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Original article

The utility of the minipig as an animal model in regulatory toxicology ☆

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and under the auspices of the Steering Group of the *RETHINK* Project

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ABSTRACT

In this article we review the value and utility of the minipig as an animal model in regulatory toxicity testing. Our review is based on detailed consideration of the comparative biology of the minipig, and of the practical features of toxicity testing in the minipig. The minipig presents a favourable profile as a non-rodent toxicology model, in terms of the similarity to man and also in terms of applicability to different study types. Studies of general toxicology can be performed in the minipig by oral, cutaneous, parenteral and inhalation routes. For reproductive toxicology studies the minipig offers numerous advantages as a non-rodent model although the lack of placental transfer of macromolecules may limit the role of the minipig in reproductive testing of biotechnology products. For safety pharmacology studies the minipig is an advantageous model, particularly as regards the cardiovascular system. The immune system of the pig is better characterized than that of the dog, making the pig an interesting alternative model to the nonhuman primate for therapeutic approaches based on manipulation of the immune system. Overall, this review leads us to believe that the minipig might be a better non-rodent toxicology model than the dog. At the present time, however, insufficient comparative data is available to permit a rigorous evaluation of the predictivity of the minipig for human drug-induced toxicities and research is urgently needed to provide experimental data for evaluation of the hypothesis that minipig studies may better reflect human drug-induced toxicities than studies performed in traditional non-rodent toxicology models. It would be of particular value to gain a better vision of the potential utility of the minipig as a model for the safety testing of new biologics, where the minipig could potentially replace the use of non-human primates in the testing of some new products.

1. Introduction

In this article we review the value and utility of the minipig as an animal model in regulatory toxicity testing, as a contribution to the RETHINK project (Forster, Bode, Ellegaard, and van der Laan (2010a,b-this issue).

Pharmaceutical and chemical compounds should be effective and safe. The data of animal safety studies contribute to the risk/benefit evaluation for humans. For safety assessment *in vitro* and *in vivo* studies are conducted. The different types of toxicology studies, which may be performed to support the development of new products, include:

- general toxicology (single dose and repeat dose toxicity testing),
- reproductive toxicity testing (fertility, embryo–fetal toxicity and peri- and postnatal studies),
- safety pharmacology studies (evaluation of undesirable functional effects on the cardiovascular, respiratory and other physiological systems),
- immunotoxicity studies
- local tolerance studies
- other *ex vivo*/*in vitro* or *in vivo* studies.

For the animal studies, typically two species are used—one rodent and one non-rodent species. There is some choice in the selection of

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the non-rodent species where dogs, non-human primates and minipigs are available. The extrapolation of animal toxicology data to humans is optimal when the most "human-like" species is used for the studies. Criteria for the selection of the non-rodent toxicology model are:

- comparable pharmacodynamic activity of the compound in humans and the selected non-rodent species
- comparable pharmacokinetic and metabolic parameters
- comparable sensitivity and profile of reactions following toxic insults.

It is recognized that it is often difficult to identify the most relevant model for prediction of human reactions, especially when no human data are available.

In this context it is clearly relevant that the pig is considered close to humans in terms of anatomy, physiology and biochemistry. More available information from domestic pigs is readily applicable and valid also for the minipig model (as discussed in more detail in Forster, Ancian et al., 2010-this issue).

In this article current knowledge about the use of the minipig as a laboratory animal model for safety testing has been reviewed with respect to:

- comparative biology of the pig and minipig and the implications for their use in safety testing
- current understanding of the value, reliability and predictivity of the minipig as a model for the toxicity of drugs and chemicals for humans,
- the potential role of minipigs in non-rodent testing strategies, compared with dogs, non-human primates and others,
- technical gaps which impede the application and wider use of the minipig in each area
- identification of opportunities for the application of 3R's

2. Comparative biology of the minipig

2.1. Cardiovascular system

The heart of a minipig shows a similar coronary artery distribution to humans with a low coronary collateral circulation. The major anatomic variation is the presence of the large left azygous vein, which drains the intercostal system into the coronary sinus which ends *directly* in the heart and not in the caval vein. The blood supply for the conduction system is delivered from the posterior septal artery, i.e. right side dominant as in humans (= auriculo-ventricular) (Table 1). In contrast to the pig the dog has a developed collateral circulation; therefore the dog is less susceptible to myocardial infarction. Furthermore, the pattern of infarction and repair of the myocardium in the pig is almost identical to humans and the wound healing characteristics of the cardiovascular system mimics human responses (Bloor, White, & Roth, 1992; Gardener & Johnson, 1988; Swindle, 2007).

Histologically, Purkinje fibers are more prominent in pigs compared to humans or other species. The aortic wall of pigs contains a *vasa vasorum* like in humans (Swindle & Smith, 1998). In terms of electrophysiology, major ion channels except I_{to} are present in pigs. $I_{Ca^{2+},L}$, $I_{\dot{N}a}$, I_{Ks}, and I_{Kr} were characterized in ventricular myocytes of 6–8 month old male Göttingen minipigs by Arlock, Mow, Lauersen, and Ganderup (2007).

All these characteristics favor the use of the minipig for cardiovascular assessments in toxicological and pharmacological studies, in particular in comparison to dog. In addition, the pig is a recognised model for several human cardiovascular diseases like atherosclerosis (Jacobsen, Lundholm, & Wingern, 1984; Jacobsson, 1989; Kobari, Koto, & Tanigawa, 1991) and myocardial infarction (Schuleri et al., 2008).

Table 1
Organ blood flow distribution.

	% of HMV	ml/100 g tissue/min
Heart	4.5	118 +/− 45
Brain	5.1	76 +/− 21
Esophagus	0.1	16 +/− 11
Stomach	2.6	59 +/− 44
Small intestine	11.0	79 +/− 49
Cecum	1.1	96 +/− 68
Large intestine	3.4	43 +/− 30
Pancreas	1.4	147 +/− 166
Spleen	3.3	297 +/− 232
Kidneys	17.0	361 +/− 86
Adrenals	0.1	82 +/− 62
Skin	5.0	8 +/− 4
Muscles	–	14 +/− 6
Fat tissues	–	11 +/− 6
Liver (total)	26	167 +/− 74
Liver (portal)	23	–
GI tract (total)	18	71 +/− 46

(16 Göttingen minipigs of 3 kg; Heart minute volume [HMV] = 717 +/− 224 ml/min; Wyler, Käslin, & Hof, 1979).

Studies which investigate the potential actions of compounds on physiological functions are known as safety pharmacology studies. These physiological functions should be investigated before the first administration of candidate drugs to humans. While rodents can often be used for the evaluation of respiratory and CNS functions, cardiovascular investigations generally use non-rodent species. The ionic mechanism of repolarization in adult rats and mice differ from larger species, including humans, rendering them less appropriate (also mentioned in van der Laan et al., 2010-this issue). The primary ion currents controlling repolarization in adult rats and mice is I_{to} and not I_{Kr} as in humans and non-rodents (see ICH guideline S7B).

The most frequently selected species for cardiovascular safety pharmacology studies is the dog, but the minipig is increasingly used for novel therapeutic agents. The utility of the minipig in cardiovascular safety studies was documented by Abadie and Ackermann (2000), presenting blood pressure and heart rate data from minipigs treated with propranolol, isoproterenol, nifedipine and clonidine and QT interval prolongation data on terfenadine (Table 2). The authors conclude that the similarities to humans in their cardiovascular physiology, size, anatomy and the perfusion distribution of blood flows make minipigs better subjects than other species.

One caveat must be kept in mind; minipigs can remain in a state of excitement for several hours after feeding, associated with a higher heart rate and this could lead to a misinterpretation of CV data. Openshaw, Purbrick, Peake, and Meecham (2008) therefore recommend that minipigs should be fed at least 4 h before starting ECG recordings to allow return of the heart rate to normal resting values.

Stubhan et al. (2008) evaluated cardiovascular and ECG parameters in the normal freely moving Göttingen minipig. Values are provided for aortic blood pressure, left ventricular pressure, LV dP/dt, heart rate, body temperature and ECGs. The authors found extremely stable patterns for all hemodynamic parameters and a good signal quality. Because of the known effects of feeding (explained above), these experimenters recommend to not feed the animals in studies in which the T_{max} of a given compound might occur very late (i.e. after

Table 2
Primary heart data.

	Minipigs	Dogs	Cynomolgus	Humans
Heart rate (BPM)	103 ± 14	125 ± 50	125 ± 50	70 ± 10
Systolic blood pressure (mm Hg)	148 ± 15	165 ± 35	150 ± 15	125 ± 10
Diastolic blood pressure (mm Hg)	96 ± 14	95 ± 25	91 ± 8	70 ± 10

(Data from 100 Göttingen minipigs of 20 kg; Glodek & Oldigs, 1981).

the feeding). In a cross-over telemetry study in six minipigs with monitoring of aortic pressure, left ventricular pressure, ECG and body temperature, Markert et al. (2009) demonstrated a dose-dependent QT prolongation when moxifloxacin was administered orally at dose producing clinically relevant plasma drug concentrations. Expected propranolol-induced effects on heart rate and myocardial contractility were also demonstrated. These and similar results (Kano et al., 2005; Purbrick, Openshaw, Peake, & Meecham, 2008; Skytte & Makin, 2008) support the use of the telemetered minipig as a relevant and valid model for the detection of drug-induced cardiovascular changes.

It is recommended that ECG recordings from the minipig should use the Nehb–Spoerri lead configuration. This system which takes account of the specificities of the heart in the pig, provides more consistent data, and is less influenced by the position of the animal (Nahas, Baneux, & Detweiler, 2002).

2.2. Respiratory system and inhalation toxicology

The anatomy of the airway system in the pig permits inhalation studies and functional respiratory studies. Differences in the anatomy between pigs and humans include apical, middle and diaphragmatic lobes as well as an additional accessory lobe of the right lung, and the interlobular fissures are incomplete. The larynx is prominent, with large vestibule and lateral and middle ventricles which leads to a caudal narrowing (Brown & Terris, 1996; Guyton, 1947; Kuckelt et al., 1981; Maggriorini, Brimioulle, De Canniere, Delcroix, & Naeije, 1998; Swindle, 2007). However, the anatomy of the pig presents certain technical problems for intubation and clinical examination may be more difficult due to its long oral cavity, angled larynx and often hypoventilated right apical lobe (Swindle, 2007).

Minipigs have been used as a model for acute respiratory distress syndrome. Also, the development of the neonatal pig lung and airways is a useful model for the human immature lung. Minipigs have a higher pulmonary vascular resistance than dogs.

The nasal cavity of the minipig is similar to humans. The two sides of the cavity are essentially separate and control and drug exposure can therefore take place in the same animals. Accordingly, minipigs are good candidates for nasal studies (Lacoste et al., 1999) although there may be some practical difficulties in administration (see Ellegaard et al., 2010-this issue). Bønløkke-Rankløve, Kaae-Thorhauge, Eriksen, and Glerup (2006) describe intranasal administration with an actuator. The aim of this study was to examine local and (because of the high nasal bioavailability) systemic effects. The minipig was chosen (and considered as the preferable species compared to rabbits, guinea-pigs, and dogs), due to the possibility of using the same device, dose and formulation as intended for humans. In addition, the weight of the pigs in this study (25–30 kg) was closer to that of humans when compared to the other species.

Metabolic activity in the olfactory epithelium and respiratory mucosa has been described for normal pigs. The mucosa contains all the components of the monooxygenase system as well as several non-oxidative enzymes (e.g. glutathione S-transferase, UDP-glucuronyl transferase, epoxide hydrolase, DT-diaphorase, benzaldehyde and propionaldehyde dehydrogenases, and various esterases). The enzyme activity is higher in the olfactory epithelium than in the respiratory mucosa (Marini, Longo, Mazzaccaro, & Gervasi, 1998; Reed, 1993).

Pigs are also characterized by the presence of a larger number of intravascular pulmonary macrophages (in addition to the alveolar and interstitial macrophages) (Warner, Barry, & Brain, 1986). Intravascular pulmonary macrophages may be beneficial to the host (scavenging cell debris, bacteria, immune complexes and endotoxins from the blood circulation), but can also elicit acute lung injury when activated by release of inflammatory mediators. Rodents, non-human primates and humans do not have such an extensive resident pulmonary intravascular component (Brain, Molina, DeCamp, & Warner, 1999).

Today, limited information is available with regard to the pulmonary administration of drugs and other chemicals in minipigs. A brief review of the three published uses of minipigs (all unrelated to toxicology) is presented by Koch, Windt, Walles, Borlak, and Clausing (2001); these authors are the first to describe methods for inhalation administration in the minipig. They focused on validating the minipig for inhalation administration by comparing the pharmacokinetics of verapamil after intravenous and inhalation (snout mask) exposure. Recently another validation study has been presented of minipigs chronically implanted with telemetry devices. The animals were exposed to lactose (102 μg/kg) or dofetilide (target doses between 25 and 300 μg/kg) using aerosols generated by a Wright Dust Feed mechanism (Purbrick, Openshaw et al., 2008; Purbrick, Moore et al., 2008).

One important gap concerning the necessary basic knowledge for using the minipig in inhalation toxicology is the current lack of data on the deposition efficiency (pulmonary distribution) of inhaled particles including droplets of different sizes. Currently, only rough estimates can be made by comparison with the data existing for dogs and non-human primates (Koch et al., 2001).

The respiratory minute volume has been determined for minipigs of 8.2 kg weight (1.9 L/min) and earlier for those of 22.8 kg weight (5.1 L/min) by Koch et al. (2001) and earlier for those of 22.8 kg weight (5.1 L/min) by Denac, Spörri, and Begliner (1977).

For Yucatan minipigs Jones, Stuart, Greufe, and Landes (1999) observed a general minimal to slight rhinitis as a common finding in the histopathological assessment, this finding might interfere with the results of inhalation studies. Such spontaneous rhinitis as part of general background pathology, however, has never been described for Göttingen minipigs (Skydsgaard, 2005).

2.3. Liver and metabolism

The liver of the minipig is constituted of six lobes and a gall bladder. In contrast to humans, primates or dogs, porcine liver histology is characterized by a prominent lobulation composed of fibrous connective tissue connecting portal triads. This characteristic is well known and does not cause any problems for histopathological evaluations.

Cytochrome P450 (CYP) enzymes do not differ significantly in their primary structure between normal pigs and minipigs (Souček, Zuber, Anzenbacherová, Anzenbacher, & Guengerich, 2001). All main metabolic activities typical for human CYP enzymes are found in porcine liver microsomes (Anzenbacher et al., 1998; Monshouwer et al., 1998; Skaanild & Friis, 1997).

An overview of specific CYP enzyme isoforms in normal pigs and their similarity to CYP isoforms identified in humans and rat is given by Myers, Farrell, Howard, and Kawalek (2001). Although these data were derived from domestic pigs the authors conclude that the CYPs from the Göttingen Minipig could be regarded as comparable (see Forster, Ancian, et al., 2010-this issue for the extent of genetic differences between common domestic pigs and minipigs).

Gender and breed-specific differences were shown by Skaanild and Friis (1997). CYP1A2 activity was 4-fold greater in female than in male Göttingen minipigs or conventional domestic pigs. Hepatic CYP3A4 activity in the Göttingen minipig was greater than in conventional pigs with a slight gender difference in both strains.

Myers et al. (2001) found constitutive levels of immuno-reactive proteins in pigs analogous to those of human or rat CYP1A2, 2A6, 2B6, 2E1 and 4A1/3.

A notable difference was seen in the molecular weight of pig CYP enzymes (1A1, 2B2, 2B1, and 2E1) when compared to rodent or human homologues.

The induction of CYP enzymes with different inducers showed similar results to CYP induction in rats. Zuber, Anzenbacherova, and Anzenbacher (2002) compared the different cytochromes from rat, dog, pig, minipig and cynomolgus macaques.

The following details are provided to facilitate the assessment of the minipig as an early model for metabolic comparison to humans.

- The microsomal fraction of minipig liver homogenate has been shown to contain the activities characteristic of human CYP3A4 (nifedipine oxidation), 2A6 (coumarin 7-hydroxylation), 2D6 (bufuralol 1′-hydroxylation), 2E1 (p-nitrophenol hydroxylation), and 2C9 (tolbutamide hydroxylation) (Anzenbacher et al., 1998).
- Souček et al. (2001) concluded that the N-terminus of minipig CYP2A is highly similar to human CYP2A6 (14 of 20 amino acids identical). Comparison of CYP2C family members may be difficult because of very high content of leucine at the N-terminus of the sequenced minipig protein (still about 50% sequence identity with the human counterpart). Moreover, the human CYP2C subfamily has at least four highly homologous clones (sequence identity at the N-terminus > 90%), and therefore the existence of other CYP2C-related genes in the minipig may be anticipated.
- The pig/minipig CYP3A and human CYP3A4 share about 60% sequence similarity (12 of 20 amino acids identical). The presence of a minipig liver CYP3A enzyme with similar activities to the human CYP3A4 has been reported earlier (Anzenbacher et al., 1998). Together with the data obtained from pig liver and intestinal microsomal systems (Bader et al., 2000; Lampen et al., 1995; Olsen, Hansen, & Friis, 1997), the results support the suitability of pigs/minipigs for modelling the biotransformation of drugs in man.
- Souček et al. (2001) also showed that pigs and minipigs have CYP2A, 2C, and 3A liver microsomal enzymes with N-terminal sequences very similar to the human enzymes.
- The observed high similarities of N-terminal sequences of minipig and human CYP2A and 2C confirm the published similarity in marker activities (Anzenbacher et al., 1998; Skaanild & Friis, 1999).

In summary, the results presented on the comparability of hepatic P450 cytochromes between pigs and humans support the usefulness of minipigs as experimental animals to predict biotransformation pathways in man (Table 3). Minipig hepatocytes and microsomes are commercially available (www.biopredic.com; http://www.celsis.com; www.3hbiomedical.com and others). More cross-species comparative data on the metabolism of reference compounds would assist in the evaluation of the potential role of the minipig in safety testing. For individual products in development, inclusion of minipig hepatocytes or microsomes should routinely be considered. We recommend that the minipig should be routinely included in metabolic profiling to establish the most suitable model predictive for humans.

Table 3
P450 test reactions: human and porcine isoenzymes involved.

Substrate	Human	Porcine	Reaction	Refs
Methoxyresorufin	CYP1A2	CYP1A	*O*-demethylation	1
Ethoxyresorufin	CYP1A2	CYP1A	*O*-deethylation	2, 1
Coumarin	CYP2A6	CYP2A6	7-hydroxylation	2–6
Nicotine	CYP2A6	CYP2A6/?	*O*-oxidation	7
Benzyloxyresulfin	CYP2B6	CYP2B?	*O*-dealkylation	8
Coumarin 7-hydroxylation	CYP2B6	CYP2B?	Dealkylation	3
Pentoxyresulfin	CYP2B6	CYP2B?	Demethylation	9–10
Mephenytoin	CYP2B6	ND	*O*-debenzylation	2
Diclofenac	CYP2C8/9	CYP2C9/?	4-hydroxylation	3,8
Tolbutamid	CYP2C9	?	4-hydroxylation	11
s-mephenytoin	CYP2C19	ND	4-hydroxylation	2–3, 8
Debrisoquine	CYP2D6	ND	4-hydroxylation	12
Bufuralol	CYP2D6	CYP2B	1-hydroxylation	3, 12
Dextromethorphan	CYP2D6	CYP2B	*O*-demethylation	12
Chlorzoxazone	CYP2E1	CYP2A/3A	6-hydroxylation	2–3, 9
Para-nitro-phenol	CYP2E1	CYP2A/?	2-hydroxylation	1
Analine	CYP2E1	CYP2A/?	4-hydroxylation	1
Dimethylnitrosamine	CYP2E1	?	N-demethylation	
Midazolam	CYP3A4	CYP3A	1 & 4-hydroxylation	13
Testosterone	CYP3A4	CYP3A	6β-hydroxylation	2–3
Nifepidine	CYP3A4	CYP3A	N-oxidation	2, 9

References: (1) Nebbia et al. (2003), (2) Skaanild and Friis (1999), (3) Bogaards et al. (2000), (4) Skaanild and Friis (2000), (5) Shimada, Yamazaki, Mimura, and Guengerich (1994), (6) Pelkonen, Rautio, and Pasanen (2000), (7) Skaanild and Friis (2005), (8) Myers et al. (2001), (9) Desille et al. (1999), (10) Behnia et al. (2000), (11) Anzenbacher et al. (1998), (12) Skaanild and Friis (2002), (13) Lu and Li (2001).

2.4. Gastrointestinal system

Both humans and minipigs are true omnivores. This is reflected in the anatomy, physiology and function of the gastrointestinal system, and despite several anatomical differences in the pig (see below) the physiology of digestion remains similar to humans.

The buccal epithelium is unkeratinized, like in humans (Shojaei, 1998), and domestic pigs have been used for studies on buccal administration of peptides (Hoogstraate, Verhoef, Pijpers, et al., 1996; Hoogstraate, Verhoef, Tuk, et al., 1996).

Domestic pigs and minipigs have a pharyngeal diverticulum which is located dorsal to the larynx in the caudal portion of the nasopharynx. For oral administration it is important to remember this diverticulum, which is part of normal porcine anatomy, since oral gavage tubes can get caught in the diverticulum during intubation. Training in appropriate technique will allow avoidance of this problem (see Ellegaard et al., 2010-this issue).

Anatomically, the stomach is similar to other monogastric species, with the exception of the mucosa. There are two types of mucosa: a non-glandular keratinized mucosa (as in the rat) and a glandular mucosa.

Minipigs are less prone to emesis than dogs, reducing problems of variable or undefined exposure after oral administration of test items. For the dog, vomiting is often a limiting factor in the selection of the species. The minipig is more robust in this respect, but nevertheless the pig is able to vomit and vomiting may be induced by some test agents. Some mycotoxins (in particular vomitoxin) are noted for provoking emesis in pigs.

The small intestine is long, with a transit time and pH very similar to humans (McAnulty, 1997). In a recent study the mean retention time in the small intestine for 30 kg pigs was estimated to be 4 h (Wilfart, Montagne, Simmins, Noblet, & van Milgen, 2007). In the dog, the small intestine is shorter, and the mean small intestinal transit time was determined as 3 h (Schulze et al., 2005). Bioavailability of orally administered drugs that are influenced by pH or transit time can be expected to be comparable in humans and pigs.

The pig shows anatomical particularities when compared to dogs, monkeys and humans, especially in the large intestine where the cecum and colon are larger. The cecum and colon are arranged in a series of coils, known as the spiral colon. The mesenteric vasculature is also different and characterized by a vascular arcade in the subserosa, rather than in the mesentery.

In summary, for safety testing, the gastrointestinal system of the minipig offers some anatomical advantages (in terms of similarity to humans) and functional advantages (in terms of absorption, metabolism and reduced tendency to vomit) when compared to dogs.

2.5. Kidneys and renal excretion

The kidneys of the pig have many similarities with those of humans in anatomy and function (McAnulty, 1997). They are multi-reniculate, multi-papillate and similar to human kidneys in size and structure (including true calices) (Brown & Terris, 1996; Pennington, 1992; Swindle, 2007; Terris, 1986). The blood supply divides transversely between poles rather than longitudinally as in humans. This feature does not affect functional characteristics of the kidney but should be remembered when performing surgery on the kidney.

The urinary bladder shows no anatomical peculiarities and is readily catheterised in females. Catheterization of the male urinary tract is not possible, because of the sigmoid flexure of the penis.

The renal system of the minipig is well characterized because of its use in transplantation surgery and as an animal model for studying urinary incontinence and renal hypertension (Conn, 2008).

2.6. Reproductive system and reproductive toxicology

Göttingen minipigs achieve sexual maturity before six months of age as shown in the table below (Table 4).

For comparison, sexual maturity is reached at 7–12 months in the beagle dog, and at 4–5 years in cynomolgus and rhesus monkeys. Sexually mature macaques are not often used in regulatory toxicology studies, as a consequence of lack of availability. Beagle dogs (aged typically 6 months at study outset) approach sexual maturity at the end of the study when used for 13-week studies. At the outset of studies, minipigs are typically aged 3.5–4.5 months; in consequence at the end of 13-week toxicology studies minipigs of both sexes are fully sexually mature and at the end of 4-week studies they are approaching sexual maturity (Mitchell & Creasy, 2007).

2.6.1. Males

The minipig shows in principle the same anatomical structures of the testis as other mammals.

Seminology data reveal similarities with humans, although there are differences in the sperm volume and seminal vesicles and the bulbourethral glands are more developed than the prostate (unlike man). The Göttingen minipig has proven to be a valid model for the detection of adverse effects on male fertility (Svendsen, 2006). The different spermatogenesis stages in the minipig have been described by Damm Jorgensen, Kledal, Svendsen, and Skakkeboek (1998) but are not as well characterized as in the rat, dog or primate. Spontaneous histological lesions are often encountered in the minipig, such as tubular hypoplasia and Leydig cell hyperplasia, which may complicate the histopathological evaluation of subtle changes.

Spermatogenesis in the pig takes 35 days (Damm Jorgensen et al., 1998), a much shorter period than the dog or rat. This has direct consequences on the duration of studies where spermatogenesis is a potential target for toxicity or for the investigation of reversibility of effects on spermatogenesis.

More detailed information concerning spermatogenesis in the boar can be found in Wagner (2005).

2.6.2. Females

The female reproductive system of the pig has a bicornuate uterus with tortuous fallopian tubes. The minipig is the closest species to humans in terms of uterine histology and estrous cyclicity (Tables 5 and 6). However females do not show menstruation, unlike the cynomolgus monkeys (Attia, 1998; Dukelow, 2005). Some comparative data from dogs, monkeys and minipigs are summarized in the tables below (further information is found in Ellegaard et al., 2010-this issue).

2.6.3. Embryonic development

Key events in embryonic development of both minipig and human embryos take place at approximately the same moment in the gestational period (Damm Jorgensen et al., 1998) (Tables 7 and 8).

Table 4
Göttingen minipigs typical growth characteristics.

Sexual maturity, male	7–9 kg (14–16 lbs)	3–4 months
Sexual maturity, female	9–11 kg (18–22 lbs)	4–5 months
Closure of growth lines	30–35 kg (60–70 lbs)	18–22 months

Table 5
Comparative sperm production.

	Sperm produced ($\times 10^6$/g testis/day)	Duration of spermatogenesis (days)
Rat	21*	51.6
Dog	20	54.4
Rhesus	23	56
Human	3.1*	37
Minipig	No data	35
Swine	21–25**	35

* Source: Johnson, L, Petty C.S., and Neaves W.B. (1980) A Comparative Study of Daily Sperm Production and Testicular Composition in Humans and Rats, Biology of Reproduction, Vol 22, 1233–1243).

** Source: Cupps PT. Reproduction in Domestic Animals (4th Ed.). New York, NY: Academic Press Inc.; 1991).

2.6.4. Placental transfer

Issues of placental transfer of test chemicals must also be considered (see van der Laan et al., 2010-this issue) (Table 9). Transplacental transfer of anti-arrhythmics has been studied in the pig and shown to be closer to the transplacental transfer in humans than the (often used) pregnant ewe model (Wiest, Swindle, Garner, Smith, & Gillette, 1996).

In several species, maternal antibodies are transferred to the fetus across the placenta during pregnancy. These species include for example rats, mice, rabbits, guinea pigs, dogs, monkeys, apes and humans. However, *in utero* antibody transfer does not occur in those mammalian species with greater numbers of interhemal placental layers and lacking a functional yolk sac placenta. In these animals (e.g. horses, pigs, cows, sheep, and goats), newborns obtain antibodies by ingestion of immunoglobulin-rich colostrum (first milk). Antibodies are absorbed by the intestinal epithelium of the newborn. (Faber & Thornburg, 1983; Rothkotter, Sowa, & Pabst, 2002).

The placenta of the pig has a six layered epitheliochorial structure and is impermeable to the passage of macromolecules such as immunoglobulins, throughout the whole period of gestation (Table 10). There are no maternal-derived antibodies in newborn piglet, and maternal antibodies are provided in the colostrum in the first 6 hours after farrowing (Wagstrom, Yoon, & Zimmerman, 2000) (Table 11). These are mainly IgG antibodies and other immunoglobulins are transmitted in negligible amounts. After cessation of colostrum transfer, mostly IgA is found in milk, providing local mucosal protection (Table 12).

For these reasons, colostrum-deprived newborn piglets are an interesting model for studies of the ontogeny of immunity, being completely immuno-naïve. Because placental antibody transfer does not occur in the minipig, it is not expected to be an informative model for the study of antibody therapeutics (or vaccines) in embryo–fetal toxicity studies.

2.6.5. Embryo–fetal toxicity studies

Where rats and rabbits are unsuitable as the species of choice for embryo–fetal toxicity studies, the selection of an alternative species can be difficult (for example, in the case of antibiotics where the rabbit cannot be used). The relatively short gestation period (114 days) and large litter size (circa 5 fetuses/primiparous litter) make the minipig an advantageous choice, when compared with dogs or primates.

Table 6
Comparative estrous cycle data.

		Periodicity of estrous phase (days)	Estrous	Ovulation
Dog	Diestrous	180	7–13 days	Spontaneous
Minipig	Polyestrous	21	47–56 h	Spontaneous
Cynomolgus	Polyestrous	28	–	Spontaneous

Table 7
Comparative gestation characteristics.

	Days	Offspring/litter	Litter/year
Dog	58–63	4–8	1–2
Minipig	114	6*	2.2
Cynomolgus	160–170	1	1

* Primiparous minipigs: 4–6 piglets, multiparous minipigs: 5–9 piglets in litter.

The following study design for a minipig embryo–fetal study is suggested:

Minipig embryo–fetal study Suggested study design

- Group size: 14–18 Göttingen minipigs per group.
- Primiparous pregnant females: age 6 to 8 months.
- Exposure: Gestation Days (GD) 11–35 (organogenesis).
- Route of administration as appropriate: (oral, subcutaneous, or intravenous etc.).
- Fetuses: Collected by caesarean section on GD 109–111.
- Examination of fetuses: (follows that for rabbits)
  - o external and visceral examination of fresh tissue at necropsy
  - o skeletal examination of Alizarin stained bones
  - o Heads from half of the fetuses are fixed in Bouin's fixative, sectioned and examined for abnormalities and described according to the terminology published by Wise et al. (1997).

Background (historical) data on variations, anomalies and malformations are available (Berggren, Jensen, & Boegh, 2008; Damm Jorgensen et al., 1998).

A range of external variations and anomalies are observed in the minipig. Some of these changes appear spontaneously in 1 to 6% of the fetuses. The most common are fetuses with domed heads, exophthalmos (protruding eye), enlarged orbital, open or slightly open eye, hyperflexion of the limbs and polydactyly. More severe anomalies are seen more rarely.

A few visceral variations and anomalies are seen in the minipig. The most common findings are malpositioned testes (seen in approx. 12% of the male fetuses) and dilatation of the ventricles of the brain (in approx. 5% of all fetuses).

A number of congenital skeletal anomalies have also been observed, including asymmetrical sternebrae, misshapen sternebrae, fused ribs, shortened ribs, a rudimentary 14th rib, cervical ribs, rudimentary cervical rib, cervical rib fused with thoracic rib, fused ulna and radius and polydactyly and absent ossification site cranial of proximal(s). There is a broad range of variations in the degree of ossification in a number of bones and more than 4% of the minipig fetuses display non-ossified or incomplete ossification of the following bones: central hyoid, cervical arches, cervical centrum, cervical processus spinosus, thoracic arches, thoracic centrum, thoracic processus spinosus, lumbar centrum, ossification center distal of fibula, ossification center proximal of the humerus, ossification site(s) distal of metacarpal(s), and tarsal bones.

The pig has been shown susceptible to the teratogenic actions of thalidomide, hydroxyurea, aminopterin, and ethanol (cited in Jørgensen, 1998a); the teratogenicity of tretinoin (Jørgensen, 1998b) and pyrimethamine (Yamamoto et al., 1984) have been demonstrated in the Göttingen minipig.

In summary, features of the reproductive biology such as the rapid sexual maturity, short spermatogenic cycle, duration of gestation and litter size make the minipig a promising alternative non-rodent species for use in reproductive toxicology studies.

Table 9
Comparative placentation.

Species	Placenta	Yolk sac development
Human	Discoidal, haemomonochorial	(+)
Monkey	(Double) Discoidal, haemomonochorial	(+)
Minipig	Diffuse, epitheliochorial	(+)
Dog	Zonary, endotheliochorial	+++
Rabbit	Discoidal, haemodichorial	+++
Rat	Discoidal, haemotrichorial	+++
Mouse	Discoidal, haemotrichorial	+++

From: D.E. Atkinson, R.D.H. Boyd and C.P. Sibley, Placental Transfer, In: Jimmy D. Neill (Ed.): Knobil and Neill's physiology of reproduction, 3rd ed., 2006. Raven Press, Page 2787ff; http://placentation.ucsd.edu/.

Table 10
IgA-concentrations of immunoglobulins (mg/ml).

Species	Serum	Colostrum	Milk	Nasal secretion	Saliva	Tear fluid
Pig	2.0	10	5.0	–	–	–
Dog	0.5	15	4.0	–	0.2–1.3	–
Man	3.0	12	1.5	1.3	0.1	0.2

2.7. Endocrine glands

The anatomy and histology of the endocrine organs in minipigs are similar to those in humans and other species.

Table 8
Comparative embryonic development.

Source	Minipigs (Becze & Smidt, 1968; Glodek & Oldigs, 1981)		Rabbits DeSesso (2006)	Rhesus monkeys DeSesso (2006)	Humans (Sadler, 1995; DeSesso, 2006)	
Event	Gestation day	Length (mm)	Gestation day	Gestation day	Gestation day	Length (mm)
Two-cell stage	1		1	1	1	
Morula	3			4	4	
Blastula	5–6			5–8	4–5	
Somite appear	14–15	3	7.75–8.25	20–21	20–21	2–3
Optic vesicle formed	16	3,5–4,5	9	23–25	24–25	3–4,5
Neural groove closing	16–18	4,5–5,5	9–10.5	25	24–27	3–5
Upper limb buds appear	18	5,5	10.5–11	25–26	26–27	3,5–5
Hind limb buds appear	20	8,5	11–12	28–30	28–30/32	4–6
Digital rays present	28/24	14–15	14.5	34–35	36–42	9–14
Nipples formed	35/28	22.5			43–49	13–22
Eyelids formed	35	30–35		36–42	43–49	13–22
					48–51	16–18
Palate folds uniting	35	30–35	19.5	44–48	54–57	23–28
End of gestation	~114		30–32	166	~280/266	

Table 11
Immunoglobulin concentration in colostrum (mg/ml).

Species	IgA	IgM	IgG
Pig	9.5–10.5	2.5–3.2	30–70
Dog	5–22	0.14–0.57	1.2–3.0
Man	12.2	0.6	0.1

With regard to endocrine function, some areas are very well characterized, others are not well studied: Endocrine function affecting reproduction and growth is well characterized in the domestic pig because of the importance for agricultural use. The hormones of the hypothalamo-pituitary-gonadal axis are well described in prenatal development as well as in adult animals (Chung, Etherton, & Wiggins, 1985; Danchin & Dubois, 1982; Molokwu & Wagner, 1973). For the minipig, endocrinology of reproductive organs is also very well reported (Glodek & Oldigs, 1981). Therefore, the minipig is a suitable model to study the impact of drugs and chemicals altering genital organ function in prenatal animals (Choi & Jeung, 2003) as well as adult animals (Tellmann, in press). The minipig may be a more suitable model for testing of drugs used for gynaecology therapy and male fertility than the dog. The estrous cycle in the minipig is closer to that of the human. The dog has been considered unsuitable for chronic toxicity studies of contraceptive steroids, in particular because of the comparatively high incidence of spontaneous mammary tumors with the pig explicitly discussed as the possible better model (Owen & Briggs, 1976). Furthermore, the dog shows some differences in hormonal control of the mammary gland because of the importance of locally produced growth hormone (Lüdtke-Handjery, in press). Finally, because the dermal administration route is common for male sex steroids, the minipig presents some advantages because of the similarity of dermal absorption and pharmacokinetics (Xing, Lin, & Chien, 1998).

The function of the endocrine pancreas is well described including methods to monitor this function, since the pig and the minipig are well-established models in diabetes and obesity research (Larsen et al., 2005, 2006; Strauss et al., 2008; Wieczorek, Pospischil, & Perentes, 1998).

Another well-studied area is the impact of glucocorticoids, mainly in the context of osteoporosis, but also with respect to impact of stress (Hizume et al., 2006; Pufe et al., 2003; Scholz-Ahrens et al., 2007).

At necropsy, the parathyroid glands are difficult to locate in the pig (and are often missed), since they are not next to the thyroid gland as in many other species but are located by the cranial part of the thymus next to the larynx.

2.8. Immune system

The porcine immune system has been the subject of substantial research efforts and much of this research has been related to infectious diseases of the domestic pig (by zoonotic pathogens such as *Salmonella* and *Campylobacter*, and by animal-specific pathogens such as Foot-and Mouth disease), driven by the economic importance of the pig in agriculture. As a result, the basic knowledge of the porcine immune system is certainly more extensive than that of the dog or old world monkeys. Multiple databases of pig immunology resources are available (http://eis.bris.ac.uk/~lvkh/welpig.htm, http://ars.usda.gov/Services/docs.htm?docid=6065, http://www.vetschool.bris.ac.uk/research/infectimmun/pig_immunology/).

The basic structure of the porcine immune system resembles that of other mammalian organisms, with some species-specific features (of the presence of chimaeric IgM/IgD (Zhao, Pan-Hammarstrom, Kacskovics, & Hammarstrom, 2003), composition of lymphocyte subsets (Saalmuller, Werner, & Fachinger, 2002), lymph node structure and lymphocyte trafficking (Binns & Pabst, 1994)). These differences have been studied in detail, and do not appear to represent biologically significant differences in the overall functioning of the immune system.

2.8.1. General anatomy

The lymph nodes have inverted morphology when compared to humans, dogs and primates but this difference does not affect the function of the lymph nodes. The inverted morphology means that there are hardly any lymphocytes in the efferent lymph system; they enter efferent lymph node venules directly and hence the venous system.

There are about 20 individual Peyer's patches in the jejunum and one long Peyer's patch in the terminal ileum. (which may have a coordinating role in the development of the humoral immune system and/or may function as a primary lymphoid organ).

2.8.2. Immunoglobulins

Pigs produce 4 major classes of immunoglobulins: IgM, IgG, IgA, and IgE and five different IgG molecules: IgG1, IgG2a, IgG2b, IgG3, and IgG4.

IgD has not been found in pigs. Serum IgA is found as a dimer as in other animals with a molecular weight of MW 350 Kd (Table 13). The ratio of kappa and lambda light chains is 1:1.

2.8.3. Swine leukocyte antigen system

The MHC system of pigs (swine leukocyte antigen: SLA system) is well characterized. The SLA system has three expressed class I loci: SLA-A, SLA-B and SLA-C; two expressed class II loci SLA-DR and SLA-DQ as well as several pseudogenes in the MHC-area (Table 14). There are more than 40 class I allotypes, an unknown number of class II allotypes as well as several class III genes (and products) which have been identified. The genomic sequence of the SLA complex has been mapped (Renard et al., 2006).

In commercial pig breeds most SLA-I haplotypes (H01–H68) are easily defined by serology using a panel of internationally standardized SLA-I alloantisera or mAbs. DNA profiles of 20 of these serotypes have been established using a SLA-I cDNA probe and RFLP (Renard et al., 1988; Ruohonen-Lehto et al., 1998).

Pigs of defined specific haplotypes (Chardon, Renard, Gaillar, & Vaiman, 2000) and Göttingen minipigs of defined SLA haplotypes can be obtained for research purposes. Haplotypes H04, H10 and DC80 can be defined using SLA-I antisera in complement-dependent lymphocytotoxicity testing, as described by Gronkjaer (2000). Haplotype DC80 is very frequent (approximately 40%), but one or more serologically blank haplotypes (i.e. for which no antisera are available) are present in these minipigs.

SLA-I serologically typed Ellegaard Göttingen minipigs have been successfully used in research on experimental lung transplantations.

Table 12
Immunoglobulin concentration in milk (mg/ml).

Species	IgA	IgM	IgG
Pig	3.0–7.0	0.3–0.9	1.0–3.0
Dog	1.1–6.2	0.1–0.54	0.01–0.03
Man	1.5	0.4	0.1

(Sources for Tables 10–12: Pastoret et al., 1998; Tizard, 2009).

Table 13
Serum concentrations of immunoglobulins (mg/ml).

Species	IgG	IgM	IgA	IgE	IgD
Pig	17–29	1.0–5.0	0.5–5.0	***	***
Dog	7–20	0.7–2.7	0.2–1.5	0.023–0.42	
Rabbit	5–20	1	3–4	***	
Man	8–16	0.5–2.0	1.5–4.0	$2 \cdot 10^{-5}$–$5 \cdot 10^{-4}$	0.1

*** Found but not quantified.

Table 14
SLA system.

	MHC-I	MHC-I	MHC-I	MHC-II	MHC-II	MHC-II	MHC-II
Name	A	B	C	DQ-A	DQ-B	DR-A	DR-B
Number of loci	1	1	1	1	2	1	1 (+1*)

DP, DO and DZ pseudogenes have been described.

Using donor and recipient minipigs that were SLA-I mismatched for one or both haplotypes, the immunosuppressive drugs tacrolimus and cyclosporine could be compared (Warnecke et al., 2005).

As in humans, pig MHC loci are polymorphic and each haplotype encodes molecules which bind and present different subsets of possible peptides. (Gerner et al., 2006). The probability that the subset presented by any haplotype will overlap to any great extent with human MHC alleles is low, but this also applies to other animal models, including the mouse.

2.8.4. Lymphocytes and subpopulations

Compared with other mammalian species, the gamma/delta T-cell count is significantly higher in certain tissues and there are higher percentage CD4/CD8 double positive T lymphocytes in peripheral blood. The function of these cell populations is unknown although a memory function has been suggested. Gamma/delta positive T-cells are divided into three categories: $CD2^-$ $CD4^-$ $CD8^-$; $CD2^+$ $CD4^-$ $CD8^-$; and $CD2^+$ $CD4^-$ $CD8^{low}$.

Porcine natural killer cells are defined as having phenotype: $CD5^-$, $CD6^-$, and $CD8^{low}$ (Table 15).

Known adhesion molecules that have been described include: LFA1 (CD11/CD18), CD44, E-selectin (CD62E), and VCAM-1 (CD106).

The information above and tabulated material are based on Pastoret, Griebel, Bazin, and Govaerts (1998), Tizard (2009) and Anon (1998).

2.8.5. Functional aspects of the pig immune system

Studies illustrating the different functional responses of the pig immune system are found in the literature. Drugs such as cyclosporin A, FK506 and prednisolone are all immunosuppressive in the pig (Gruessner et al., 1995). The T-cell dependent antibody responses to KLH are well documented in the pig and breed differences in response have been described (see Joling et al., 1993). Lymphocyte subsets are defined and reagents are available for the principal porcine lymphocyte populations. The role of porcine cytokines in pathophysiological responses to infectious diseases and in disease models has been characterized (Souza, Cheetham, Azevedo, Costantini, & Saif, 2007; Steinberg et al., 2005; van Gucht, van Reeth, & Pensaert, 2003; van Reeth & Nauwynck, 2000). Some data are available relating to the reactivity of pigs to human cytokines (Batten, Yacoub, & Rose, 1996) (Table 16).

With this background of functional responses of the pig, the evaluation of immunotoxicity in the minipig can be addressed (Table 17). Considerable information is available concerning the minipig immune system and immunological tools are available for pigs, although this is not all yet available in the open literature (Hinton, 2000). After adaptation of several immunotoxicological endpoints for use in minipigs, they have been successfully used in a subacute immunotoxicity study in Göttingen Minipigs® using the classical immunosuppressive compounds cyclosporin A and dexamethasone (personal communication, A.H. Penninks and G. van Mierlo, TNO). Next to various quantitative (immuno)toxicological endpoints such as clinical signs, body weight, lymphoid organ-weights and -histopathology, haematology, and lymphocyte subset analysis in peripheral blood mononuclear cells (PBMC) also immune function parameters were determined. Potential effects on the function of the immune system were measured by the T-cell-dependent antibody response (TDAR) to KLH, the delayed type hypersensitivity (DTH) response upon intradermal KLH injection, the ex vivo mitogen and KLH-induced lymphocyte proliferation, and the Natural Killer (NK)-cell activity in peripheral blood mononuclear cells (PBMC). From the results obtained, it was clear that the different assays showed variable sensitivity in assessing immunosuppressive effects of the selected immunosuppressive compounds and that, apart from some further improvement of some immunological tools (e.g. a better marker for B cell determination), immunotoxicology assessment in the minipig is indeed feasible. (A.H. Penninks and G. van Mierlo, in preparation).

Table 15
Porcine T-cell receptors.

Number of constant (C) TCR gene segments			
TCR α-chain	TCR β-chain	TCR χ-chain	TCR δ-chain
1	1	3 (maybe 4)	1

Number of V-gene segments not yet determined.

Table 16
CD groups on defined porcine leucocyte subpopulations.

T-cells	CD2, CD3, CD4, CD5*, wCD6, wCD8, SWC1, SWC2
Activated lympfocytes	CD25
B cells	wCD1, wCD21, SWC7
Macrophages	SWC3, CD14, SWC9
Null cells	CD2 negative, gamma/delta TCR positive T lymfocytes: SWC 4–6.
Lymphoid cells	CD16, CD18, wCD29, SWC8, wCD44, CD45.

2.8.6. Pig immune system and biotechnology products

Improved understanding of the functioning of the immune system has opened up many opportunities for novel therapeutic approaches based on targeted interventions or manipulation of the human immune system. Many such projects are currently under development and some products are already in therapeutic use (e.g., Herceptin, Rituxan, Tysabri, Betaferon and others).

As a general principle, the toxicology testing of such products should be performed in animal models in which the (immuno)-pharmacological action is reproduced (since many toxic actions of drugs are related to their intended pharmacological reactions). It will therefore be increasingly important to have access to laboratory animals which can model the reactions of the human immune system to interventions of different kinds. Rodents have often proved inadequate models for such products, and this reason is often used to justify the use of nonhuman primates. If the minipig is going to be a relevant toxicology model for the future, it will therefore be important to establish the extent to which it can contribute to the evaluation of this category of biotechnology products.

A significant gap in our knowledge concerns the extent to which homologous molecular targets in porcine tissues are reactive to human biologics (cytokines, monoclonal antibodies and others). In a survey of current practices (described below), as regards the development of monoclonal antibodies, only 2 out of 22 surveyed companies stated that they include the minipig in the screen for species relevance (cross-species target binding affinity/relative potency) prior to selection of species for safety assessment. In order to gain a better vision of the potential utility of the pig/minipig as a model for the safety testing of new biologics, it is crucial to generate data of this kind. In a prospective initiative, companies should be encouraged to include minipigs in the early screening for selection of

Table 17
CD numbers identified in pigs.

CD1	CD4	CD8	CD18	CD29	WC1	WC4	WC7
CD2	CD5	CD14	CD21	CD44	WC2	WC5	WC8
CD3	CD6	CD16	CD25	CD45	WC3	WC6	WC9

species responsive to the pharmacodynamic actions of new biologics. In a retrospective initiative, a survey of the responsiveness of porcine molecular targets of existing biologics would be a valuable and informative exercise which could make a useful contribution to our understanding of the future role of the pig in safety assessment.

2.8.7. Immunology conclusions

Pig immune system shows analogous structure and function to human immune system. Some differences are known and have minor impact (with possible exception of gamma delta cells and greater role of innate immune system). The porcine immune system has been well studied and is probably better characterized than that of the dog or the Macaque monkey. Nevertheless, little effort has been dedicated to evaluation of the relevance of the pig immune system specifically as a model for therapeutic interventions (or toxic actions) in humans. This certainly hinders the greater (informed) use of the pig for the evaluation of new therapeutic approaches based on immune system manipulation.

2.9. Central nervous system

Since the first use of minipigs as a model of MPTP-induced Parkinsonism (Mikkelsen et al., 1999) and in a model of diffuse brain injury with relevance for Alzheimer's disease (Smith et al., 1999), a considerable amount of knowledge has accumulated. In their extensive review of the use of pigs in neuroscience, Lind et al. (2007) conclude that the gyrencephalic pig brain is more similar to the human brain than the brains of common small laboratory animals. In addition they state that a considerable amount has been learned about pig brain anatomy and neurochemistry, but cortical function is not yet sufficiently well described.

Various research tools concerning the central nervous system are available for pigs. This includes a number of stereotactic atlases (e.g. Anderson & Reiner, 1990; Felix et al., 1999; Salinas-Zeballos, Zeballos, & Gootman, 1986; Watanabe, Andersen, Evens, Gjedde, & Cumming, 2001) and the increasing availability of pigs with transgenic manipulations of neural genes (cf. Lind et al., 2007). The large size of the pig brain permits the detailed identification of cortical and subcortical structures by imaging techniques such as positron emission tomography (PET) or magnetic resonance imaging (Lind et al., 2007). In addition, various behavioral testing methods are also established for pigs, including open field, anxiety and social behaviour testing (reviewed by Lind et al., 2007), automated video tracking of locomotor activity (Lind, Arnfred, et al., 2005; Lind, Gjedde, et al., 2005; Lind, Vinther, et al., 2005), and operant learning in a test battery similar to a battery already used for rats, primates and humans (Ferguson, Gopee, Paule, & Howard, 2009). Yet another learning test, a spatial delayed non-match to sample task, has recently been described for the Göttingen minipig (Nielsen et al., 2009).

Although many of the neurobehavioral methods are not immediately relevant for regulatory toxicity studies the accumulating knowledge in this area has toxicological significance for two reasons. The first reason is the requirement that whenever possible the selected species should be responsive to the primary pharmacodynamic effect of the substance (Note for Guidance on Repeated Dose Toxicity, CPMP/SWP/1042/99). In other words, a species which represents a good pharmacological model for brain disorders (to be used for the development of drugs to treat them) is from this perspective also good model for toxicity testing. The second reason is the potential use of minipigs in postnatal reproductive toxicity and juvenile toxicity studies. Behavioral testing is an important part of such studies. Therefore it is very helpful that meanwhile a number of behavioral tests are well-established in the (mini)pig (see above). In addition, brain development of the neonatal pig appears in many respects more similar to the human newborn infant than that of young rodents or dogs (Lind et al., 2007), including the important "brain growth spurt" originally described by the group of Dobbing (Dickerson & Dobbing, 1966; Dobbing & Sands, 1979).

2.10. Anatomy and biology of the eye

After the primates, the porcine retina, pupil, and lens bear the closest resemblance to human eyes and the porcine eye anatomy shares many similarities with the human eye. As in humans, the macula is composed only of cones in pigs. It is located in the visual axis of the bulb, thus enabling good visual acuity in daylight and excellent color vision in these species. The advantages of the porcine model are the holangiotic retinal vasculature (fully vascularised, unlike the rabbit), the choroidal blood flow and the absence of a tapetum. In addition, the thickness and surface area of the porcine sclera make the porcine eye a suitable model for studying transcleral drug delivery. Pigs do not have a true macula, but they have a narrow horizontal *area centralis* with photoreceptors, in a region that mimics the primate macula (Olsen, Sanderson, Feng, & Hubbard, 2002; Prince, Diesem, Eglitis, & Riskell, 1960).

In spite of the smaller axial length, data on refractive error and corneal power in the Göttingen minipig are comparable to human values. A lack of accommodative reflex is probable. This information may be of importance when using minipigs as models for human ocular diseases or in research involving vision, e.g. in cognitive tasks (Nielsen & Lind, 2005).

Background data on ophthalmological findings and electroretinography in the minipig are available (Ausburger et al., 2009; Loget & Saint Macary, 2000).

2.11. Skin and dermal toxicity studies

Porcine skin resembles human skin. As in humans, the hair coat is sparse. The skin is thicker in older minipigs, and is less vascular than in humans (Mortensen, Brinck, & Lichtenberg, 1998). The thickness in older minipigs compared to humans is due to a thickening of the dermis. The thickness of the stratum corneum and epidermis remains fairly constant after minipigs have reached three months of age.

Göttingen minipig skin is lightly pigmented as a result of the "dominant white" genetic status (see Simianer and Köhn, 2010-this issue). In contrast to rodents, dogs, rabbits, porcine skin also has fine intersecting lines and rete ridge structures (it is closely attached to the underlying structures). Apocrine sweat glands are absent in pigs (Swindle & Smith, 1998). Similar to humans, pigs have lipid biophysical properties, epidermal turnover kinetics (1 month), and an aptitude for tanning (Meddahi, 1994).

Based on the similarities of the minipig and human skin the minipig is the species of choice in dermal studies to evaluate both local tolerance and possible systemic toxicity after dermal application. Moreover, the abundant surface area of the minipigs allows multiple-site and long-term testing. Since visual assessment of skin reactions may not be fully consistent between studies and observers, non-invasive instrumental measurements (e.g. reflectance measurements with a chromameter, high-resolution ultrasonography, transepidermal water loss due to skin lesions by an evaporimeter and monitoring of skin blood flow and cutaneous vasoconstriction by laser Doppler flowmetry) may be applied for a quantitative evaluation of local treatment-related effects (Vogel, Kolopp, Singer, & Cordier, 1998); these types of examinations are more easily performed in minipigs than in smaller species or animals with dense fur coat.

Chemical compounds, depending on their physicochemical properties and the area of dermal administration, generally show a high percutaneous penetration through the skin of densely haired animals, whereas permeability and metabolic properties of the porcine skin appear to be more comparable to man (Lin, Hou, Hsou, & Ye, 1998; Poet & McDougal, 2002; Ngawhirunpat, Opanasopit, & Prakongpan, 2004). The skin penetration of several compounds was found to be

similar between pig and human skin, whereas no significant correlation existed between human skin and hairless dog or nude mouse skin (Reifenrath, Chellquist, Shipwash, & Jederberg, 1984). Furthermore, low intra- and inter-variations were evident for different substances in *in vitro* permeability studies using Göttingen minipig or domestic pig skin in comparison with human skin (Qvist, Hoeck, Kreilgaard, Madsen, & Fokjaer, 2000).

The similarities between human and minipig skin with regard to clinical, histological and immuno-histological findings further support the minipig as a model for the investigation of immuno-pathological mechanisms and pharmacological intervention (Vana & Meingassner, 2000). A pig model has also been established for the investigation of inflammatory skin lesions and anti-inflammatory treatment (Nair et al., 1993). A more exotic administration route is on the upper snout to investigate the neurotoxic potential of anticancer compounds on subcutaneous cranial nerves (Mahl et al., 2006).

2.12. Musculoskeletal system

2.12.1. Skeletal muscle

Like in all mammals, striated muscle of pigs is derived from three different areas of mesoderm: The so-called myotomes of the somites, the branchial arches, and locally formed mesenchyme not derived from somites (Table 18). The skeletal muscle fibers are postmitotic syncitia derived from fusions of embryogenic precursor and therefore multinucleated.

It is widely accepted, that mammalian skeletal muscle is composed of different fiber types, which are made up of a distinct set of structural proteins (e.g. myosin) and metabolic (glycolytic and/or oxidative) enzymes (Schiaffino & Reggiani, 1996) (Table 19). At the end of fetal development, the first differences between muscle fibers are already found which allow to differentiate between (light-stained, slow-twitch) type I fibers and (dark-stained, fast twitch) type II fibers (Brooke & Kaiser, 1970). Slow fibers acting for postural functions, are tonically active and fatigue resistant, and have an oxidative metabolism as the dominant source of ATP synthesis. Fast fibers, on the other hand, are phasically active, for which glycolytic metabolism is functionally appropriate (Peter, Barnard, Edgerton, Gillespie, & Stempel, 1972). Most of the proteins involved in contractile function, for example the myosin heavy chain (MyHC), and the sarco(endo)plasmic reticulum Ca^{2+}-adenosine triphosphatase, exist as multiple isoforms (Schiaffino & Reggiani, 1996), which are usually distributed in a myofiber type specific and coordinated manner (Hämäläinen & Pette, 1995). Fiber typing based on acid and alkaline denaturation of myosin molecules by using myofibrillar adenosine triphosphatase histochemistry leads to a further subdivision of the myofibers (Peter et al., 1972): Type I fibers: SO (slow oxidative), Type IIa fibers: FOG (fast oxidative glycolytic) and type IIb fibers: FG (fast glycolytic). On basis of monoclonal antibodies and of mRNA content of the respective fiber type, a subdivision of type II fibers into types IIa, IIx and IIb can be made, without considering the existence of further subtypes, intermediate and mixed type variation or possible posttranslational shifts of fiber types (Schiaffino & Reggiani, 1994, 1996).

These different fibers contain different myosin heavy chain (MyHC) isoforms. So far, eight isoforms of myosin heavy chain (MyHC) in skeletal muscle of mammals are known (Weiss et al., 1999). In porcine muscle, four myosin isoforms have been characterized: one cluster contains type I (or β) MyHC on chromosome 7, whereas the second cluster contains the embryonic, IIa, IIx, IIb, neonatal, and extraocular MyHC on chromosome 12 (Davoli et al., 2002). In postnatal growing pigs, only four isoforms, type I, IIa, IIx, and IIb MyHC, are expressed in skeletal muscle (Lefaucheur, Ecolan, Plantard, & Gueguen, 2002).

In humans, striated muscle, fiber type and myosin heavy chain (MyHC) expression is extremely heterogenous (Schiaffino & Reggiani, 1994), e.g. the relative distribution of type IIb fiber in the M. vastus lateralis can vary from 0 to 44% with a coefficient of variation of ~66% within a sedentary population (Simoneau & Bouchard, 1989).

In pigs, MyHC composition differed between muscle types and breed. In most breeds as well as in wild boar, the relative number of white fibers (IIx and IIb types) was between 80 and 85% (Wimmers et al., 2008). Type IIb fibers, the isoform exhibiting the most rapid speed of contraction, are the predominantly isoform in fast-contracting muscles of pig (Lefaucheur et al., 2002). The differences in histochemical properties between breeds were, however, smaller in M. adductor than in M. longissimus dorsi. In addition, the variation in muscle fiber composition in pigs within the breeds was larger than the average variation between the breeds (Ruusunen & Puolanne, 2004). Only the native Vietnamese Mongcai breed shows lower proportions of type II fibers (47%) when compared to other conventional breeds (Wimmers et al., 2008). In the cross breed Duroc × Berlin Miniature pigs, type II muscle fibers have contributions of 70% type IIb and IIx and 15% type I measured as relative MyHC isoform transcripts or as histochemical differentiation (Wimmers et al., 2008).

Overall, there is a considerable correlation between contractile, metabolic, and morphological features in skeletal muscle of minipigs. This correlation allows discrimination of muscle fibers according to their MyHC content into a system of discrete types. In consequence, the minipig muscle does not exhibit dramatic variations in phenotypic expression of their myofiber properties with regard to those previously reported in other small and large species of mammals or in humans with either similar or different expression of MyHCs (Schiaffino & Reggiani, 1996).

Daily muscular training by treadmill-running in Yukatan Minipigs led to an increase in citrate synthetase enzyme activity in various skeletal muscles. The number of type IIB fibers decreased after the training, whereas the type IIxX fibers increased in numbers, indicating a transformation of fiber types and that skeletal muscle of Yucatan minipigs adapts to endurance exercise training in a manner similar to muscle of humans with increased oxidative capacity (McAlister & Laughlin, 1997).

The calcium release channel (CRC) gene mutation responsible for halothane-sensitivity and "porcine stress syndrome" in domestic pigs has not been demonstrated for Göttingen minipigs.

Table 19
Relative distribution of skeletal muscle fiber types.

Fiber type	Human	Pig	Minipig	Dog	Rat
I	50–65%	9–11%	20–45%	18–28%	5–28%
II	IIa: 30% IIx: 10–20% IIb 0–44%	IIa: 4–12%; IIx: 18–20%; IIb: 60–71%	IIa: 15–40%; IIx: 10–50%; IIb: 25–55%	IIa: 43–67% IIx: 15%	IIa: 19–50% IIb: 8–75%

Table 18
Principal characteristics of skeletal muscle fiber types.

Property	Type I	Type IIa	Type IIx	Type IIb
Morphology and color	Slighted, red	Huge, white	Huge, white	Huge, white
Oxidative capacity	Low	High	High	low
Glycolytic capacity	Low	High	High	High
Contraction time	Slow	Moderately fast	Fast	Very fast
Metabolism	Aerobic	Anaerobic (long-time)	Anaerobic (short-time)	Anaererobic (short-time)

2.12.2. Bones and joints

Bones consist of organic matrix (=osteoid) with incorporated minerals (mainly Ca and P). During the pre- and postnatal period, bones are developed from connective tissue (desmal ossification, i.e. skull) or from preformed hyaline cartilage (peri- and enchondral ossification, i.e. long bones) (Table 20). By birth most of the shafts of the bones are ossified but the ends are still cartilagenous. From birth to puberty, secondary ossification centres appear in the cartilagenous ends of the bones. Eventually, the whole bone will be mineralized except for the articular surfaces which remain covered by cartilage. A thin zone of cartilage – the growth or epiphyseal plates – persists between the end of the bone and its shaft. Long bones growth by the interstitial growth of the epihyseal plate in which chondrocytes are produced by repeated cell division. The chondrocytes form palisades and are growing towards the bones end and approach the diaphyseal side of plate, where they enlarge and their matrix becomes mineralized. Cells next to the marrow cavity die, their matrix disappear and leave "tunnels" invaded by vessels and osteoblasts. The osteoblasts build bony lamellae on the inner wall of those tunnels until only a narrow channel (Harvesian channel) remains and the osteoblasts become osteocytes. Growth in width is achieved by deposition (appositional growth) of new bone on peri- and endost (Hochberg, 2002). The skeleton is mature when epiphysis, metaphysis and diaphysis are mineralized and fused (Gilli, 1996), that is at an age of over 2.5 years in minipigs (Reinwald & Burr, 2008).

In minipigs, the peak bone mass is reached between 3 (Bouchard et al., 1995) and 6 years (Tsutsumi, Katagiri, Morimoto, et al., 2004), in humans at an age of 30–40 years (Soyka, Fairfield, & Klibanski, 2000). Peak bone mass is the maximal bone mineral density present at the end of the skeletal maturation, is an important determinant of osteoporotic fracture risk.

The histomorphometric examination of bones of miniature pigs showed that the bone apposition rate and trabecular thickness in minipigs is similar to that in humans (Honig & Merten, 1993). With the help of freeze-fracture scanning electron microscopy (SEM) Kääb, Gwynn, and Notzli (1998) could demonstrate that the collagen fiber arrangement in pig and dog articular cartilage is very similar to the leaflike arrangement found in humans, whereas cow, sheep, rabbit or rat present a columnar pattern.

The presence of true osteonal remodelling is another desirable characteristic in the Göttingen minipig model. Remodelling means the mechanism by which the basic multicellular unit, of old bone or damaged bone is removed and replaced with new bone without alteration in the bone's shape. Modelling of bone means bone growth and shaping.

The remodelling and especially the forming of secondary osteons is a characteristic of porcine bone that is relevant to humans and is an improvement on the rodent model with its lack of secondary osteons (Martiniakova, Grosskopf, Omelka, Vondrakova, & Bauerova, 2006). Animal models may be classified as either modelling (rats) or true remodelling (i.e. pigs, dogs, ewes, and primates). In the rat, epiphyseal growth in the long bones continues for 2 years and longer, and there is no plate closure, in the sense of remodelling and disappearance (Jowsey, 1968). Rat cortical bone is made of primary lamellar bone (periosteal and endosteal lamellae) which is less affected by bone remodelling compared to human or porcine bone which contains secondary osteonal bone ("harvesian bone") (Hillier & Bell, 2007). Therefore, rat cortical bone is not necessarily a suitable model for studying the changes of mechanical property due to changes in bone remodelling.

Table 20
Basic bone data of different species.

	Human	Minipig	Dog	Rat
Skeletal maturity (years)	18–25	>2.5	1.3	>2/lifelong
Maximum bone density (years)	30–40	6	6	1
Remodelling Time (month)	8–10	3–6	3	35 days

Due to the similarity of skeletal parameters and bone-healing rates the minipig is a well-established model in bone and joint surgery. The GMP is also used for studies on bone metabolism (Tsutsumi, Katagiri, Takeda, et al., 2004) dental and craniomaxillofacial surgery (Honig & Merten, 1993), osteoporosis (Ikeda et al., 2003), bone and fracture repair (Merten, Wiltfang, Honig, Funke, & Luhr, 2000), implant fixation (Wong, Eulenberger, Schenk, & Hunziker, 1995), healing of articular cartilage defects (Hunziker & Rosenberg, 1996), chondral and osteochondral defect repair (Gotterbarm et al., 2008) and total hip replacement (Thomsen et al., 1997) in orthopaedic research.

2.12.3. Musculoskeletal system: Gaps of knowledge

There is limited knowledge on the occurrence of spontaneous lesions of the musculoskeletal system known from swine, which are attributed to the enormous growth potential of commercial slaughter pigs and include exertional myopathies like the porcine stress syndrome and skeletal disorders like "leg weakness" and osteochondritis. Such lesions should be less frequent or absent in minipigs, as the body weight gain is much lower than in commercial pigs.

3. Regulatory toxicology testing

3.1. Species-specific toxicity

Species-specific toxic actions are occasionally encountered in regulatory safety testing and account must be taken of this species-specificity in extrapolating to man, and risk evaluation. Examples are given below to illustrate some advantages and disadvantages of regulatory toxicology testing in the minipig as a consequence of species-specific reactions.

As reviewed by Dogterom, Zbinden, and Reznik (1992) and Lehmann (1998), dogs are particularly sensitive to sympathomimetics and hypotensive drugs, showing cardiotoxicity at doses close to the ED_{50}. As a consequence, it is difficult to assess systemic toxicity other than exaggerated pharmacodynamic activity in this animal model (Dogterom et al., 1992). In the example presented by Lehmann (1998), a benzodiazepine piperidinyl derivative with sympathomimetic activity caused tachycardia of similar extent in both dogs and minipigs. However, myocardial necroses were seen in the dog, but not in the minipig. The minipig was therefore considered more predictive of human responses. Dogterom et al. (1992) proposed the use of more suitable species like the pig as an alternative to dogs for the safety evaluation of vasodilators and positive inotropic/vasodilating drugs.

Active renal clearance mechanisms for organic acids are ubiquitous in mammalian species, but the dog is known to have a much lower capacity of this saturable carrier system for the excretion of organic acids from the kidney than other species. This leads to an increased sensitivity of dogs to organic anions, making the dog an unsuitable non-rodent model for human risk and safety assessments of organic acids (reviewed by Timchalk, 2004). It should be noted that excretion by this active transport mechanism is not affected by protein binding, i.e. organic acids bound to plasma proteins are readily transported across the proximal tubule epithelium. Consequently, comparison of the phamacokinetics of phenoxyacetic acids demonstrated that pigs (and rodents) were better models for the toxicity evaluation of these compounds than dogs (Timchalk, 2004).

Elliott, Purmalis, VanderMeer, and Denlinger (1988) suggested that gastrointestinal lesions provoked by proprionic acid nonsteroidal anti-inflammatory drugs (NSAIDs)–the most important (dose-limiting) adverse effect of this class of drugs are caused by systemic exposure rather than local irritation. This was concluded, because such lesions have been seen after both parenteral and oral administration. The order of sensitivity to NSAID's was described as dog>rat>non-human primates. Minipigs were not mentioned in this paper. The

question arises whether the dog-specific lower capacity to excrete organic acids described above might be involved in the exaggerated sensitivity of the dog towards NSAIDs. This idea was subsequently taken further by Lehmann, who used the minipig as the non-rodent toxicology model for the development of the NSAID meloxicam (Yabe et al., 1997).

In contrast, proquazone, another NSAID compound which is not a propionic acid, was more toxic in minipigs than in dogs (van Ryzin & Trapold, 1980). In a 15-month oral (capsule) study using beagle dogs, proquazone induced gastrointestinal irritation causing ulceration, peritonitis and death at 40 and 80 mg/kg, whereas doses up to 75 mg/kg were tolerated without effects in a 14-week study. In a 26-week study in Hormel minipigs, proquazone was administered with small amounts of food at doses of 0, 6, 15, 38 and 94 mg/kg. The death rate increased dose-dependently from 1/12 pigs at 6 mg/kg to 10/12 pigs at 94 mg/kg, and all deaths were attributed to gastric ulceration and its consequences.

Another example for pig specific toxicity was provided by Henne-Bruns, Arthwohl, Broelsch, and Kremer (1988). The attempt to establish a pig model of acetaminophen-induced acute hepatic failure was unsuccessful. The pigs developed methemoglobinaemia before hepatic failure, because of acetaminophen's capability to oxidize hemoglobin. This observation is commensurate with the known low capacity of the pig's methemoglobin-reductase, (i.e. approximately one third of the human capacity), whereas the estimated spontaneous methemoglobin-reductase in the dog is more than two-times higher in the pig (Smith, 1996).

Spontaneous (background) pathology must not be confused with species-specific reactions. Four types of background lesions in barrier-bred Göttingen minipigs should however be kept in mind during the histopathological assessment of tissues from toxicity studies (Svendsen, Skydsgaard, Aarup, & Klastrup, 1998): (a) vasculitis, which may occur in different arteries or arterioles, (b) tubular hypoplasia in the testis, (c) serous atrophy with a decrease cellularity in the bone marrow (restricted feeding may be involved, cf. Bollen & Skydsgaard, 2006), and (d) the so-called hemorrhagic syndrome. These spontaneous lesions are rare, most likely because of the high microbiological quality of barrier-bred Göttingen minipigs (Skydsgaard, 2005).

3.2. Juvenile toxicology studies

For non-rodent species such as the dog or nonhuman primate, juvenile toxicity studies present a number of practical and logistical challenges. Examples of these challenges include smaller litter sizes in comparison with rodents, long gestation periods, long pre-weaning periods, space requirements and cost. Compared with other non-rodent animal models such as the dog or nonhuman primate, the biological characteristics of the minipig are already favourable: the litter size is relatively large (5 newborns per sow), the rate of growth is very rapid, sexual maturity is achieved at an early age and piglets are accessible for handling. Weighing about 450 grams at birth, they are amenable to laboratory procedures.

There are three basic approaches to the study design. One is to use the whole litter as the basic group or experimental unit. The second is to treat animals within a litter differently from each other. The third approach is to randomise the allocation of offspring to mothers at birth (cross-fostering) and then to treat all animals allocated to one mother as a group. All three approaches have advantages and disadvantages, but the cross-fostering approach offers the most potential benefits.

Cross-fostering in minipigs is possible without difficulty and is routinely done at the production facility. Randomised cross-fostering of piglets may be performed on Day 1 after birth. The piglets may be born on site or they may be born at the breeders and transported, with their mothers, on the day after birth to the experimental laboratories. Oral gavage dosing can be performed from Day 1. Vascular access ports can be implanted in 5–7 day old minipigs and have been shown to remain functional for four weeks. The ports used were intended for rats and despite the rapid growth during the first four–five weeks after birth these ports remained functional. During the second week after birth, the piglets may be subjected to electrocardiographic and ophthalmological examination. Blood and urine may routinely be collected from week 1 for haematology, clinical chemistry, bioanalysis and urinalysis examinations.

The data obtained for the various experimental parameters have been compared with background data from older minipigs. There were several notable findings. For instance, at one week of age, the values for red blood cell parameters (hemoglobin, red cell count and haematocrit) were all lower than historical values for 4 to 6 month old animals. However, at 4 weeks of age the values for these parameters had moved into the same range as the older group. In comparison with older animals, serum values for alkaline phosphatase, bilirubin, gamma-glutamyl transferase, cholesterol, triglycerides and urea were elevated. The mean values for several of these parameters at week 4 were more in line with those of older animals. The reasons for these differences are easily explained in terms of the animal age, diet, etc. However, they do underline the importance of having adequate background data for the age and strain of animals used in any study.

It is concluded that cross-fostering of minipigs for performance of juvenile toxicology studies is a practical option. However, there needs to be allowance in the number of animals allocated to the study in case of early deaths; this complication is also well known from juvenile animal studies in rats or dogs. Also, it is important that the mothers are allowed to give birth at the site where the study will be conducted. The importance of having adequate background data for the animals is highlighted.

The Göttingen minipig is a promising non-rodent animal model for juvenile toxicity studies, because of advantageous characteristics such as a large mean litter size (5 newborns per sow), high rate of growth and rapid achievement of sexual maturity. The piglets are accessible and amenable to handing (unlike for example, nonhuman primates, where the neonates are carried by the mothers) and cross-fostering is feasible. A number of behavioral tests are well-established in the pig (see Section 2.9). In comparison with other non-rodent animal models such as the dog or cynomolgus monkey, the minipig appears to be an eminently practical choice for this kind of study.

3.3. Predictivity of minipig toxicity studies

In this article the potential benefits and disadvantages of the minipig as a suitable model predictive for humans have been analysed and the comparative anatomy, physiology and biochemistry of the pig and minipig have been reviewed. Some areas of species-specificity of the minipig and other non-rodent toxicology models have also been discussed.

Numerous examples illustrating the close similarities of porcine and human anatomy, physiology and biochemistry have been identified in almost every organ system or physiological process that was considered (including the dermal, cardiovascular and renal systems, functionality of the gastrointestinal and immune system, comparable hepatic biotransformation etc.).

- The cardiovascular conditions are similar, humans and minipigs show comparable anatomy, comparable electrophysiological functions with the major ion channels and comparable reactions to harmful influences.
- The comparability of hepatic P450 cytochromes between pigs and humans support the usefulness of minipigs as experimental animals to predict biotransformation pathways in man.
- The gastrointestinal system of the minipig offers some anatomical and functional similarities in terms of absorption and metabolism.
- The dermal structure and dermal reactivity is similar; the minipig is also important for transdermal penetration studies.

– The basic structure of the porcine immune system resembles that of other mammalian organisms including humans.
– Due to the similarity of skeletal parameters and bone-healing rates the minipig is a well-established model in bone and joint surgery and has been used for studies on bone metabolism and osteoporosis. Minipig muscle and myofiber properties are comparable to other mammalian species.

These similarities suggest that the minipig will be a useful model in safety testing of new products. In addition to these broad similarities to humans, there are characteristics and features of the biology of the minipig that make it an attractive model in toxicology testing. A good example is given by reproductive toxicology testing, where the early achievement of sexual maturity, short estrous cycle length and large litter size make the minipig an attractive alternative to existing (non-rodent) models for reproductive toxicity testing. In other areas, the similarity of the skin structure and large surface area make the minipig attractive for cutaneous studies, the reduced propensity for vomiting make the minipig a useful alternative for some classes of orally administered drugs.

A greater role for minipigs in testing could thus bring advantages. We may anticipate that the similarities of the minipig to man result in a similar profile of toxic reactions in the minipig and man. Hence testing in minipigs may bring a more accurate prediction of adverse and harmful reactions in man. This is a conclusion that would bring significant economic and public health benefits.

We were not able to address and evaluate this issue of predictivity in the Safety Issues Working Group, since insufficient published material and comparative safety testing data is available to permit a rigorous evaluation of this hypothesis. Altogether our comparative biology analysis supports the hypothesis that the pig or minipig should be a good model for predicting human undesirable reactions. On the other hand, we have scoured the literature and could not identify sufficient material for systematic or carefully conducted studies to test this idea fully. It is therefore one of our conclusions that research is need to allow evaluation of the hypothesis that minipig studies may better reflect human drug-induced toxicities than studies performed in traditional non-rodent toxicology models (such as the dog).

There is a further issue in the application of the minipig to regulatory safety testing of biotechnology products (and hence to the replacement, in part, of primate studies). A significant gap in our knowledge concerns the extent to which homologous molecular targets in porcine tissues are reactive to human biologics (cytokines, monoclonal antibodies and others). In a survey of current practices as regards the development of monoclonal antibodies, only 2 out of 22 surveyed companies stated that they include the minipig in the screen for species relevance (cross-species target binding affinity/relative potency) prior to selection of species for safety assessment. The scientific literature is equally devoid of information and examples on this point. In order to gain a better vision of the potential utility of the pig/minipig as a model for the safety testing of new biologics, it is crucial to generate data of this kind. In a prospective initiative, companies should be encouraged to include minipigs in the early screening for selection of species responsive to the pharmacodynamic actions of new biologics. In a retrospective initiative, a survey of the responsiveness of porcine molecular targets of existing biologics would be a valuable and informative exercise which could make a useful contribution to our understanding of the future role of the pig in safety assessment.

In conclusion, insufficient comparative data is available to permit a rigorous evaluation of the predictivity of the minipig for human drug-induced toxicities. In addition, for minipigs to play a more significant role in the testing of biotechnology products and biologics, data is required on the pharmacodynamic responsiveness of minipig molecular targets to human biologic drugs.

3.4. Criteria for selection of non-rodent species

3.4.1. Individual company experience

A retrospective survey of the use of non-rodent species in 57 projects over the course of 10 years was performed by one of the pharmaceutical companies. Although drug classes are not disclosed here, this survey reveals the rationale for species selection in the past. In addition, a strategy is described for the current approach essentially in line with the requirements/recommendations of CPMP and ICH guidelines.

According to the survey species selection was based on the following considerations.

•Practicability (dog use)	30%
•Toxicology/pharmacodynamics	30%
•Drug metabolism	12%
•Inhalation (dog)	9%
(primates)	1%
•Proteins (use of primates)	4%
•Unknown reasons	14%

Currently non-rodent species selection at this company takes into account the following aspects based on experience and on data collected at an early stage of drug development:

- Species-specific inadequacies (e.g. exaggerated sensitivity of dogs e.g. concerning cardiovascular effects, emesis and histamine release);
- Animal PK and Drug Metabolism (adequate exposure, presence of human key metabolite[s], if any)
- Avoidance of primate use whenever possible with due regard of scientific and ethical justification
- Technical considerations (e.g. exposure techniques in inhalation toxicology)
- In case of similar suitability of dogs and minipigs various aspects of resource availability are taken into account.

Ideally the following information would be available for species selection prior to the start of the first non-rodent toxicity studies:

- Pharmacodynamic activity in the different non-rodent species;
- Exploratory toxicity data from the rat;
- Cardiovascular data after low single doses in telemetered animals of the different species;
- Information from front runner compounds (if applicable);
- in vitro metabolic stability in liver microsomes and/or hepatocytes of rat, dog, minipig, non-human primates, and man
- Active drug principle in vivo po/iv plasma profiles: rat, dog and/or monkey and minipig
- iv/iv correlation for clearance early assessment of metabolite(s) if identified as major carrier of action.

As a conclusion we recommend early consideration of the inclusion of the minipig when looking for the best model for predicting side effects in humans.

3.4.2. Current practice survey/results of a questionnaire

In order to better understand the way in which the minipig is currently used in pharmaceutical safety assessment, the way in which decisions are made regarding its use and the perceived barriers to the use of the minipig, a survey of current practice was undertaken. A questionnaire on "*The use of minipigs in safety assessment*" was prepared and submitted to pharmaceutical industry companies; this questionnaire and the detailed results are found in the Appendix A to this article. This questionnaire was distributed to the pharmaceutical industry via the following industry organizations: US PhRMA, BioSafe, EFPIA and ABPI. Responses to the questionnaire were received from 23 organizations.

The results of this survey showed that experience with safety assessment studies using minipigs was in safety pharmacology studies, in general toxicity studies (using dermal, oral and parenteral routes) and also in reproductive and immunotoxicity studies. The experience in each individual company was limited.

Literature sources and communications via the questionnaire revealed that the minipig is used for dermal, oral and parenteral administration. Studies by the intravenous route are possible, but as the result of technical difficulties are less frequent. Dermal application in particular is considered to be advantageous in minipigs.

A minority of companies (6/22) routinely include the minipig in the screen for pharmacological activity and/or metabolic clearance profiles used to aid selection of the non-rodent species for safety assessment of low molecular weight products.

For monoclonal antibodies, only 2/22 companies stated that they include the minipig in the screen for species relevance (cross-species target binding affinity/relative potency) prior to selection of species for safety assessment.

Six companies who used minipigs for safety assessment used this species as the single non-rodent species (for the drug project), and five companies used minipigs as a second (supporting) non-rodent species. Four companies had experience of using minipigs for safety assessment both as the only non-rodent species and as a second non-rodent species. The reasons stated for conducting studies in minipigs as a second non-rodent species are outlined below:

- To try to reproduce a finding observed in humans but not in monkeys
- Clarification of organ toxicity to determine species-specificity
- Not tolerated in dogs
- Clarification of effects
- General tolerability issues
- Metabolism issues
- Consideration of possible metabolic differences
- Poor exposure in dogs, special susceptibility of dog to vomiting caused by test substance
- Occasionally for dermal programs to supply supporting data when another non-rodent species has been used
- Dermal toleration for dermal product/cutaneous patch programs.
- GI tolerability of a new formulation
- Pigs are used only if they offer a more relevant model of human physiology, disease or drug disposition/metabolism.

The majority of companies (14/22) perceived hurdles to the use of minipigs as a more general non-rodent species in safety assessment. These perceived hurdles fell into 5 main categories:

1. the size of minipigs and corresponding high compound requirement, particularly for chronic studies;
2. lack of experience in the use of minipigs and issues relating to historical control data;
3. animal husbandry, supply and logistics;
4. technical aspects and staff training;
5. lack of reagents and diagnostic kits.

About half of the companies stated that the lack of commercial kits for laboratory diagnosis and immune function testing for minipigs influenced their decision not to use minipigs more generally in safety assessment.

In summary, the results of the survey illustrate that many companies are already using the minipig as a first or second line non-rodent toxicology model in regulatory studies for the registration of new drugs. The reasons for selecting the minipig are various, but scientific in nature. The “hurdles” that are cited to the greater use of the minipig illustrate the need for further availability of training and scientific support for the model and development of reagents.

4. Gaps and research opportunities

The gaps in knowledge and/or research opportunities are presented under two general headings of results that would better characterise of the model, and results that would permit the proper deployment of the minipig in safety testing.

4.1. Characterisation of the minipig model

In reviewing the comparative biology of the minipig, many topics have been described where further research would bring greater understanding. In presenting these recommendations, we have limited our proposals to areas where further understanding or more detailed knowledge of the minipig would catalyse more informed and/or broader exploitation of the minipig model for regulatory safety testing. These areas are as follows:

1. *Inhalation toxicology and respiratory administration*: this is currently an area of weakness for the minipig model. There are few publications and methods are not well-established for inhalation toxicology or respiratory function studies. Until inhalation approaches are better established, the minipig will not be seen as an alternative model for products administered by this route. A program of work to provide basic information in this area (RMV values, deposition efficiency) and to consolidate methods (inhalation administration, respiratory function measurements) would open the way to the use of the minipig for such products.
2. *Reproductive toxicology*: A number of actions would strengthen the role of the minipig in reproductive toxicology. These include (i) further characterisation of the different stages of spermatogenesis in the minipig boar, (ii) extended background data on spontaneous fetal variations and anomalies in the minipig and (iii) information on the placental transfer of macromolecules. It is known that antibodies do not cross the placental barrier in pigs, but very little hard data is available regarding other large molecules and macromolecules. This point requires clarification in order to better understand the product categories where the minipig can be a relevant model for embryo–fetal toxicity testing.

4.2. Deployment of the minipig model

Proposals for research that will permit the proper deployment of the minipig model are as follows:

3. *Collaborative programs*: some issues are best addressed through collaborative programs rather than focussed research actions. Data that could be collected in this way that would better define the role of the minipig in regulatory toxicology testing includes:

    a. Cross-species comparative data on metabolism providing a database and case-histories where the minipig does or does not provide a close model of drug metabolism and disposition in humans.
    b. It is generally believed that the minipig provides a better model of human cutaneous reactions than other animal models such as the rabbit or the rat. For percutaneous absorption data is available to demonstrate the value of the mini(pig) model, but for skin tolerance we do not have quantitative data. It is known that the correlation between rabbit and human irritant responses is poor for weak irritants (Steinberg, Akers, Weeks, McCreesh, & Maibach, 1975), but the corresponding data is not available for the pig. This is an important point to establish, both in the interest of drug safety and also since it could open the way for highly predictive porcine skin-based *ex vivo* models

4. *Predictivity of the minipig for human toxicities* (discussed in the following section):

   a. A predictivity testing program is urgently needed in which an adequate number of compounds or drugs (for example ten) of well characterized toxicity would be tested in both the minipig, the dog and the monkey in order to demonstrate that predictivity in the minipig is at least as good as the monkey and better than the dog. Such a result would be a significant finding and should provoke rethinking about the role of monkeys in the testing of new drugs.

5. *Safety testing of new biologics*: (discussed in the following section). In order to gain a better vision of the potential utility of the pig/minipig as a model for the safety testing of new biologics, it is crucial to generate data on the responsiveness of porcine molecular targets to human biologics. Two proposals are made to address this point:

   a. In a prospective initiative, companies should be encouraged (by guidance documents or by research initiatives) to include minipigs in the early screening for selection of species responsive to the pharmacodynamic actions of new biologics.
   b. In a retrospective initiative, a survey of the responsiveness of porcine molecular targets of existing biologics would be a valuable and informative exercise which could make a useful contribution to our understanding of the future role of the pig in safety assessment.

5. Discussion and conclusions

5.1. Current use of the minipig in toxicity testing

In this article current knowledge about the use of the minipig as a laboratory animal model for safety testing has been reviewed with respect to the comparative biology of the pig and minipig and the implications for their use in safety testing.

Minipigs are already routinely used for regulatory toxicity testing, typically for general toxicity studies. In Europe the Göttingen minipig is selected most frequently for such studies. There is some interest amongst toxicologists in the use of the minipig in reproductive toxicity testing and in the performance of juvenile animal studies, although the routine use of the minipig for these studies is not yet widespread. The current number of minipig regulatory toxicology studies performed in Europe is estimated at circa 150 studies per annum (Estimate from Ellegaard Göttingen Minipigs ApS).

In general it can be stated that studies performed in minipigs are acceptable to regulatory authorities. This question is reviewed in detail in van der Laan et al. (2010-this issue), where examples of products in commerce which were developed using the minipig as the non-rodent toxicology model are also given.

Current use of the minipig in regulatory toxicity testing and attitudes of the toxicology community to the minipig were explored through a questionnaire which was distributed to representative organizations including ABPI, EFPIA, PhRMA (DruSafe) and BioSafe. From the responses to the survey received from 22 companies, the following general points emerged:

- Minipigs are used principally for toxicity studies with small molecules rather than biologics and macromolecules
- The minipigs are considered to be a useful model for cutaneous toxicity studies, general toxicology studies and safety pharmacology.
- In terms of testing strategy, the minipig is used both as first line or second line non-rodent species. It is considered useful in cases where:
  - species-specific toxicity is encountered in the non-rodent model
  - the test item is poorly tolerated (e.g. vomiting in dogs) or
  - other species have divergent metabolism from humans
- Handling of minipigs requires an investment in training of technicians
- As a consequence of the body weight of the minipig, compound requirements for the studies are substantially greater than in the primate or dog.

5.2. Review of the comparative biology of the minipig

A large part of the present article has been dedicated to a detailed review of the comparative biology of the minipig, taking physiological systems in turn. In particular the minipig was compared with the two non-rodent species commonly used in toxicity testing, the dog and the macaque monkey. This review not only demonstrates the general utility of the minipig as a non-rodent model in toxicology. It also documents numerous examples where the pig and/or minipig is considered to have close similarities with humans or to be an excellent model for human physiology and responses. The review has also provided the opportunity to highlight some features of minipig that facilitate toxicity testing or that make the minipig a particularly convenient or relevant model for toxicity testing.

Many of these points are presented in Table 21 below and may be summarized as follows:

- Studies of general toxicology can be performed in the minipig by oral, cutaneous, parenteral and inhalation routes
- For reproductive toxicology studies he minipig offers numerous advantages as a non-rodent model. Nevertheless, the lack of placental transfer of antibodies (and of macromolecules with molecular weight exceeding 5000) may limit the role of the minipig in reproductive testing of some categories of biotechnology products.
- For safety pharmacology studies the minipig is an advantageous model.

Overall the minipig presents a very favourable profile as a non-rodent toxicology model, in terms of the similarity to man and also in terms of applicability to different study types.

5.3. Opportunities for the application of 3R's

The safety assessment of pharmaceutical or chemical products is based today on the combination of *in vitro* and *in vivo* testing. Regarding *Replacement*, as long as there is no regulatory or scientifically justified chance to replace *in vivo* assays by *in vitro* studies, there will be no option to replace animal studies. In such circumstances it does not matter which animal model one selects to use, rodents or non-rodents, as has been described in Webster et al. (2010-this issue).

Regarding the contribution for *Refinement*, the analysis presented in this article stresses the importance of selection of the relevant and most predictive animal species for extrapolation to humans. The minipig has a number of advantages as outlined above. The selection of appropriate species may contribute to improvement of the predictability of toxic actions and adverse events in humans and therefore improve the refinement of investigations by offering better anatomical, functional, metabolic conditions and meaningful biomarkers to monitor and evaluate drug-induced changes.

Regarding *Reduction*, there are options to reduce the number of animals used for the preclinical safety evaluations, if current strategies are changed and improved. Today, minipigs are often the second choice when results from dog studies seem insufficiently predictive for humans. In contrast, if the minipig is included in early orientating, dose-range finding and metabolic studies, as an optional species for selection of the best model for humans, it should contribute to the avoidance of unnecessary and/or irrelevant animal studies and in this way support initiatives to reduce the conduct of irrelevant assays in inappropriate animal models.

Table 21
Utility of the minipig in regulatory toxicology.

Study type	Remarks on application and utility of the minipig
In vitro toxicology	Pig organs and tissues for in vitro studies can be obtained from abattoirs, avoiding the need to sacrifice animals only to obtain biological materials. Minipig tissues can be obtained from the Ellegaard Göttingen Minipigs. Minipig hepatocytes and microsomes are commercially available, facilitating evaluation of minipig as second (toxicology) species.
Pharmacokinetics, toxicokinetics and DMPK studies	There is a high degree of similarity between human and porcine hepatic CYP P450 cytochromes (although there is a lack of published examples or case-histories showing the similar pharmacokinetic behaviour of drugs in pigs and humans).
General toxicology Oral administration	Broad similarity of humans and porcine digestive tracts. Both species are true omnivores, and GI tract has many similarities to man including intestinal pH values, transit times and drug absorption. Drug administration can be achieved by oral gavage, capsule or dietary admixture. Technician training is required for gavage administration. Minipigs are much less prone to emesis than dogs.
General toxicology	Inhalation administration can be performed in the minipig. Stronger data is required for supporting background information (such as respiratory minute volume and pulmonary distribution of inhaled particles of different sizes).
Nasal and respiratory tract administration (Inhalation)	Nasal administration can be performed in the minipig, but administered product may be lost through sneezing or contaminated by material from "grubbing". Pulmonary tissue reactions may be influenced by the large number of intravascular pulmonary macrophages present in the pig.
General toxicology	Single dose intravenous administration of up to 5 mL is achieved through the ear vein.
Parenteral administration	Repeated intravenous administration requires catheterisation (and as a consequence, individual housing). Cannulation of the deep vessels for more than a month can be performed on a routine basis.
General toxicology	Porcine skin is an excellent model for human skin in terms of anatomy, physiology, biochemistry and immunology.
Cutaneous administration	There is a good correlation between transdermal absorption and local tolerance in the minipig and in humans. The abundant surface area of skin and scarce hair follicles of the Göttingen minipig permit long-term cutaneous testing and multiple-site cutaneous testing. It is also adapted to the testing of patches and depot preparations. The "dominant white" skin of the Göttingen minipig permits easy evaluation of cutaneous reactions. During toxicity studies by cutaneous administration, minipigs will usually require individual housing.
Reproductive toxicology Segment I (fertility) studies	Minipigs offer a convenient model since they rapidly achieve sexual maturity (before six months of age) and the spermatogenic cycle is of relatively short duration (35 days). The different spermatogenesis stages are not as well characterized as in the rat, dog or primate.
Reproductive toxicology	Compared with other "second species" the minipig has a short gestation period (113 days) and large litter size (5–6 fetuses/litter) making it an attractive model for Segment II studies. Initiatives are underway to increase the existing database of fetal variations and anomalies. Previous studies have demonstrated the sensitivity of this model to drugs shown to be teratogens in humans. Available data does not support concerns expressed in ICH guideline S5, relating to malformation clusters in pigs (Berggren, Jensen, & Boegh, 2008).
Segment II (embryofetal toxicity) studies	Placentation in the pig is dissimilar to humans. Available data suggests that placental transfer of "small drugs" is similar in pigs and humans, but there is less transfer of macromolecules, making the minipig an inappropriate model for Segment II studies with macromolecule biotechnology products. For monoclonal antibodies and vaccine products the minipig is not an appropriate model since there is no placental transfer of antibodies in this species.
Juvenile animal toxicology and Segment III studies	Compared with other "second species", the Göttingen minipig has a large mean litter size (5 newborns per sow), and the piglets show a high rate of growth and rapid achievement of sexual maturity. The piglets are accessible and amenable to handing (unlike for example, non-human primates, where the neonates are carried by the mothers) and cross-fostering is feasible. A number of behavioral tests are well-established in the pig. The minipig appears to be an eminently practical choice for this kind of study. Some experience has already been gained in the performance of juvenile studies in the Göttingen minipig.
Safety pharmacology Cardiovascular	The minipig has been shown to be a useful model. Ion channels controlling repolarisation are similar to those in humans. ECG recordings are best made using the Nehb–Spoerri lead system which takes account of pig anatomy. Post-prandial influences on cardiovascular parameters should be taken into account. The long QT interval of the pig does not influence the utility of the minipig as a model for QT prolongation. The minipig T-wave can show variable pattern and polarity but using minipig specific algorithms most beats can be captured by available computerized ECG waveform analysis programs(e.g. Notocord, EMKA).
Safety pharmacology Respiratory function	Respiratory function can be evaluated in the minipig if a large animal is needed; for approaches based on plethysmography rodents are generally practicable and sufficient.
Safety pharmacology studies CNS	A number of behavioral tests are well-established in the pig.
Safety pharmacology Other	For renal studies, urinary catheterisation is not possible in male minipigs.
Bone studies	Minipig reaches early skeletal maturity and is a true bone remodelling species. As in humans, bone density is influenced by estrogens.
Immunotoxicity studies	The immune system of the pig has anatomical and organisational specificities, but is functionally similar to other mammalian species.

5.4. Value, reliability and predictivity of the minipig in toxicity testing and the potential role of minipigs in non-rodent testing strategies

What conclusions can be drawn about the value, reliability and predictivity of the minipig as a model for the toxicity of drugs and chemicals for humans? And the potential role of minipigs in non-rodent testing strategies?

Everything that has been reviewed in this article leads us to believe that the minipig is a better non-rodent toxicology model than the dog. This is based on the numerous similarities to man that make the minipig a relevant model, and also on the characteristics of the minipig that make it a flexible and convenient animal model for the performance of general toxicology, reproductive toxicology and safety pharmacology studies.

Numerous anatomical, physiological and biochemical similarities between pigs and humans suggest that the minipig will be a useful model in safety testing of new products, giving a profile of reactions to

toxic actions of drugs similar to those in humans. As a consequence it seems probable that testing in minipigs could provide a more accurate prediction of adverse and harmful reactions in man than rodents or dogs. If true, this is a conclusion that would bring significant economic and public health benefits, and therefore deserves further investigation.

At the present time, however, there is very little comparative data to evaluate this question and insufficient comparative data is available to permits a rigorous evaluation of the predictivity of the minipig for human drug-induced toxicities. It is therefore a conclusion of this project that research is need to provide experimental data that allows evaluation of the hypothesis that minipig studies may better reflect human drug-induced toxicities than studies performed in traditional non-rodent toxicology models (such as the dog).

(i) A testing program to provide this data could be proposed in which an adequate number of compounds or drugs (for example ten) of well characterized toxicity would be tested in both the minipig, the dog and the monkey in order to demonstrate that predictivity in the minipig is at least as good as the monkey and better than the dog. Such a result would be a significant finding and should provoke rethinking about the role of monkeys in the testing of new drugs.
(ii) An alternative to a testing program would be the accumulation of data through a prospective validation exercise ("learning by doing").

There is a further issue in the application of the minipig to regulatory safety testing of biotechnology products. There would be significant interest if the use of the minipig for safety testing of biologics permitted the replacement, even in part, of primate studies. In order to evaluate the utility of the minipig for biologics and biotechnology products there is a significant gap in our knowledge; this concerns the extent to which homologous molecular targets in porcine tissues are reactive to human biologics (cytokines, monoclonal antibodies and others). In order to gain a better vision of the potential utility of the pig/minipig as a model for the safety testing of new biologics, it is crucial to generate data of this kind.

Two proposals are made to address this point:

(i) In a prospective initiative, companies should be encouraged (by guidance documents or by research initiatives) to include minipigs in the early screening for selection of species responsive to the pharmacodynamic actions of new biologics.
(ii) In a retrospective initiative, a survey of the responsiveness of porcine molecular targets of existing biologics would be a valuable and informative exercise which could make a useful contribution to our understanding of the future role of the pig in safety assessment.

6. Conclusions

It is therefore a major conclusion of this project that research is need to allow evaluation of the hypothesis that minipig studies may better reflect human drug-induced toxicities than studies performed in traditional non-rodent toxicology models such as the dog and the macaque monkey.

In order to gain a better vision of the potential utility of the pig/minipig as a model for the safety testing of new biologics, it is crucial to generate data on the responsiveness of porcine molecular targets to human biologics. It is a strong recommendation of this project that a survey of the responsiveness of porcine molecular targets of existing biologics should be undertaken. This would be a valuable and informative exercise which could make a useful contribution to our understanding of the future role of the pig in safety assessment.

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Appendix A. Pharmaceutical Industry Questionnaire on the use of minipigs in safety assessment

A.1. Introduction

A.1.1. Pharmaceutical Industry Questionnaire on the use of minipigs in safety assessment

At the beginning of the RETHINK project, the Working Group members had little information on the use of minipigs for safety assessment for human pharmaceuticals apart from general information from Ellegaard Göttingen Minipigs ApS, the suppliers of minipigs. Working Group members also considered the potential reasons that might be perceived as hurdles to the use of minipigs for safety assessment and it was considered that there was value in collecting more information on the use of minipigs in safety assessment directly from the pharmaceutical industry. This led to the questionnaire in Appendix A being distributed to the pharma industry via the following industry organizations: US PhRMA, BioSafe, EFPIA and ABPI. Only pharma companies who are members of one of more of these organizations would have received the questionnaire but this represents most of the major pharma and biotechnology-based companies.

Responses to the questionnaire were received from 23 organizations, 22 pharma companies and one CRO. Only the general comments received from the CRO were included in the analysis of responses to preclude the potential for double counting of responses. Responses were received from most of the big pharma companies. A summary of the questionnaire analysis is provided in Section 3 below.

Over half (13/22) of the respondents have experience of using the minipig in safety assessment for low molecular weight medicinal products, but only one company had experience of using the minipig for the safety assessment of protein therapeutic/monoclonal antibodies. A second company responded that although there was no experience of using minipigs for protein therapeutics/monoclonal antibodies, the respondent had "experience in a former life in a CRO of using domestic pigs extensively, mostly for transplant, dermal and cardiovascular products; most of the (many) mAbs encountered did not cross to pigs—if they had, there would have been difficulty identifying reagents and assays for the necessary immunophenotyping for these immunomodulatory products". When asked if there companies had experienced any problems in conducting studies in minipigs as a result of immunogenicity to a protein therapeutic product, one company responded YES, pigs (domestic) are permissive hosts for hAd-5 and form significant humoral and cellular immune responses when exposed. When testing hAd-5-related gene therapy products in pigs, immunogenicity was often encountered. Domestic pigs were also used for evaluation of PK properties of PEGylated IL-2 administered SC and IV with lymphatic catheters for sampling. In that study immunogenicity did not seem to be an issue, though that may reflect the fact that dosing was limited.

The type of safety assessment studies in which minipigs have been used is outlined in Table A1.

Although over half (13/22) of the pharma companies had experience if using minipigs for safety assessment, the experience within each company was limited: only one company had experience of performing more than 10 studies using minipigs, 3 companies had experience of performing 5–10 studies, and 9 companies had

performed only several safety assessment studies (<5) using minipigs.

Table A1
Study types where minipigs used.

Type of study	Number of companies
Safety Pharmacology—cardiovascular	5
<3m dermal repeat dose toxicity	7
>3m dermal repeat dose toxicity	4
<3m oral repeat dose toxicity	12
>3m oral repeat dose toxicity	5
<3m parenteral repeat dose toxicity	4
>3m parenteral repeat dose toxicity	0
Reproductive toxicity	1
Immunotoxicity	0
Other	1—model for adverse event, Bridging studies to compare formulations (requested from FDA if clinical dermal irritation study is positive) 1—GI tolerability

A minority (6/22) companies routinely include the minipig in the screen for pharmacological activity/metabolic clearance/profiles used to aid selection of the non-rodent species for safety assessment of low molecular weight products. An additional company did include the domestic pig in such a screen but this was based on the nature of products in the company portfolio. A further 2 companies sometimes included the minipig in such screening for low molecular weight products, depending on the nature of the product, for example for dermal products. Companies were asked to comment if they did not include the minipig in such screening and the comments received are outlined below:

Table A2
Comments on use of minipig.

- We include the minipig in our metabolite screening to aid the selection of the non-rodent species
- YES but not at the first pass
- Dermal products only
- The dog is the default second, non-rodent species
- Our default has been dog or NHP for non-rodents
- Dog and primate considered standard
- Unlikely choice over dog or non-human primate
- Normally we include dog, monkey and rabbit for metabolic clearance/profiles but have so far not routinely included minipig. I guess this is based rather on tradition than on any specific scientific rational
- We prefer the monkey
- Other non-rodent species provide adequate coverage/data
- Currently, not an accepted model
- Has not been perceived as necessary.
- No intention to use minipigs in DSE programs
- Lack of understanding of species-specific pharmacology and gene function, lack of assays, experience and historical data
- No historical control data base—only used for 1 GI tolerability study once

For monoclonal antibodies, only 2/22 companies stated that they include the minipig in the screen for species relevance (cross-species target binding affinity/relative potency) prior to selection of species for safety assessment. Several comments were also received in relation to this question: 1) have routinely screened domestic pigs—they were typically not cross-reactive with the mAbs under evaluation; 2) it is assumed that there will be immunogenicity issues.

Six companies who used minipigs for safety assessment used this species as the only non-rodent species, and 5 companies used minipigs as a second non-rodent species. Four companies had experience of using minipigs for safety assessment both as the only non-rodent species and as a second non-rodent species. The reasons stated for conducting studies in minipigs as a second non-rodent species are outlined below.

Table A3
Reasons stated for use of minipig.

- To try to reproduce a finding observed in humans but not in monkeys
- Clarification of organ toxicity to determine species-specificity
- Not tolerated in dogs
- Clarification of effects
- General tolerability issues
- Metabolism issues
- Consideration of possible metabolic differences
- Poor exposure in dogs, special susceptibility of dog to vomiting caused by test substance
- Occasionally for dermal programs to supply supporting data when another non-rodent species has been used
- Dermal toleration for dermal product/cutaneous patch programs
- GI tolerability of a new formulation
- Pigs are used only if they offer a more relevant model of human physiology, disease or drug disposition/metabolism

The majority of companies (14/22) perceived hurdles to the use of minipigs as a more general non-rodent species in safety assessment. These perceived hurdles fell into 5 main categories: 1) the size of minipigs and compound requirement, particularly for chronic studies; 2) lack of experience in the use of minipigs and issues relating to historical control data; 3) animal husbandry, supply and logistics; 4) technical aspects and staff training; 5) lack of reagents and diagnostic kits. About half of the companies stated that the lack of commercial kits for laboratory diagnosis and immune function testing for minipigs influenced their decision not to use minipigs more generally in safety assessment. Further details about the perceived hurdles can be found in the analysis of responses in Section 3.

A.2. Questionnaire

EU Framework 6 Project RETHINKMinipigs as models for toxicity testing of new drugs & chemicals

Questionnaire on the use of minipigs in safety assessment

This is a simple questionnaire with the objective to obtain a rapid and high level response from the pharmaceutical industry regarding the use of minipigs in safety assessment. Please return your questionnaires either to your industry association (EFPIA, BioSafe or phRMA DruSafe) or directly to Dr Jenny Sims (jenny.sims@astrazeneca.com) who is a member of the EU RETHINK project. Please reply by May 18th.

1. Has your company used minipigs for the safety assessment of low molecular weight human pharmaceutical products?

   NO YES
   If no, please give reasons:

2. Has your company used minipigs for the safety assessment of protein therapeutics/monoclonal antibodies?

   NO YES
   If no, please give reasons:

3. If you answered yes to one or both of Q1/2 please specify the type of studies in which minipigs were used

   Safety Phrmacology (specify nature of study)

   General repeat dose toxicolgy study

Dermal	< 3months duration	> 3months duration
Oral	< 3months duration	> 3months duration
Parental	< 3months duration	> 3months duration

   Reproductive toxicity
   Immunotoxicity study
   Other (please specify)

4. If you answered yes to one or both Q1/2 please give an estimate of the number of products for which the minipig has been used as a non-rodent species for safety assessment (e.g. several, <5, 5–10, >10 etc.)

5. For low molecular weight products, does you company include the minipigs in the screen for pharmacological activity/metabolic clearance/profiles used to aid selection of non-rodent species for safety assessment?

   NO YES
   If no, please give reasons:

6. For monoclonal antibodies does your company include the minipig in the screen for species relevance (cross-species target binding affinity/relative potency) prior to selection of species for safety assessment?

   NO YES
   If no, please give reasons:

7. For products which used minipigs for general repeat dose toxicity studies and/or reproductive toxicity were minipigs the only non-rodent species used in the safety assessment programme or an additional non-rodent species? Only non-rodent species second non-rodent species

   NO YES
   If no, please give reasons:

8. If minipigs were used as an additional non-rodent species to dogs or non-human primates please specify the usual reasons for conducting studies also in minipigs

   NO YES
   If no, please give reasons:

9. If you answered Yes to Q2, have you experienced any problems in conducting studies in minipigs as a result of immunogenicity to the product?

   NO YES
   If, yes, please provide more details, on an additional sheet if necessary

10. Do you perceive any hurdles to the use of minipigs as a more general non-rodent species in safety assessment

    NO YES
    If, yes, please provide more details.

11. Does the lack of commercial kits for laboratory diagnosis and immune function testing for minipigs influence your decision not to use minipigs more generally in safety assessment

    NO YES
    If, yes, please provide more details.

12. Are there any other reasons that have prevented you from using minipigs more generally in safety assessment?

    NO YES
    If, yes, please provide more details.

13. Any other comments?

A.3. Analysis of questionnaire responses

Responses were received from 23 organizations (22 Pharma companies and one CRO)

- Abbott
- Altana
- Amgen
- Archemix
- AstraZeneca
- Bayer
- Boehringer Ingelheim
- BMS
- GSK
- Lilly
- Merck
- Merck Serono
- NMS
- Novartis
- Novo
- Organon
- Orion
- Pfizer
- Roche
- Sanofi-Aventis
- Solvay
- Wyeth
- CRO: HLS (The comments that were only received from the CRO were included in the questionnaire analysis to preclude double counting).

Question 1: Has your company used minipigs for the safety assessment of low molecular weight human pharmaceutical products?

- 13/22 respondents have used the minipig in safety assessment for low molecular weight medicinal products
- Comments:
  - No, but we have used domestic (Yorkshire) pigs for testing the pharmacologic properties of oligonucleotide products (aptamers) in animal models of human disease. In the US, many CROs offer domestic pigs rather than minipigs. For relatively short-term studies this is preferable because of their larger size (that facilitates surgical instrumentation).

Question 2: Has your company used minipigs for the safety assessment of protein therapeutics/monoclonal antibodies?

- 1/22 respondents have used the minipig
- Comments:
  - No. In my former life in a CRO we used pigs extensively, mostly for transplant, dermal and cardiovascular products. Most of the (many) mAbs I have worked with did not cross to pigs. If they had, there would have been difficulty identifying reagents and

assays for the necessary immunophenotyping for these immunomodulatory products.

Safety pharmacology (specify nature of study)		
cardiovascular: 5 companies		
General repeat dose toxicology study		
Dermal	<3 months duration:	7 companies
	3 months duration:	4 companies
Oral	<3 months duration:	12 companies
	>3 months duration:	5 companies
Parenteral	<3 months duration:	4 companies
	>3 months duration	
Reproductive toxicity: 1 company		
Immunotoxicity study		
Other (please specify)		
• Model for adverse event, Bridging studies to compare formulations (requested from FDA if clinical dermal irritation study is positive)		
• GI tolerability		

Question 3: If you answered yes to one or both of Q1/2 please specify the type of studies in which minipigs were used

Question 4: If you answered yes to one or both Q1/2 please give an estimate of the number of products for which the minipig has been used

• 9 companies	Zero
• 9 companies	<5 studies
• 3 companies	5–10 studies
• 1 company	>10 studies

as a non-rodent species for safety assessment (e.g. several, <5, 5–10, >10 etc.)

Question 5: For low molecular weight products, does your company include the minipigs in the screen for pharmacological activity/metabolic clearance/profiles used to aid selection of non-rodent species for safety assessment?

• Yes	6/22
–1/6 screen domestic pigs (nature of products in company pipeline)	
• No	14/22
• Sometimes	2/22
• We include the minipig in our metabolite screening to aid the selection of the non-rodent species	
• YES but not at the first pass	
• Dermal products only	
• The dog is the default second, non-rodent species	
• Our default has been dog or NHP for non-rodents	
• Dog and primate considered standard	
• Unlikely choice over dog or non-human primate	
• Normally we include dog, monkey and rabbit for metabolic clearance/profiles but have so far not routinely included minipig. I guess this is based rather on tradition than on any specific scientific rational	
• We prefer the monkey	
• Other non-rodent species provide adequate coverage/data	
• Currently, not an accepted model	
• Has not been perceived as necessary.	
• No intention to use minipigs in DSE programs	
• Lack of understanding of species-specific pharmacology and gene function, lack of assays, experience and historical data	
• No historical control data base — only used for 1 GI tolerability study once	

Question 6: For monoclonal antibodies does your company include the minipig in the screen for species relevance (cross-species target binding affinity/relative potency) prior to selection of species for safety assessment?

• Yes	2/22
• No	20/22
• Comments:	
Yes, I have routinely screened domestic pigs. They were typically not cross-reactive with the mAbs I was evaluating	
No—because it's assumed that there will be immunogenicity issues	

Question 7: For products which used minipigs for general repeat dose toxicity studies and/or reproductive toxicity were minipigs the only non-rodent species used in the safety assessment programme or an additional non-rodent species?

• Only non-rodent species	6 companies
Used for repro	
We used domestic pigs and rats to evaluate several different human adenovirus-5 vector gene therapy products.	
• Second non-rodent species	5 companies
• Examples of both	4 companies

Question 8: If minipigs were used as an additional non-rodent species to dogs or non-human primates please specify the usual reasons for conducting studies also in minipigs.

- To try to reproduce a finding observed in humans but not in monkeys
- Clarification of organ toxicity to determine species-specificity
- Not tolerated in dogs
- Clarification of effects
- General tolerability issues
- Metabolism issues
- Consideration of possible metabolic differences
- Poor exposure in dogs, special susceptibility of dog to vomiting caused by test substance
- Occasionally for dermal programs to supply supporting data when another non-rodent species has been used
- Dermal toleration for dermal product/cutaneous patch programs.
- GI tolerability of a new formulation
- Pigs are used only if they offer a more relevant model of human physiology, disease or drug disposition/metabolism

Question 9: If you answered Yes to Q2, have you experienced any problems in conducting studies in minipigs as a result of immunogenicity to the product?

- No (but unsure whether this response refers to a protein therapeutic or DNA-based product(s)
- Yes. Pigs are permissive hosts for hAd-5 and form significant humoral and cellular immune responses when exposed. When testing hAd-5-related gene therapy products in pigs, immunogenicity was often encountered. I have also used domestic pigs for evaluation of PK properties of PEGylated IL-2 administered SC and IV with lymphatic catheters for sampling. In that study immunogenicity did not seem to be an issue, though that may reflect the fact that dosing was limited.

• Yes	14/22
• No	5/22
• Comments:	
No: Technicians are trained and can handle either dogs or minipigs. Clinical pathology can also evaluate blood samples from both species under GLP	
• Note: comments from question 12 have been incorporated into the analysis of question 10	
Question 12: Are there any other reasons that have prevented you from using minipigs more generally in safety assessment?	

Question 10: Do you perceive any hurdles to the use of minipigs as a more general non-rodent species in safety assessment?

Comments relating to size:

- Size, compound requirement
- Size
- Their size
- Amount of API
- Need for larger amount of test material(s)
- Compound needs (especially for long-term studies)
- High weight (studies of >3 months duration)
- Minipigs are larger than other species which will require more API
- Compound requirements are greater than in primate (or dog in longer term studies)
- Greater quantities of test compound needed for chronic studies
- For some companies their relatively large size will be an issue, with respect to test article requirements
- Excessive drug supplies for such a large animal.
- More compounds are needed due to higher body weight (generally in the range of 8–16 kg).
- In order to use sexually mature animals for development of biologics, this would represent a significant increase in requirements for drug substance (mature male cynos = 5–9 kg, mature female cynos = 3–6 kg) which would add significant delay to progress of novel biotherapeutics to humans.

Comments relating to historical control database, experience, regulatory acceptance etc.

- Lack of historical control database
- Lack of background data
- Historical background data for controls is not as extensive as other species
- Availability of historical datasets
- Historical database
- The lack of (and expense) of obtaining historical control data may be an issue
  - I have not seen published studies describing the use of pigs as a species for reproductive toxicity testing, though that seems useful
- General lack of familiarity resulting in project reluctance to use the species
- Limited historical data base for safety endpoints
- Lack of vast historic controls
- Gene function, pharmacology, historical data plus poor understanding of spontaneous disease/pathology in lab-based minipigs
- Lack of background data e.g. reproductive toxicology
- Lack of familiarity with their use
- Poor understanding of predictivity for humans
- For biologics, a better understanding of immunological profile and how it relates to humans is vital
- The dog is always the first choice; the minipig is only ever considered as a fall-back if dog and monkey are unacceptable
- Sponsors are unsure of using minipigs for multiple reasons
- Some sponsors generally only think in terms of dog and primate
- Dog is a “more comfortable” species
- Perceived lack of acceptability with regulators
- Regulatory acceptance
  - Request to duplicate the study(ies) with more conventional preclinical species (i.e. dogs, NHPs)

Comments relating to logistics etc.

- Logistics for housing and husbandry
- Housing and husbandry are not routine in our lab
- Housing and handling
- Primarily husbandry
- Facilities, facility planning—minipig would compete for dog housing
- In order to conduct pharmacology and early discovery/safety studies in minipigs in-house, major re-configuration of vivaria and associated management practices would be required
- Need to adapt in-house facilities or place studies at CRO
- Limited number of CROs and no in-house capabilities
- Availability of minipigs from vendors has been problematic on occasion in the past
- Animal sources limited (limited breeders)
- Facilities (non-spf) in the neighbourhood of pig farms

Comments relating to technical aspects, training

- Dosing procedure
- Handling, dosing
- Compared to dogs minipigs are more resource-consuming and challenging regarding handling (blood sampling, test item administration)
- Repeat dose intravenous dosing is problematic in minipigs with compounds affecting the coagulation
- Training is required to facilitate handling/dosing
- Technical staff not trained to dose, evaluate for abnormal clin obs, bleed, etc.
- Veterinary staff not experienced to recognize common background infections or treating ailments
- Technically and regarding handling mini pigs prove to require more attention and specialized skill, compared to beagle
- Animal handling
  - serial blood sampling for PK often results in quite some tissue damage
  - handling (requires more strength than most female biotechnicians have)

Comments relating to diagnostic kits/reagents

- Lack of diagnostic reagents
- Available assays
- Immune reagents
- Reagents not available for many of many parameters that are frequently evaluated (i.e. immunotox and troponin)
- Availability of pig kits in clin. path., historical database limited, lack of experience in labs

Question 11: Does the lack of commercial kits for laboratory diagnosis and immune function testing for minipigs influence your decision not to use minipigs more generally in safety assessment?

Question 13: Any other comments?

- For training reasons, we already performed a 4-week oral placebo study in minipigs including clinical pathology and histopathology.

• Yes	9/22
• No	10/22
• Lack of kits for immune function testing (and lack of experience, data) would present significant issues for the use of this species in developing immunomodulators, in addition to assessing unanticipated immunotoxicity for other molecules	
• No reference values. Also, lack of commercial kits is already an issue for immunotoxicity testing in dogs.	
• Availability of pig kits in clin. path., historical database limited, lack of experience in labs	
• This is not the primary concern, since current use is primarily dermal programs. It will become a greater concern, if use of pigs is more prevalent for a greater variety of programs (requiring many types of assays)	
• For example, pigs are used extensively for evaluating the role of complement activation with certain product-related hypersensitivity responses, yet there are essentially no reagents available to directly quantify the various components of the complement pathways. There are few reagents to allow traditional immunophenotyping also, assuming that one had a product (presumably a biologic) that crossed to a target expressed in pigs	
• Problem for laboratory diagnosis—no experience with immune function testing	

- In the future, we plan to use minipigs for safety assessment in pilot and dose-range finding studies (up to 2 weeks) in case dogs are not an adequate species
- We see a great opportunity for using minipigs as a second non-rodent species and the number of enquiries and studies we have conducted is currently burgeoning. This is generally for transdermal products and NCEs
- The immunological profile of the minipig requires further characterisation before we can determine its value in developing biologics
- We would definitely consider minipig as an option for safety assessment of dermal products and also for some products for cardiovascular treatments
- Expense and availability
- We would normally consider dog as the first option for oral small molecular weight compounds. Based on some pilot evaluation in one of our projects the bioavailability of the compound was very poor in the minipig which probably discouraged us to consider minipig as a real option
- Pigs are monogastric, omnivorous animals with GI and CV systems which are generally considered similar to humans. They are domesticated and will display clinical signs in the presence of humans which humans can recognize and interpret. Greedy pigs—a pig which doesn't eat all of its food quickly is a sick pig. Can you say that of a marmoset or a cyno?? Pigs ought to be considered prior to selecting NHP!

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