven phenotyping is the possibility to run the analysis under challenge conditions [13]. This may apply to cases, in which phenotypic alterations are expected to occur only under environmental challenges (e.g. specific diets or physical activity) or after treatment with novel compounds or combinations of drugs. For mutant lines or projects with specific working hypothesis, one of the above-mentioned specialized pipelines may serve as starting point for in-depth phenotypic characterization.

The design of the phenotyping pipelines is based on the experience of more than 15 years mouse phenotyping, and implies considerations about capacity, logistics and legal issues as well as the principle of the 3Rs (reduction, replacement, refinement). The GMC obtained a license from the local authorities to run projects using all the experiments mentioned in this article. Within this framework, there is also flexibility for project-specific adaptations to a limited extent. The phenotyping strategy of the GMC is summarized in Figure 1. In the following part, the different phenotyping pipelines of the German Mouse Clinic are presented and described in more detail.

**2. Screening pipeline**

The screening pipeline is the standard phenotyping procedure for mouse lines without or with incomplete knowledge of phenotypic alterations. In the screening pipeline, disease relevant key parameters for all research areas in the GMC are assessed in a specially designed pipeline of tests. The screening pipeline evolved during the last 15 years of phenotyping activities in the GMC, and underwent permanent cycles of improvements (e.g. by discussions with our partners within international consortia), where new technologies were implemented and old-fashioned methods or tests with minor hit rates were omitted. The present version of the screening pipeline is very similar to the adult-phenotyping pipeline of the IMPC, but covers a few more methodologies that represent the scientific focus of the GMC-scientists. For projects on aging research (also in the framework of IMPC), another variant of the screening pipeline has been recently defined but does not constitute the focus of this article.

The GMC standard screening pipeline (Table 1) starts at the age of 8 weeks, and comprises tests for behavior (open field, acoustic startle and its pre-pulse inhibition) and neurology (SHIRPA-protocol, grip strength, rotarod and later on in the pipeline hotplate test and a test for hearing ability via auditory brainstem response). Analysis of morphology and bone health is represented by a dysmorphology protocol, DEXA measurement and X-ray analysis. Energy metabolism, substrate utilization and home cage activity are assessed via indirect calorimetry, body composition analysis (by qNMR, Bruker MiniSpec), body surface temperature measurement, glucose tolerance test and a fasted blood analysis (cholesterol, HDL-cholesterol, triglyceride, NEFA, glycerol, glucose). In another blood sample (at the end of the pipeline), clinical chemistry and hematology as well as immunological analysis and allergy parameters are examined. The allergy screen is completed with the analysis of trans-epidermal water loss (TEWL). In addition, cardiac function (ECG and echocardiography) is analyzed and a complete picture of eye-morphology and eye function is examined (by Scheimpflug analysis for cornea and lens transparency, laser interference biometry to assess the eye size, optic coherence tomography for retinal morphology, and an analysis of visual acuity via the virtual drum). Expression analysis is carried out for one selected organ. The primary workflow is completed with a comprehensive pathological analysis of the mice. This screen at the end of the primary phenotyping pipeline performs a standardized study of the morphological changes in the body as a whole and in the visceral organs. Each of the 28 organs is scrutinized for histological alterations in the tissues and in cell structures and considered for the identification of aspects of etiology and pathogenesis taking into account common strain-, age-, or sex-dependent background findings. A comprehensive evaluation of different organ systems (Figure 2) is performed and supported by standard and (when required) special tissue stainings, morphometry and immunohistochemistry, and measurement of absolute and normalized organ weights. The whole-mouse approach permits not only the detection of alterations in anticipated target tissues and organs (based on the genetic manipulation), but uncovers deviant morphological phenotypes suggesting new gene-related functions that were *a priori* not expected. For instance, the phenotypic characterization of a mouse model for juvenile neuronal ceroid lipofuscinosis (JNCL) revealed an early onset of sensorimotor processing abnormalities that long preceded neuronal cell loss and alterations in hematopoiesis and epididymal biology (vacuolated structures) [14]. Therefore, the pathologic analysis plays an important role in contextualizing data from neurological and behavioral *in vivo* tests in mice by enabling the discovery of morphological changes in terms of structure of the central nervous system (CNS) and other organ systems.

**3. Emotionality**

When existing data, for example, from human studies or primary mouse phenotyping analyses, indicate a possible relationship of a gene to human neuropsychiatric disease or alterations in specific brain areas, we apply the emotionality pipeline (see Figure 3a) to detect relevant CNS dysfunction. Aspects of diseases such as post-traumatic stress disorder (PTSD), other anxiety disorders, depression, schizophrenia, autism and attention-deficit hyperactivity disorder (ADHD) can be assessed with these tests in the GMC.

Locomotor and exploratory activity, as well as anxiety-related behavior, is analyzed using the automated open field test. This plexiglas arena is traversed by criss-crossed infrared light beams. The light beam breaks caused by the movement of the animal are transduced by software into measures of horizontal and vertical activity. The amount of time spent in the center of the open arena is used as an index of anxiety. To further characterize a possible anxiety-related phenotype, the open field test may be complemented by the light–dark box test, the elevated plus-maze and the social interaction test. Each of these tests exploits different ethologically-relevant anxiety-related vulnerabilities of the mouse from brightly lit open spaces (light-dark box), exposed elevated platforms (elevated plus maze) to social phobia (social interaction test; all protocols described in [15]). Ideally, when an anxiety phenotype is hypothesized, a cascade of these corroborative tests should be applied to gain a more rounded appraisal of the effect and establish an anxiety phenotype that would be perhaps only detectable under comparatively more aversive circumstances. To evaluate depression-related behavior, the tail suspension test can be employed. It exposes the mice to a stressful situation from which escape attempts are futile. The degree of behavioral despair (time spent immobile) is then quantified and interpreted to reflect depression-related behavior. As an endo-phenotype associated with schizophrenia, sensorimotor recruitment and sensorimotor gating can be assessed in mice that have intact hearing ability using the acoustic startle reflex and its pre-pulse inhibition. Pre-pulse inhibition is a measure of how a weak pre-stimulus can mitigate the response to a subsequently stronger stimulus and is an index of pre-attentive sensory processing. When there is a clear hypothesis supporting a genotype effect on stress responsivity and hypothalamic-pituitary-adrenal axis activity, we apply our acute restraint stress protocol [16].

These tests can be confounded, to varying degrees, by impaired vision, olfactory and motor ability, alterations in metabolism and so on. Thus, for accurate assessment of an emotion-related phenotype, abnormalities in other systems should be excluded and/or considered in parallel. Ideally, given the sensitivity of these tests to prior handling of the animals, behavioral phenotyping should be carried out before more invasive manipulations are performed. Furthermore, in case of a positive phenotyping result, the outcome of the analysis should be confirmed, for example with complementing tests.

**4. Learning and memory**

The neurocognitive tests, and related analyses, are applied when existing data or hypotheses indicate a possible relationship of a gene to impaired cognitive function and/or alterations in relevant brain areas e.g. hippocampus (see pipeline learning and memory, Figure 3a). This can be seen in neurodegenerative diseases such as Parkinson’s disease, Alzheimer’s disease, Huntington’s disease, stroke or prion diseases but also in neuropsychiatric diseases as well as during the aging process.

The Y-maze spontaneous alternation task is used to assess working memory and is a test that exploits a rodent’s natural tendency to investigate novel environments. A mouse with intact working memory will tend to explore the least recently visited arm of this three-armed maze producing a pattern of spontaneous alternation. A reduction in the number of spontaneous alternations translates as impaired working memory. The social recognition task is applied to analyze social memory and olfactory function, and the object recognition task is used to assess object memory (all protocols detailed in [17] ). Social recognition relies on the ability of the mouse to distinguish between a novel conspecific versus one previously encountered. Using a similar premise, the object recognition task relies on the ability of the mouse to distinguish between a novel object versus an object previously encountered. We use the Intellicage™ system to assess place and reversal learning. This large home-cage system permits continuous assessment of these cognitive functions over several weeks using four software-controlled identical operant conditioning units placed in the corners of the cage and transponders that are implanted into each animal. In this context, place learning means that the mice can freely enter all four corners, but must learn that they can only gain access to water in one of them. With a simultaneous smell discrimination olfaction task, we can also detect graded differences in the olfactory abilities of mice. This test uses the ability of the mouse to associate an odor with an appetitive stimulus and subsequently, by altering the amount of the odor in the presence of another odor, measure the olfactory discrimination ability. The results of the overall neurocognitive analysis can often yield a hypothesis about the brain areas or neuronal circuits involved in the observed phenotype. Thus, it can ideally be complemented by both functional neuroanatomical investigations (such as assessing adult hippocampal and olfactory bulb neurogenesis in fixed brain tissue sections) and appropriate pharmacological challenges (for example, targeting neurotransmitter systems) for further clarification of the mechanism involved.

As with the tests of emotionality described previously, abnormalities in other bodily systems should be excluded as they can confound accurate assessment of a neurocognitive phenotype. Furthermore, in case of a positive phenotyping result, the outcome of the analysis should be confirmed, for example with complementing tests.

**5. Motor deficits**

Motor deficits can be analyzed by different tests dependent on the phenotypes either expected after prior analysis or based on model information (see Figure 3a). Assessment of basic motor function is followed by more sensitive but also more complex tests to specify the functional impairment.

The modified SHIRPA analysis with grip strength testing offers an overview and a checklist for visible alterations in reflexes, posture or movement together with the assessment of muscle function. Muscle strength is analyzed with a force meter allowing for direct measurement of applied force to a grip. Latencies on an accelerating rotarod give information about motor coordination and balance. This can be further specified by using balance beams of different diameters to measure traversing times, but also to be able to record number of falls and especially foot slips. While a balance beam is apparently especially sensitive towards balance problems, the beam ladder focuses more on fine skilled movements. In this test, the mice are required to traverse a ladder of small beams at different distances and slips of front and hind paws can be monitored for grasping deficits. The vertical pole test uncovers difficulties performing more complex movements requiring turning and descending a vertical pole. Mice that are placed on a grid, which is then turned upside-down require muscle strength to support their body weight attached to the grid. The time they are able to stay attached is an indicator for muscle strength. In contrast to direct grip strength testing the inverted grid analysis is usually negatively correlated to body weight. More precise but also more complex information about motor deficits can be obtained in automated gait analyses by quantifying video-taped foot step recordings either using voluntary walkways or using fixed speed treadmill paradigms. From these a multitude of temporal, spatial and variability parameters can be derived and analyzed, as in human gait analysis. All tests employed in this pipeline require to a certain extent collaboration of the mice and alterations in behavioral traits could interfere with motor test results and vice versa, thus additional information about changes in anxiety or activity is helpful for data evaluation.

**6. Sensory impairment**

Basic neuronal dysfunctions often cause widespread alterations in different central as well as peripheral nervous tissues and also affect sensory functions (vision, hearing, touch, taste, smell, balance) relevant for several human diseases. Thus, the analysis of sensory functions integrates tests for hearing and vision, but also electrophysiological analyses for basic neuronal functions (Figure 3a).

Hearing sensitivity, examined by auditory brainstem response, measures in anesthetized mice sound pressure levels needed to elicit a physiological response to different auditory stimuli. This is independent of mouse behavior and highly translatable to human conditions. Since several commonly used mouse strains are prone to an early age-related hearing loss [18], the age of the mice should be carefully considered. To further characterize sensory impairment, the hotplate assay offers the measurement of thermal latencies for assessment of the integrity of nociceptive pathways. Mice are placed on a warm surface and reaction latencies (hind paw shaking or licking) are recorded up to a maximum of thirty seconds.

As sensory problems may include visual loss, the visual impairment is an important assessment request as it has influences on data interpretation of an emotion-related phenotype. Being a part of the peripheral nervous system, the retina contains a high density of neuronal cells with a laminar structure outside the brain. During the visual process, light is detected by photoreceptor cells. The signals will then be relayed by the cells in the inner nuclear layer (INL) to retina ganglion cells (RGCs). The nerve fibers of RGCs converge into the optic nerve fibers where signals are further transmitted to the visual cortex for visual processing. RGCs hold the responsibility of conducting the signal to the brain. The vulnerability of RGCs to stress factors such as inflammatory factors, high intracellular calcium ions, glutamate, or free radicals, or high intraocular pressures may lead to irreversible blindness. To study ocular diseases in mice, there are different tools available that we use in the screening pipeline: OCT, Scheimpflug, LIB, virtual drum. Additional technologies to study physiology are electroretinography (ERG [19]) and the analysis of intraocular pressure (IOP). Mouse mutants with visual deficits will be subject for a special physiological investigation serving as a guideline in the sensory impairment pipeline. The ERG serves for the assessment of the activity of the retinal neurons and the IOP is a major risk factor for glaucoma development. Studies investigating retinal damages in neurodegeneration mouse models show that neurons of retinal ganglion cell layer (RGCL) and INL are affected which makes the utilization of the ERG and IOP as sensitive approaches to observe neuron cell alteration in the retina [20], the last one being a tool to confirm eventual glaucoma pathological process. The sources of the ERG arise in various layers of the retina and are recorded by using electrodes placed on the surface of the cornea and the reference electrode is placed on the forehead. By ERG it is possible to identify the existence of a degenerative disease in the retina as well as monitoring sensory performance during and/or after therapeutic interventions. Changes in IOP levels are detected by measurements with a tonometer based on a rebound measuring principle. The mouse is gently restrained by hand on an adjustable stand, and the eye is oriented in such a way as to align the probe tip with the optical axis of the eye at a 1-2 mm distance. Six consecutive IOP readings are averaged. IOP readings obtained with Tonolab have been shown to be accurate and reproducible in different mouse strains [21]. After finalization of this pipeline, histological and immune-histochemical analyses can be performed to have a more detailed view on the underlying changes leading to the alterations in ERG and IOP.

Motor as well as sensory nerve conduction velocities are measured to evaluate basic neuronal functions but also to test peripheral neurons affected e.g. by demyelinating diseases like Charcot-Marie-Tooth or functional neuropathies e.g. diabetic neuropathy. For respective hypotheses, identification of drug target effects or respective susceptibility genes seizure thresholds can be analyzed by application of pentylenetetrazole (PTZ, acting via GABA-A receptors and calcium channels). The concentrations used here do not affect healthy individuals but induce the occurrence of pre-seizure behaviors in susceptible mice. This can be combined with the use of telemetric encephalography where electrical activity can be recorded from implanted surface electrodes.

**7. General neuro-behavioral assessment**

Knowledge about basic neurological functions like motor functions, hearing and vision abilities are a prerequisite for the evaluation of behavioral analyses. Furthermore, several neurodegenerative diseases are syndromes involving different pathophysiologies. For instance, Huntington’s and Parkinson’s disease e.g. are characterized by motor deficits, but also present with cognitive changes and psychiatric symptoms [22-26]. Such systemic alterations e.g. caused by and occurring early in neurodegenerative processes are analyzed by a series of tests covering different aspects of CNS function including locomotor activity, exploratory behavior, emotionality, learning and memory abilities (see Figure 3a). This is particularly interesting in (potentially slowly) progressing diseases, because robust early disease markers are important for early diagnosis and therapeutic intervention. Especially in degenerative diseases, attempts to halt disease progression already in the prodromal stage are meaningful goals for therapy development.

**8. Kidney function**

The renal function analysis has been developed to investigate kidney function in mice with suspected alterations in urine production and excretion. Elevated urea and/or creatinine levels, which are the classical signs of renal failure, can be hints towards an altered renal function within the screening pipeline. However, changes in plasma electrolyte levels (sodium, potassium, chloride), mineral concentrations (calcium and inorganic phosphorus) or protein abundance, as well as signs of changes in water homeostasis such as altered hematocrit values can also be related to kidney dysfunction. The renal function pipeline (Figure 3b) can therefore be used to check mouse models showing altered renal parameters and confirm whether the above findings are associated with altered renal excretion of water and urinary solutes.

Two to three days before urine collection a blood sample is collected to determine blood levels of solutes excreted via the kidneys for later calculation of creatinine clearance and fractional excretion rates. Quantitative urine collection is performed in metabolic cages for single mice during 24-48 hours allowing us to measure daily water uptake and urine production and calculate daily solute excretion and fractional excretion rates [1]. If food uptake and feces production are quantified and feces composition is analyzed, additional data on energy uptake and food digestion can be generated in parallel. In cases of observed polyuria, part of the investigation can be carried out with water withdrawal to test urine concentration ability in affected mice. A direct effect on renal function can be concluded on, if there is a specific hyper-excretion or retention of single solutes found. The interpretation of decreased or increased urinary concentration or generally increased excretion of all solutes is more difficult, since it could also be secondary to altered feeding and or drinking behavior in the new environment during the test. Without the water deprivation challenge, it is sometimes not possible to draw a final conclusion whether increased or decreased urine excretion is a primary effect or a secondary effect of altered feeding and/or drinking behavior, as for example in the case of Scube3-mutant mice [27]. A final organ collection for patho-histological analyses is recommended. However, especially in cases of tubular dysfunction, histological alterations in H&E stained sections often cannot be seen. Immunohistochemistry and electron microscopy could in these cases add additional information, as previously reported in *Umod* mutant mice [28].

A combination of neurological and behavioral phenotyping with renal function analysis can be of interest for several reasons. Neurological alterations can be secondary effects of renal dysfunction in both mice and men [29-31]. Several systemic diseases, like for example diabetes or excessive erythrocytosis [32, 33], affect both neurological and renal function, and furthermore several genes and cellular mechanisms have been shown to be relevant in both systems [34-37].

**9. Glucose metabolism**

Disorders of glucose metabolism, like diabetes, belong to the most important common diseases today. We have implemented a pipeline to investigate effects on glucose metabolism in mice and thereby contribute to the understanding of diabetes and other disorders of glucose metabolism (see Figure 3b). In the screening pipeline plasma chemistry analyses, body mass and body composition analysis, a glucose tolerance test and indirect calorimetry are implemented, which could give first hints towards altered glucose metabolism. Fasting or fed hyper- or hypoglycemia, altered glucose tolerance and alterations of respiratory exchange ratio indicating differences in substrate utilization can be detected.

For an in-depth characterization of animal models with altered glucose metabolism, a regular monitoring of blood glucose and insulin levels is applied to investigate temporal dynamics or age-related dysfunctions. Additionally, blood levels of glucose, lipids and relevant hormones in fed and fasting state as well as body weight and body composition analysis give an overview of the metabolic state of the animal. To identify specific alterations in the response to a high glucose load challenge, a glucose tolerance test is performed [38]. However, since observed differences in glucose tolerance in this test can be due to a number of different reasons, for example altered insulin production, maturation, secretion, or structure on the one hand or insulin resistance of tissues on the other hand, additional tests, as for example the insulin tolerance test, provide additional insight. An insulin tolerance test shows the blood glucose level response to a high insulin dose as an indicator of whole body insulin sensitivity. Glucose clamps are widely seen as the gold standard to investigate β-cell function (insulin secretion) or insulin action (glucose metabolism on the whole-body or organ level). Two different clamp types are usually applied: (1) hyperglycemic clamp to address insulin secretion and (2) the hyperinsulinemic-euglycemic clamp to address glucose uptake in tissues [39]. In addition, the clamp set up allows measuring tissue specific glucose uptake rates by using labelled glucose. Even though glucose clamps are invaluable to fully assess disturbed glucose homeostasis both in humans and rodent models, the procedure of mouse glucose clamps is rather elaborate because it involves substantial expertise for example in micro-surgery for the cannulation of the jugular vein or carotid artery, postsurgical control, the clamp itself due to fine-regulation of glucose infusion, and finally in data analysis.

**10. Energy metabolism**

We designed a special pipeline focusing on parameters characterizing whole-body energy balance regulation in mice (see Figure 3b). All phenotyping assays comprise *in-vivo* testing of parameters that affect energy homeostasis. On the whole-body level, energy homeostasis depends on balanced energy fluxes both on the energy intake (food uptake and gastrointestinal energy absorption) as well as the energy expenditure side (metabolic rate). Imbalanced energy fluxes result in changes of endogenous energy depots stored as lipids, glucose, glycogen, proteins, etc. [40]. In mice, only small imbalances can result in obesity or cachexia but still in some cases it may be reasonable to further stress energy balance regulation by adding challenge tests. Feeding a high calorie diet, exposing mice to low ambient temperatures, or stimulating energy expenditure by adding running wheels or a treadmill are well-defined experimental challenges. To address the highly dynamic nature of energy balance regulation it is useful to continuously monitor body mass, body composition, blood glucose levels, or other suitable blood parameters. This can be facilitated or refined by implanting transponders that monitor physiological parameters such as core or peripheral body temperature, locomotor activity, or allow the online monitoring of blood glucose levels.

The energy metabolism pipeline comprises some tests that are also implemented in other pipelines such as the screening pipeline. Analysis of blood samples for energy metabolism related compounds such as glucose, triglycerides, lactate, etc., indirect calorimetry and qNMR based body composition analysis are described in the respective sections. Implantation of transponders to monitor physiological functions requires substantial training of the responsible people. Surgery, analgesia and the monitoring of successful recovery are important prerequisites for the preparation of a study. In addition, sufficient recovery time has to be allowed for before continuing the study due to the acute total impact and the time required for wound healing. Both surgery as well as the implantation of a transponder of considerable size and weight limits the options to which mutant lines to select. It is advised that general health status and body weight should be appropriate for mice to successfully cope with the burden. Therefore, costs and benefits of transponder implantation need to be carefully traded-off. The tests for energy balance regulation and indirect calorimetry concentrate on classical parameters of energy balance. Energy balance regulation is designed to monitor food uptake of single caged mice over a period of days. In parallel, all egested feces is collected, desiccated, and energy content is determined by bomb calorimetry. Alternatively, energy content and feces composition can be determined by FT/IR based reflectometry. The aim of the test is to generate exact data on food consumption, energy uptake and apparent energy assimilation efficiency characterizing hunger control and major functions of the gastrointestinal tract. Indirect calorimetry allows the monitoring of locomotor activity and rearing behavior in addition to data on metabolic rate and substrate utilization. Similarly to the calculation of lipid and carbohydrate oxidation rates from gas exchange data during the indirect calorimetry, direct measurement of exhaled volatile, mainly organic molecules, has recently opened a new and non-invasive window into mouse (and human) metabolism (breath gas analysis, [41]). It could be shown that exhaled volatile organic compounds (VOC) such as short-chain fatty acids, ketone bodies, or alcohols can be used to assess the metabolic status (fasting state, glycemia, obesity, [42]). Finally, this pipeline offers the opportunity to vary the ambient temperature. In brief, we suggest two protocols one exposing mice to a moderate drop in ambient temperature from 22°C (optional 30°C) to 16°C during a 24-hours indirect calorimetry trial. The second more sophisticated protocol aiming to determine major features of thermogenic functions in mice at various ambient temperatures ranging between 30°C and 6°C (upper and lower critical temperature, and thereby the thermos-neutral zone, conductance, and basal metabolic rate). As mice are rather sensitive to low ambient temperature because of the required endogenous thermogenesis to maintain normothermia at ambient temperatures below about 30°C, cold challenges can be very effective in functional testing of physiological thermogenic properties.

**11. Imaging technologies**

Skeletal abnormalities in mouse models are often discovered using means that permit for high throughput *in vivo* work, including planar X-ray and DEXA. While X-ray enables the examination of changes in skeletal anatomy, such as deformed or missing skeletal structures, DEXA allows for quantification of altered bone mineral density. Particularly in combination, these techniques have been proven to be powerful means for the identification of skeletal abnormalities due to gene mutations, thus generating hypotheses with respect to the potential functions of a particular gene in skeletal development and homeostasis. However, the use of X-ray and DEXA imaging also has limitation. For example, planar X-ray imaging generates 2-dimensional images at approximately millimeter resolution. Because bones are 3-dimensional structures and mouse trabeculae are about 30-50 micrometer in width, initial *in vivo* X-ray imaging is complemented in our pipeline by subsequent ex vivo micro-CT imaging on dissected bones. This well-established technique [43] generates images of mineralized tissues such as bones in 3-dimensions at single digit micrometer resolution, resulting in morphometric measures of bone architecture. Standard procedures analyze both cancellous and cortical bone often in either the distal femur or proximal tibia. Morphometric measures can also be combined with BMD measures, hence facilitating BMD measures on defined skeletal structures, for example cancellous or cortical bone. Upon micro-CT detection of a skeletal abnormality, subsequent use of two other techniques may be indicated. First, as architectural changes in bone can affect its mechanical properties, mechanical testing, for example three-point bending or compression tests, deliver important additional information with respect to skeletal function. Second, histological assessment including dynamic histo-morphometry, performed for example on a matching contralateral bone, does not only yield morphometric parameter sets that validate the micro-CT measures but also quantifies important *in vivo* parameters such as the bone formation rate. Lastly, we emphasize that a meaningful examination of a skeletal abnormality in a mutant mouse model needs to extend beyond micro-CT measures and include the assessment of skeletal cells to begin to pinpoint underlying mechanisms.

Magnetic resonance imaging (MRI) is a non-invasive imaging modality enabling morphological and functional studies of living systems. The excellent soft tissue contrast offered by MRI enables valuable insights into morphological alterations of biological tissues. Furthermore, physiological parameters of organs can be evaluated as well. For example the perfusion of white/grey matter, of liver and of muscle tissue can be assessed. Besides imaging the magnetic resonance phenomenon renders another key application in life science. Magnetic resonance spectroscopy (MRS) provides insights into the major constitute of living systems, which is hydrogen (1H) as well as into specific less abundant nuclei such as phosphor (31P), sodium (23Na) and fluoride (19F).

The non-invasive imaging pipeline has the potential to independently study phenotypes of interest. Distinct questions raised by preliminarily results of the primary units can be addressed, contributing additional morphological information. MRI experiments, for instance, provide detailed data of the fat distribution within the phenotype as well as within specific organs in conjunction to the primary fat/lean studies. The screening of certain pathologies can be conducted based on the quantification of the magnetic relaxation times (longitudinal relaxation time T1, transversal relaxation time T2). The application of diffusion weighted MRI renders the analysis of the micro-structural organization of biological tissue by studying the water diffusibility. Perfusion weighted MRI studies reveal perfusion related data of distinct organs. MRS experiments offer the potential to access complex metabolic processes and specific neurochemicals.

MRS and MRI are both non-invasive techniques and such permitting the screening at minor stress level along longitudinal studies as well as at single time points. Immobilization through anesthesia is required for *in vivo* studies of phenotypes, *in vitro* experiments on single organs or on tissue samples can be performed after appropriate fixation. In summary, preclinical MRI and MRS renders the possibility to monitor maturation effects, to study the development of pathological processes, to identify morphological variations and to observe specific metabolic pathways in vivo.

**12. Immunity**

The immune system orchestrates imperative host defence mechanisms that protect against infectious microorganisms, malignant cell growth as well as auto-immunity. A great diversity of immune cell types has been identified and associated with two major parts of the immune systems: innate and adaptive immunity. The dynamic interaction between these two systems finely maintains a healthy balance. A wealth of studies has been revealed that distinct mutations on candidate genes can cause complex immunological diseases [44, 45]. In order to identify potential mutant genes that are related to human immune disorders, the screening pipeline is performed to evaluate the integrity of immune cell composition via flow cytometric analysis as well as to monitor the profile of immunoglobulin subclasses in the peripheral blood of mutant mice. Subsequently, identified aberrant phenotypes attributed to the immune system can be further characterized by application of detailed secondary analyses on lymphoid organs. We have established a series of standardized examinations that comprise flow cytometric analyses of primary (bone marrow, thymus) and secondary (spleen, mesenteric lymph nodes) lymphoid organs, and allow identification of a broad range of cellular parameters: T cell subsets in the thymus undergoing different maturational stages; hematopoietic stem cells, lineage progenitors and developing B cells in the bone marrow; a vast variety of subpopulations derived from lymphocytes and myeloid cells in spleen and lymph nodes.

In many cases, phenotypic screening based on the surface maker signature alone is not able to estimate the severity of the immunologic defect. Therefore, *in vivo* challenges, like infection with intracellular bacterium *Listeria monocytogenes*, provide detailed insights into the quality of innate and adaptive immune functions. *Listeria* has been widely used as laboratory mouse infection models with predictable response patterns. It induces an inflammatory response that substantially restricts bacterial growth and is essential for early survival of infected mice [46]. Assessment of bacteria loads within three days after *Listeria* infection represent a sensitive readout for the competence of innate immunity. Competent adaptive immune system is detectable around day seven post infection [47, 48]. The *Listeria*-specific CD8+ T cells are promoted to reach peak expansion and secrete effector cytokines, which are crucial for bacterial clearance [46, 49]. In order to evaluate long-term protective immunity governed by T cell-mediated immune responses, mice are first immunized with a low dose of *Listeria* *monocytogenes* followed by a secondary re-challenge with a high-dose infection within the memory phase several months later [46, 47, 50]. Antigen-specific CD8+ T cells can be analysed in more detail via utilization of the so-called MHC-I multimer technique. Moreover, additional characterization of effector and memory surface markers as well as cytokine production can be examined in response to re-stimulation of specific antigenic peptides. With successful establishment of the *Listeria* infection platform in the German Mouse Clinic (see Figure 3c), we are able to evaluate the quality of *in vivo* immune response in mutant mouse lines [51-53].

**13. Allergy**

The *in vivo* inflammation challenge platform (see Figure 3c) offers the opportunity to clarify or enhance immune phenotypes from the screening pipeline where findings were unclear or subtle. Furthermore, the functionality of genes over the immune system cannot always be addressed by using an organism in a steady state (e.g., unchallenged). We assume that *in vivo* challenge screening will disclose new genes that are implicated in the mechanism of action or signaling networks affecting immune system functions. Thus, we established a straightforward animal *in vivo* test in the GMC to disclose possible implication over pathways associated to inflammatory processes.

The murine allergen airway inflammation model implicates both humoral and cell-mediated immune responses and consists in two phases: the sensitization of mice (by a potential allergen e.g. ovalbumin) and the challenge (e.g. aerosol exposure to allergen). The exposure to an aerosolized allergen triggers the infiltration of cells into the airways. This inflammation is the protecting response to a harmful stimulus, and it activates a wide range of immune parameters such as cytokines, immunoglobulins isotypes and the recruitment and proliferation of immune cells. Such biomarkers can be straightforwardly monitored in blood, lung or broncho-alveolar lavage fluid.

The allergy airway inflammation model is a terminal test. It is easy to implement and, compared to other alternatives such as infection models, requires no special safety condition or regulations. The model is very suitable for large-scale experiments enabling both high-throughput pipelines and robust statistical analysis for hit selection. The allergic airway inflammation test pipeline was already successfully applied for the effective selection of genes predisposing to an altered airway inflammatory response from a large compendium of mutant mouse lines [54].

**14. Lung function**

With the increasing importance and necessity for translational research, it is of great relevance today to accurately measure the lung function of mouse model systems and be able to relate this to human disease pathologies as an initial screen for potential pulmonary phenotypes. Primarily two common systems can be used – the FlexiVent® forced oscillations methods from Scireq© and the Forced Pulmonary Maneuvers® methods from Buxco Research Systems©. For the detailed analysis of alterations in lung function, there are five options: Either a general lung function screen for the measurement of lung volume, resistance and gas exchange capacity as an initial screen for potential pulmonary phenotypes. Furthermore, we are using the LPS-induced acute lung injury (ALI) mouse model to investigate the inflammatory reaction after injury and its resolution. The inflammatory reaction will be monitored by broncho-alveolar lavage at several days after the LPS challenge. An acute (3 days) and chronic (120 days) cigarette smoke mouse model implicates both inflammatory and pathological response. The exposure of cigarette smoke triggers the infiltration of cells into the airways (acute) and in parenchymal tissue with decreases of gas-exchange area and loss of elastic fibers (emphysema). Further, the elastase-induced emphysema mouse model provides more detailed insights into regeneration and repair capabilities of lung tissue. Additionally, the bleomycin-induced lung fibrosis mouse model can be used to investigate bleomycin-induced inflammation and for the assessment of its fibrotic properties. Lung function systems are providing to monitor and diagnose mouse lung models induced pathological changes caused physiological and mechanically alterations.

As all the mentioned tests are final tests, only one of the described tests can be performed with the same cohort of animals.

**15. Progression analysis**

Many human diseases have a strong progression in severity over the age of the patient. In order to monitor the progression of phenotypes in mouse models, this pipeline is designed to follow up individual parameters over a certain period. A closer look at blood parameters (e.g. glucose levels) might be particularly interesting for diabetes models. All non-invasive tests from before-mentioned pipelines in screening or hypothesis-based pipelines may be performed and parameters are consistently recorded. As special focus imaging technologies may be included in this particular pipeline.

**16. Conclusion**

The described phenotyping pipelines of the German Mouse Clinic cover the most relevant areas of research for the use of mutant mice as models for human diseases and the understanding of mammalian gene function. Depending on the focus of the targeted project, a broad systematic, standardized mouse phenotypic analysis in a screening pipeline, as well as hypothesis based detailed characterizations of mice can be designed using the 14 phenotyping pipelines developed at the GMC. Thus, the GMC is able to follow up the need of the scientific community for more hypothesis-driven mouse phenotyping with the goal to thoroughly understand disease mechanisms and gene functions. Contact for scientific collaborations with the GMC can be made via www.mouseclinic.de.

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**Figure Legends**

Figure 1: Phenotyping strategy in the German Mouse Clinic (GMC): mouse lines with unknown or incomplete prior knowledge enter the screening pipeline of the GMC for a broad systemic characterization of phenotypes. Specialized pipelines are hypothesis-driven, and can be combined with challenges. Final aim is the publication of the results.

Figure 2: Schematic diagram illustrating some of the histopathological analyses performed in mouse phenotyping. The target organs are illustrated in blue and the techniques used in light pink. The arrows indicate an interaction between different organs. All techniques are performed in sections of formalin-fixed paraffin embedded tissues. (A) Luxol fast blue (LFB) staining is commonly used to detect axonal degeneration and demyelinating neuropathy in the peripheral nervous system (PNS). Immunohistochemistry (IHC) for glial fibrillary acidic protein (GFAP) is performed in the central nervous system (CNS) to visualize for example astrocytes after CNS injury. (B) The distribution of testis-specific variants of histones is used to study mouse testis germ and somatic cells. Granulosa cell survival and proliferation during folliculogenesis is analyzed using IHC for proliferative markers such as Ki-67. (C) In the kidney, observation of mesangial matrix expansion in more than 50% of the glomeruli using Periodic acid–Schiff (PAS) staining and sclerosis using IHC for collagen IV are used to diagnose glomerular injury. (D) Double IHC for insulin and glucagon secretory granules in β- and α-cells, respectively, is used to examine islets of Langerhans composition in the pancreas. (E) In the liver, the non-alcoholic fatty liver disease (NAFLD) activity score (NAS), with or without inflammation, is used to identify pre-diabetic mouse models. The adipophilin IHC is performed for better visualization of the lipid droplets in hepatic steatosis. The increased number of Kupffer cells, visualized using IHC for F4/80, is associated with steato-hepatitis. (F) Automated adipocyte cell size analysis is performed in the white adipose tissue (WAT) due to its role in lipid metabolism and insulin sensitivity. (G) Physiological contexts such as dyslipidemia, hypertension and obesity contribute to myocardial damage and are commonly visualized with Masson's trichrome and Sirius Red stainings to detect collagen fibers orientation and fibrosis levels in the heart.

Figure 3: Hypothesis-driven pipelines of the GMC. A) Pipelines for neurology and behaviour, B) Pipelines for physiology, energy metabolism & diabetes, C) Pipelines for immunology, allergy and lung diseases.