EFFECTIVENESS OF MEDICAL ALERT DOGS TO RESPONSE TO HYPOGLYCEMIA IN PATIENTS WITH TYPE 1 DIABETES.

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Appreciation

I want to thank all the professors who, during these five years, have always had patience and love in their teaching, so that they will always keep it. Special mention to Domingo Navarro Bosch, for helping me with the work in this final stretch of the career, we have formed an amazing team.

I would also like to thank my family for always supporting me, whether the times were against or in favour of the current; my partner, for his unconditional support and finally my friends, because the shared laughs are always the best. Without them this would not have been possible.

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Summary

Combining the dog's great ability to smell with its mutual love relationship with man, the use of dogs to assist people in various activities has become a matter of course. Dogs have long been used for hunting and ranching. More recently, they have been used to assist people with disabilities, for rescue and for the detection of thousands of volatile organic compounds (VOCs) related to drugs, narcotics, explosives, food and even diseases.

In recent years, the number of medical assistance tasks assigned to domestic dogs has increased. Diabetic Alert Dogs (DADs) have been shown to improve the quality of life of owners with type 1 diabetes. These dogs are a non-invasive method that can assist in blood glucose control. The canines can detect VOCs characteristic of hypoglycaemia allowing them to ``alert´´ to this condition with an average sensitivity of 80%. Some dogs are also capable of detecting hyperglycaemia and can even alert to people other than their owners and to different pathological conditions.

DADs still present several limitations. These include the enormous economic cost, lack of official certification, poor standardization of training, variability between breeds, within breeds and between individuals, variety in warning signals, ability of the owner to interpret the animal's alertness, and lack of follow-up and evaluation of the dog once it is placed with its new owner. The results of the studies are still very heterogeneous and further research is needed, but eliminating these limitations could make DAD an incredibly efficient method for diabetes management.

Keywords: Medical Alert Dogs, Diabetes Mellitus type 1, Hypoglycaemia, Olfaction.

Introduction

The domestic dog (*Canis lupus familiaris)* has been man's best friend for centuries, bringing great benefits to people with its company, both physical and psychological.

It has been shown that keeping a dog as a pet has a great impact on the health of the owner, affecting different routes, including the cardiovascular, endocrine and autonomic nervous system. Reducing stress, lowering heart rate and blood pressure, and increasing social-emotional function (Welles, 2019).

The possession of this pet has been related to an increase in the performance of physical activity, fewer medical consultations (Headey & Grabka, 2007) and in general, in the improvement of the owner's quality of life (Hughes *et al*., 2020). Thus, the interaction with these animals has also been related to an increase in the concentrations of beta-endorphins, prolactin, beta-phenylethylamine and dopamine (Nagasawa *et al*., 2015).

More specifically, the influence of this interaction with the regulation of decreased activity of the Hypothalamic-Pituitary-Adrenal (HPA) axis and the activation of the Parasympathetic Nervous System (PNS) and oxytocinergic system (OTS) was evidenced (Theo *et al*., 2022).

Interactions with dogs demonstrated a lower predisposition to depression, as well as an increase in the psychological well-being of people, decreasing the feeling of loneliness and increasing the capacity for empathy (Sable, 2013).

Dog ownership has been linked to the creation of family routines (Díaz Videla *et al*., 2015) and, according to several studies on the interactions between pets and owners, the aforementioned positive effects were evidenced after certain actions such as kissing the dog (Handlin *et a*l., 2012), physical contact (Rehn *et al*., 2014) and the holding of the gaze between the animal and the owner (Nagasawa *et al.,* 2009).

Apart from the general benefits of having a dog, they have been shown to help in the therapy of people with post-traumatic stress, (Lass-Hennemann *et al*., 2018) reducing their stress during crises, in the communication of children on the autism spectrum (London *et al.,* 2020), people with depression, etc.

In several hospitals, Assisted Therapy Dogs (ATD) have been included in various units such as paediatric oncology, intensive care (ICU) and palliative care to help the well-being of patients.

Since 2019, at the ``Hospital Universitario del 12 de Octubre´´ in Madrid, it has implemented the `` Huellas de Colores´´ program, in which dog-assisted interventions are carried out on children in the UCI and in Paediatric Resuscitation (REA) in hand-held of the PsychoAnimal association. According to the study, children experience a decrease in pain of up to three points on the pain scale, decreased anxiety and an increase in general mood.

All these benefits make the dog an excellent candidate not only to provide us with company, but also as a work animal due to its noble and mouldable character.

The best-known working dogs are those used for hunting, military assistance, herding, detection of bombs, drugs and other substances, rescue, assistance and medical alert dogs (MAD).

The MAD are those dogs trained to detect odours associated with different diseases in people and that can alert the owner before any crisis occurs.

Dogs have been used for medical support and assistance since the 1920s, when the first guide dog was trained (Reeve *et al.*, 2021). Since then, they have been seen in increasing numbers accompanying people with disabilities and other illnesses.

More than 20 years ago, certain organizations began training dogs to live with and alert people with unpredictable epileptic seizures 10-45 minutes before an episode. Studies suggested that owners experienced a decrease in the frequency of attacks (Strong *et al.*, 1999).

Due to the number of studies on MADs, more charities have begun to train dogs for these purposes with a view to using them in the future for early diagnosis of diseases through the establishment of biomarkers and to help the development of technologies based on their abilities, such as bioelectronics noses.

The first published case of a dog detecting cancer was that of a British woman who went to her dermatologist because her pet insistently barked, bite and licked a mole lesion on her leg. Subsequently, the malignancy of the tumour was confirmed (Williams & Pembroke, 1989).

Like this, there are numerous documented cases of dogs that "alert" their owners to the existence of cancer in a specific area, before the onset of seizures (Martos Martinez-Caja *et al.*, 2019), migraines (Marcus & Bhowmick, 2013), lowering blood glucose levels (Rooney *et al.*, 2013), etc. Owners claimed to see behavioural changes in their dogs even before they recognized their own symptoms.

This predictive behaviour of dogs is best understood by studying the canine olfactory system. This is made up of more than 220 million olfactory receptors that makes them capable of detecting odours in parts per trillion (Uemura, 2015), being ideal subjects for training in the detection of certain substances.

The immense olfactory acuity of these animals makes them capable of detecting volatile organic compounds (VOCs) associated with changes in the physiology of their owners (Rudnicka *et al*., 2014) and that form the "odour signature" of certain diseases. There are reliable VOC profiles associated with asthma, various types of cancer, liver disease, pre-eclampsia, kidney disease, tuberculosis, diabetes mellitus, and even SARS-CoV-2 (Jendrny, Twele, Meller, Schulz, *et al*., 2021; Shirasu & Touhara, 2011). These VOCs can be found in the feces, breath, blood, skin, saliva, sweat, urine, and vaginal secretions of affected individuals.

Although the greatest relevance of these animals at a medical and scientific level is focused on the detection of different types of cancer in people, their ability to perceive variations in blood glucose levels is currently being demonstrated.

The appearance of diabetic alert dogs (DADs) is very recent, with the first published case of a dog alerting its owner with type 1 diabetes to an episode of hypoglycaemia in 2003. Since then, there have been numerous published cases of dogs alerting their owners during hypoglycaemics episodes at night or while driving, which could be fatal.

Diabetes mellitus is a chronic disease that currently affects 537 million people worldwide, 10% of them being type I diabetes. In people with this type of diabetes, hypoglycaemia is the most common complication of insulin treatment, which can be fatal, with serious neurological and cardiovascular consequences. Fear of this complication can determine that patients do not administer the correct insulin dose and decrease their quality of life.

This study is even more relevant in the Canary Islands, since the prevalence of both types of diabetes mellitus reaches levels of around 15.6% in our population, doubling the incidence nationwide. Although 92% of these data refer to type II of this pathology, largely associated with nutritional disorders and age, the percentage of the population that suffers from the most serious and early form of this disease, type I, is noteworthy (Parcan, 2022).

Known for being a physiological alteration since the first century, the term “diabetes” refers to the main symptom of this disease, that is, polyuria and the consequent polydipsia, in addition to others. It was Tomas Willis, back in 1679, who gave it a clinical entity describing its symptoms, adding the term "mellitus" to the original definition of the disease, when verifying the sweet taste of the urine of his patients.

Type I diabetes mellitus is a disease characterized by insufficient or absent insulin production by pancreatic β cells. Although it most commonly presents in childhood or adolescence, it can even manifest itself in adulthood. Autoimmune in nature in most cases, today there is no treatment available to solve this disease, there are only palliative remedies that avoid the dangers of insulin deficiency, the hyperglycaemic symptoms, that in the most severe cases they could lead to the death of the patient.

For these reasons, there are a wide variety of technologies available for people with type I DM, such as the glucometer or continuous glucose monitors. However, there is also an effective and non-invasive method that is the DAD. This is trained to give alert signals with specific behaviors when the owner's blood glucose is out of the marked range (OOR). In this way the owner can measure glucose levels and act as necessary, either by administering insulin or eating, to return glucose levels to normal.

The mean sensitivity of DADs in alerting their owners when blood glucose is out of range is 81%. There is variability between individuals, and some dogs can reach a sensitivity of 100% (Rooney *et al*., 2019).

The objective of this review is to determine the actual effectiveness of DADs, the advantages they provide over other glucose monitoring techniques, and the selection criteria for candidates.

Material and methodology

The sources used for the development of this bibliographic review have been mainly scientific databases such as PubMed, ResearchGate, Elsevier and Google Scholar with articles between 2005 and 2022 in Spanish and English.

The information search was performed using the following keywords: "Diabetic Alert Dogs", "Medical Alert Dogs", "Detection Dogs" or "canines" in combination with "hypoglycaemia" or " 'Diabetes".

The development of the project has been elaborated in a coherent way, first defining the olfactory physiology of dogs and, later, developing the interest of its application in the field of human medicine, especially in the field of hypoglycaemia detection.

Main text

Canine olfaction

Anatomy and Physiology

The canine olfactory system is formed mainly by the nose, with its corresponding nostrils and nasal wings, the nasal cavity, the olfactory epithelium and its receptors, the vomeronasal organ, the olfactory bulb and the olfactory cortex of the brain (Jenkins *et al*., 2018).

The nasal cavity is divided medially by the nasal septum. Each hemicavity includes a nasal vestibule lined with cutaneous mucosa, a respiratory and an olfactory region with a small number of olfactory neurons and the ethmoturbinate, which serves to increase the olfactory surface area of the mucosa.

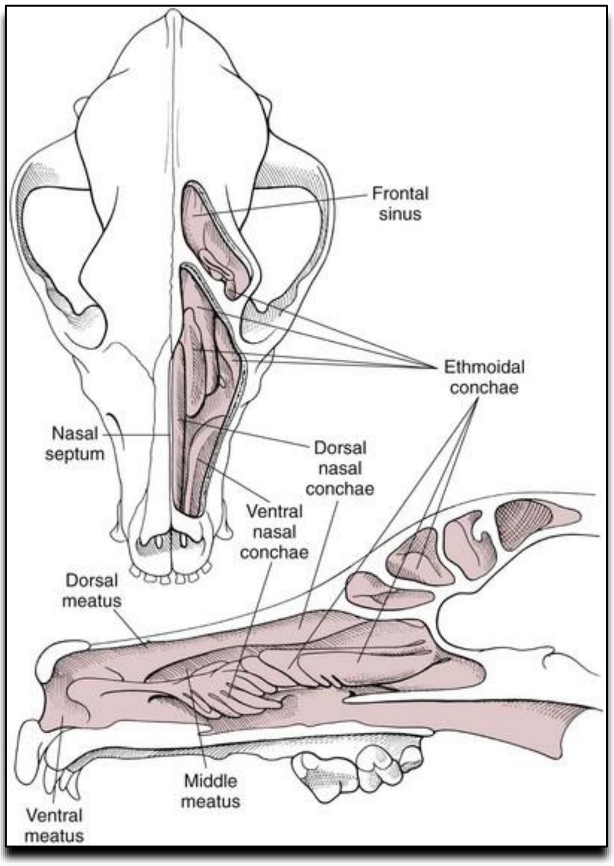

Figure 1. *Anatomy of the canine nasal cavity (VeterianKey, 2016).*

There are three turbinates that divide the nasal cavity into three meatuses, the ventral meatus being responsible for respiration, the dorsal meatus responsible for conducting signals to the olfactory organ, and the middle nasal meatus which ends in the paranasal sinuses (Jenkins *et al*., 2018).

Figure 1 shows the general structure of the canine nasal cavity. All components between the lumen of the nasal cavity and the cribriform plate are shown in simplified form. The lining of the nasal cavity has the function of separating odour molecules by their partition coefficients in the mucosa (MOZELL, 1964) and create different flow dynamics to deliver odour molecules to receptors, thus modelling odorants (Moulton, 1976). While the respiratory epithelium consists of a multi-rowed ciliated epithelium with goblet cells, the olfactory epithelium (MOE) involves a pseudostratified columnar neuroepithelium consisting mainly of millions of olfactory receptor cells (ORCs), and olfactory receptors (ORs) (Mori & Yoshihara, 1995).

ORCs are bipolar neurons that extend into the airspace to interact with odorants and whose lifespans are only a few weeks. Emerging thus new ORCs of pluripotent basal cells capable of differentiating into ORCs or sustentacular.

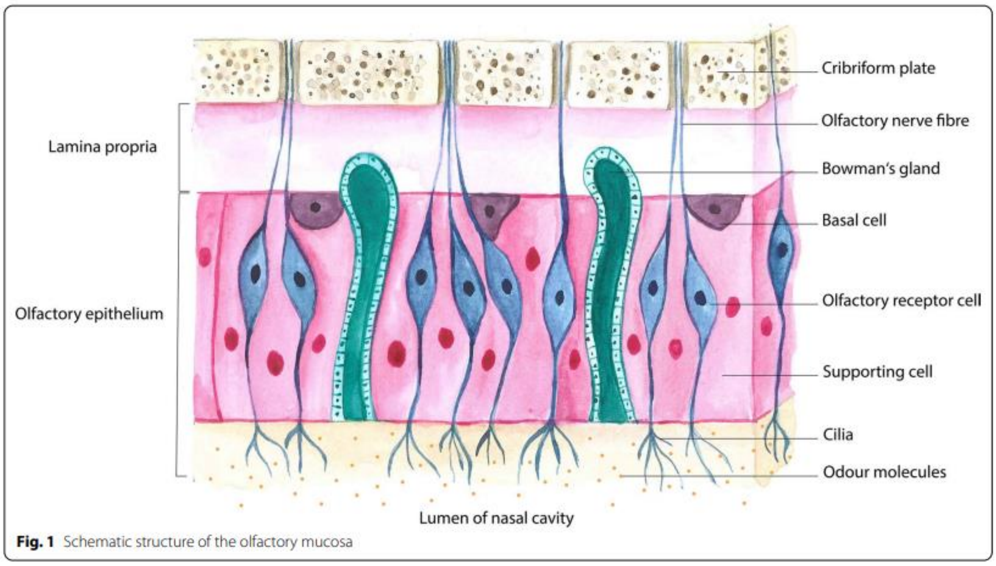

***Figure 2**. Schematic structure of the olfactory mucosa (Jendrny, Twele, Meller, Osterhaus, et al., 2021)*

Sustentacular cells (i.e., support cells) surround the ORCs, providing support and participating in the phagocytosis of dead neurons and in the transformation of odorants and xenobiotics (Liang, 2020).

In the olfactory epithelium we also find Bowman's glands, which are responsible for the production of secretions that dissolve odorants and play a role in the protection of the neuroepithelium against harmful agents that reach the nasal cavity with inhaled air.

Basal cells are cells located adjacent to the lamina propia in the olfactory epithelium and which, together with Bowman's glands and sustentacular cells, build a mucous layer that maintains nasal moisture and captures odours (Jenkins *et al*., 2018). Regular olfactory perception depends on the lamina propia, which is adjacent to the bony cribriform plate and is traversed by olfactory nerve fibres (axons of the ORCs) (Hawkes & Doty, 2009).

In the dog we find an additional olfactory system, the Vomeronasal Organ (VNO). This organ is located between the nasal and oral cavities, along with the vomer bone, on the roof of the oral cavity. The nasopalatine duct lies caudal to the maxillary incisors and connects the mouth to the VNO, an elongated tubular organ, separated by the nasal septum (Dzięcioł *et al*., 2020). The VNO is not only the main structure in pheromone recognition but can also be used to recognize other low volatility substances (McGann, 2017).

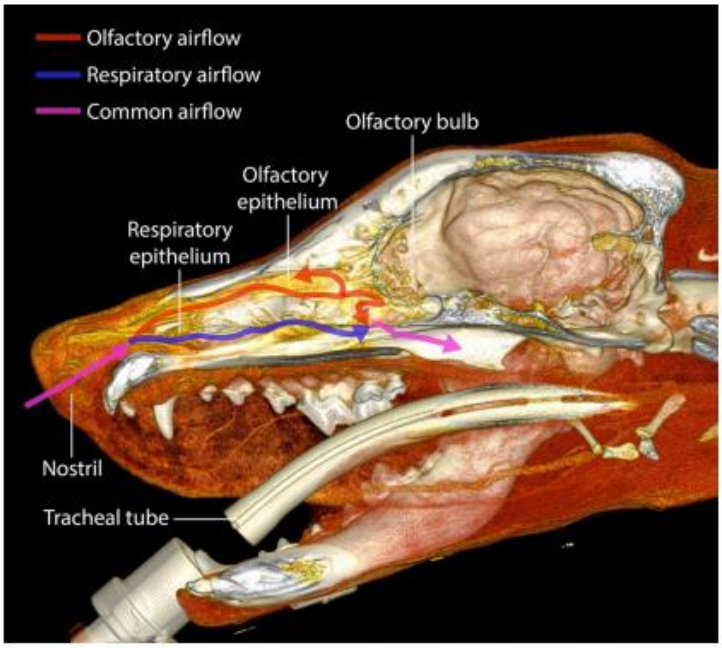

Figure 3. *Three-dimensional computed tomographic reconstruction of a canine skull (Paula Jendrny et al. 2021)*

In *Figure 3* we can see the two different paths that the passage of air takes through the dog's nostrils. The upper flow path, approximately 12-13% of each breath, goes directly to the olfactory region, where odour molecules settle and accumulate, avoiding being exhaled. The rest of the air, in the lower airway, flows through the pharynx to the lungs. Nasal airflow turbulence is a consequence of anatomical and physiological factors. These factors influence humidification, heating and the trajectory of the inspired air, guiding a portion of the air towards the olfactory epithelium (Patel & Pinto, 2014).

The impulses generated by the olfactory cells because of the detection of odours are transmitted through the cribriform plate of the ethmoid bone by the olfactory nerves (creating the cranial nerve 1-CNI).

The structure that makes up the next level of the olfactory pathway is the olfactory bulb (OB), which lies below the frontal lobes (Kavoi & Jameela, 2011). The OB contains glomeruli created from bundles of nerve fibres and is the site where the axons of incoming receptor cells contact the dendrites of mitral neurons, and it is their axons that transfer impulses to other areas of the brain. The OB plays a modulating and sensory role. It is involved in the initial processing and filtering of olfactory information, allowing discrimination between odours, as well as improving the sensitivity of odour detection and filtering out background odours (Jenkins *et al*., 2018).

From the OB, olfactory signals are transmitted to the olfactory cortex, which contains the anterior olfactory cortex, the piriform cortex, the peritonsillar cortex, and the entorhinal cortex. The first three areas transmit the olfactory signal to the frontal cortex and thalamus, while the entorhinal cortex sends the impulses to the hippocampal formation, which is involved in memory recognition of odours.

In the dog, as well as in people and other species, the acquisition of separate odour samples in each nostril occurs during inhalation, allowing bilateral comparison of stimulus intensity and odour source localization. Compared to other senses, where the sensory track is crossed, the olfactory pathways lead ipsilaterally from the sensing area located in the nasal cavity to the sensing area in the brain, meaning that the right nostril is the source of signals for the right cerebral hemisphere, and receptors located in the left nostril transmit impulses to the left hemisphere.

The phenomenon of olfactory lateralization observed in this animal is important and must be explained. Dogs have a preference for the right nostril, as this is where they begin to sniff. Then, if the odour is familiar, heterospecific, or non-aversive odours like food, they switch to using the left nostril. Also, they will use this pit if it is about target scents in detection dogs (Jezierski *et al*., 2016). However, if the stimulus turns out to be novel, threatening, or exciting, such as adrenaline, the dog continues to use only the right nostril. These findings are consistent with the theory reviewed by Vallortigara et al, observing that the right hemisphere controls the processing of new information and that the left hemisphere takes charge of the behavioural responses to familiar stimuli (Vallortigara *et al*., 1999).

Behavioural lateralization directly reflects asymmetries in brain function, which may confer a better understanding of the training process and thus the need for new training methods for detection dogs (Siniscalchi *et al.,* 2016).

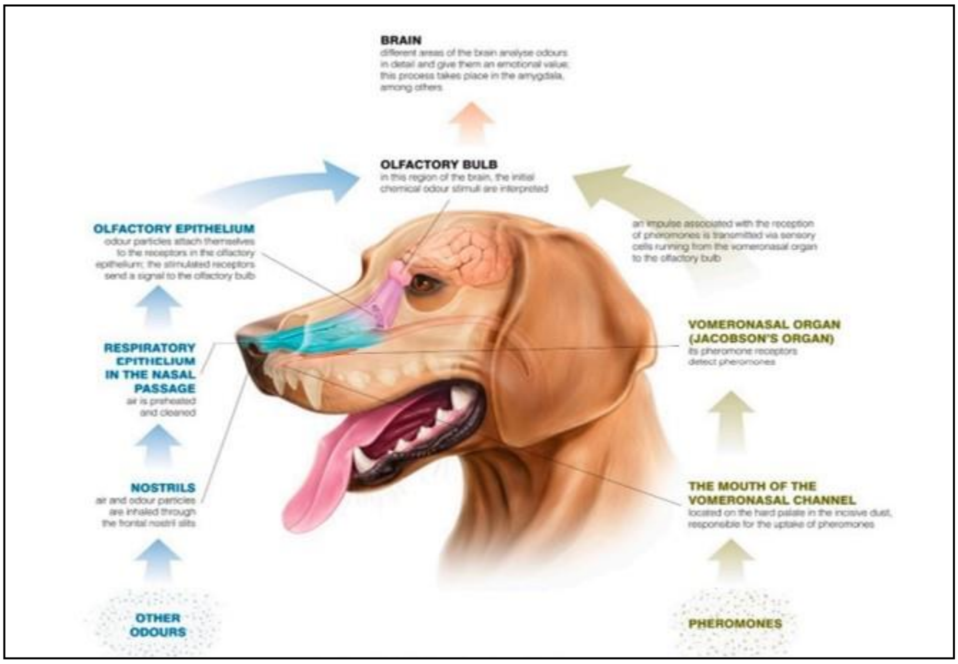

Figure 4. *This schematic shows the traditional structure of the chemical substances detection system. Currently, the distinction between the so-called nose and the VNO in terms of the detection of odours and pheromones is not so obvious, and researchers tend to believe that both the "nose" receives pheromone signals and the VNO receives stimuli, with low- volatility odorant compounds suspended in the liquid phase (Kokocinska-Kusiak et al., 2021).*

Compared with humans, dogs can detect significantly lower concentrations of odorants due to a combination of olfactory neuron density and number, nasal airflow modification, and specificity of central processing.

The proper identification of an odour seems to involve the activation of a unique combination of ORs. It is believed that the number of ORCs activated is responsible for determining the intensity of the odour (Jenkins *et al.,* 2018), although the maximum intensity is limited and the relationship between odour intensity and the number of ORCs activated is not linear. In addition, the intensity of the odour depends on additional external factors, such as the duration of exposure to the odour and its concentration. The intensity of the odour could also be influenced by the phenomenon of adaptation (Sirotin *et al*., 2015).

Apart from neurotransmitter receptors, ORCs also have receptors for hormones, and studies in humans and rodents have shown that odour discrimination abilities could be influenced by circulating levels of hormones (eg, leptin or ghrelin) (Loch *et al.,* 2015). It was concluded that when animals are fasting, ghrelin increases and there is an increase in their olfactory abilities, observing that populations of cells that did not respond to certain odours did respond under these circumstances (Kokocinska-Kusiak *et al*., 2021)

Unlike in humans, where nasal detection of volatile chemicals is mediated by the olfactory and trigeminal systems (Jacquot *et al.*, 2004), in the dog the detection of odours is carried out solely through the olfactory neuroepithelium, even though some odours can stimulate the endings of the trigeminal nerve of the nasal mucosa (detecting sensations such as heat or cold) (Jenkins *et al.,* 2018).

Factors affecting olfactory ability.

1. ***Genetic Implications and Breed.***
   OR genes form the largest gene families in mammalian genomes. The genetic evaluation carried out by Tacher et al. between breeds of dogs focused on the genetic polymorphism of the first element of the olfactory pathway: the olfactory receptors. They discovered that "some alleles are breed specific" (Tacher *et al*., 2005), which could explain the interspecies variability.

Later, Robin et al. correlated in their study a high general level of polymorphism with the binding capacity of the ligand of the receptor, which supports the previous hypothesis. However, the results of the research on the determination of the most sensitive breeds to smell are not entirely consistent (Robin *et al*., 2009).

These studies might suggest that, in addition to genetic predisposition, some behavioural attributes, such as inherent motivation, eagerness to learn, trainability, and the ability to work with people, might also significantly influence canine general olfactory ability. According to the study carried out by Polgár et al., hounds are the breed considered to have the greatest olfactory acuity, with 300 million ORCs. However, in the real context, the most used breeds for scent work are Malinois, other breeds of shepherds or Labradors. These dogs are selected for their ability to smell with appropriate cognitive and motivational behaviour, making them popular breeds for biomedical screening (Polgár *et al*., 2016).

Brachycephalic breeds showed the lowest sensitivity in odour detection, suggesting that changes in the structure of the olfactory system could influence olfactory acuity (Hall *et al*., 2015). This seems to confirm once more that in the use of canine olfactory abilities, the behavioural aspect is just as important as the purely genetically determined olfactory ability.

2. ***Age and sex.***

Like the other senses, olfactory abilities may decrease with age due to atrophic changes, with degeneration of the olfactory epithelium and fewer cells especially in dogs older than 14 years of age (Hirai *et al.*, 1996). However, compared to young dogs, adult individuals may have a much stronger long-term memory for odours and can deal with more complicated odour information.

There are differences in olfactory abilities between the sexes, indicating the results of Wei et al. that the cells of the olfactory bulbs of bitches are more active than those of males (Wei *et al*., 2017).

3. ***Environmental conditions.***

Regarding the effect of environmental conditions, humidity has been found to be an important factor in improving olfactory abilities in dogs, probably due to improved nasal moisture and odour capture. According to Gutzwiller, the increase in humidity could be responsible for a greater intensity of the odour, which positively influences the tracking efficiency of the dogs (Jenkins *et al*., 2018).

Higher temperatures can also negatively influence canine smell. Brauer and Blasi showed that this was due to a reduced ability of the dogs to work, leading to decreased search performance (Brauer & Blasi, 2021). Among the environmental factors that could influence olfaction performance, Jenkins et al. lists dietary components as factors acting negatively on olfactory acuity, such as coconut oil, or positively, increasing olfactory acuity, such as millet oil, omega-3 fatty acids of marine origin (EPA, DEA and DPA), proteins of animal origin, etc. In other words, olfactory work consists of a combination of physical and mental work and requires large amounts of energy that must be provided in the diet.

Faced with complex detection tasks, Ramaihgari et al. were able to show that zinc nanoparticles can enhance olfactory sensitivity, potentially regulating both activity and connectivity (Jia *et al*., 2016). This finding is important particularly in environments where very low concentrations of odours would not otherwise be detected (Ramaihgari *et al*., 2018).

4. ***Conditioning, handling and training.***

Exercise and condition deficiencies are described as physical stressors that can affect dogs' sense of smell directly or indirectly (Jenkins *et al.*, 2018). Physical exercise affects the sense of smell in detection dogs by decreasing detection rates, especially in dogs with poor physical condition (CT Angle *et al*., 2014). Therefore, a working dog must be well trained to be in optimal physical condition.

Odour detection training techniques can improve odour sensitivity and discrimination. Housing and general management can also influence detection work of dogs by affecting learning ability. Lower levels of stress due to social contact and a safe, enriched environment have been shown to improve cognitive performance (Byosiere *et al*., 2019).

Positive rewards with particularly tasty treats, as well as a specific toy in some dogs, increase their work motivation, while aversive training methods decrease motivation and have negative effects on their physical and mental health (Ziv, 2017).

Also, the olfactory capacity can be affected by some drugs such as metronidazole, a common antibiotic for the treatment of infections caused by strict anaerobic bacteria and certain protozoan parasites that, in high doses, can affect the olfactory performance of the dog (Jenkins *et al.*, 2016).

All these factors are important and must be especially considered when discussing medical alert dogs. It is essential to know what factors affect their olfactory ability, since this can affect their performance and have fatal consequences for their owners.

What do diabetic alert dogs perceive?

The incredible acuity of the canine olfactory system plays a fundamental role in the identification of certain organic compounds (ie the so-called "volatiloma") generated in different pathological processes (especially in cancer, epilepsy, hypoglycaemia, infections and psychiatric diseases) (C. Angle *et al.*, 2016). Volatiloma, understood as the composition of both the volatile organic compounds (VOCs) in an organism and the VOCs that reflect its unique current metabolic state (including the influence of infection), can be used to detect disease and the presence of pathogens specific.

Most odours detected by dogs through inhalation are VOCs in different compositions that reside in the air (C. Angle *et al.*, 2016). VOCs can differ in magnitude, volatility, and concentration. The concentration of odour in air is correlated with the concentration of its source, volatility, odour release surface area of the source, volume flow rate, ambient air movements, and diffusion rate within the air. Gas chromatography and mass spectrometry studies showed that each human odour consists of a combination of volatile components produced from the skin and differ in proportion from person to person, along with some compounds that are unique to certain individuals (Curran *et al*., 2007).

Biogenic VOCs are isoprene and monoterpenes (the most prominent compounds), as well as alkanes, carbonyls, alcohols, esters, ethers, and acids that have a strong odour and are produced and emitted by people, animals, plants, and microorganisms. The VOC pattern of an organism is signed by VOC-producing cells or tissues and largely determined by its physiological or pathophysiological metabolism, the latter subject to exogenous influences such as infections, skin fumes, and even smoker's breath (Kesselmeier & Staudt, 1999).

Different diseases cause the appearance and emission of specific VOC patterns (Shirasu & Touhara, 2011), which can be used as diagnostic olfactory biomarkers. VOCs are released from various tissues and body fluids. The most common body fluids or tissues for diagnostic testing are skin fumes, urine, blood, saliva, and feces that differ in their VOC composition (*Amann et al.,* 2014). However, when we talk about diabetic alert dogs it is common to use exhalation samples.

Pathological processes influence body odour by either producing new VOCs or by changing the pattern of VOCs that dogs can detect. Knowing this, and with the goal of reproducing the natural detection ability of dogs, Siegel et al. carried out a study in which they collected VOCs from the breath of 52 type 1 diabetics and analysed them with gas chromatography/mass spectrophotometry (GC/MS) (Siegel *et al*., 2017). The result of the analysis showed that the hypoglycaemic episodes were associated with very characteristic signatures, showing a sensitivity of 91% and a specificity of 84%.

In another recent study, Saidi et al. collected respiratory samples from six diabetic patients and compared their profiles with other non-diabetic subjects using GC/MS techniques (Lippi & Plebani, 2019). The results obtained in diabetics were very different, showing higher concentrations of benzaldehydes, toluene, methane, aniline and chloroformic acid (Saidi *et al*., 2018).

Other studies were able to identify a putative number of VOCs during exhalation in hypoglycaemic episodes, which are then detected by the dog. These basically include isoprene (Neupane *et al.,* 2016) and another great variety of substances that increase in hypoglycaemia such as Ketones (acetone) hydrocarbons (ethane and pentane), acetaldehydes, methanol and ethanol (Qiao *et al*., 2014).

The process by which hypoglycaemia could increase isoprene is not clear and there are numerous studies that suggest higher levels of this compound in hypo than in euglycemia *(Figure 5)*.

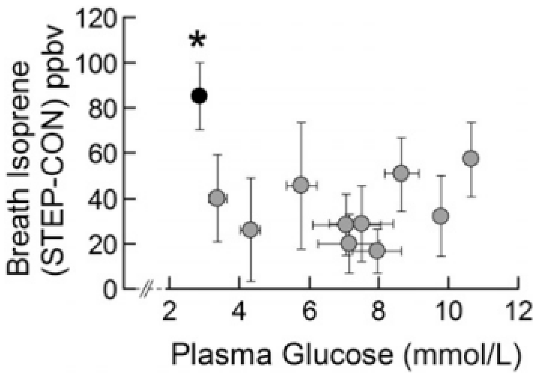

Figure 5. *Exhaled breath isoprene during studies. *P<0.01 compared with nonhypoglycemia (Neupane et al., 2016)*

Despite being one of the most common VOCs in human breath, the endogenous source of isoprene remains undetermined. To this day, the only thing that is known is that isoprene is a product of cholesterol biosynthesis (Stone *et al*., 1993). In patients with diabetic ketoacidosis, we could also find high levels of ketones in the exhaled sample, such as 3-hydroxybutyrate, acetoacetate or acetone. This is due to the inability of type 1 diabetics to synthesize insulin (Buszewski *et al.*, 2007). Cells use fatty acids as a source of energy instead of glucose, whose catabolism creates these ketones that lead to ketoacidosis of the blood and are excreted via the urinary and respiratory pathways, allowing their measurement.

One of the biggest challenges in DADs is the low concentration of these volatile compounds associated with hypo- and hyperglycaemia compared to the "normal" volatile compounds present in liquid samples (eg, blood, urine, sputum). Efforts to determine the limits of canine olfactory detection can help identify dogs with superior potential for medical detection and can help monitor the daily reliability of trained dogs.

Regarding the detection limits of known odorants, Concha et al. concluded that dogs can detect concentrations as low as 1.5 parts per trillion, making these animals extremely sensitive to physiological variations that would be undetectable by any other diagnostic method (Concha *et al.*, 2019).

Training of the DADs.

Election of the DADs.

The selection criteria for service dogs consists of choosing those that have characteristics of sociability, adaptability, trainability and motivation. DADs must demonstrate independence when making decisions and have problem-solving ability (Marshall-Pescini *et al.,* 2009). Dogs not suitable for this job are those who are fearful and anxious and are unable to focus their attention.

Optionally, to choose the best candidates, the Dog Mentality Assessment can be done as a behavioural test to evaluate their sociability, playfulness, fearlessness and multiple other aptitudes. However, the results are greatly influenced by external factors, as well as the score of the jury (Saetre *et al*., 2006).

Although breeds with elongated noses and bred for detection work are associated with better scent detection performance (Polgár *et al*., 2016), Hall et al. found that pugs outperformed German shepherds in learning and in discriminating scents at decreasing concentrations (Hall *et al.,* 2015). This finding is surprising considering the anatomical differences between these breeds and the popularity of the German Shepherd in detection work. This discovery implied that other factors could influence the performance of odour detection, the behavioural ones. Behaviour is so important that despite their superior olfactory acuity, scent hounds are rarely used in detection work due to their poor trainability (Jamieson *et al.,* 2017) and breeds such as the Labrador Retriever, English Springer Spaniel, or Border Collie are used. However, it is a very ambiguous issue since there is enormous variability between individuals of the same breed, concluding that the individual character, conditioned by training, life experiences, environment and genetics, is more important than the genetics itself.

Training method.

The training of these animals begins at an early age and is based on positive reinforcement. It lasts for 8-10 months; being the dogs fully trained by the age of approximately one year.

The target odour and the required behavioural response (which will be the future alert signal) are combined with rewards, being the animal rewarded with food or games each time it displays the expected response in the presence of the target odour. Little by little the dog is trained to distinguish between increasingly similar but different scents, until they are only capable of alerting to the target scent.

It is important in training biomedical detection dogs that they are not conditioned to the individual odours of the subjects or the environment where the samples were produced (eg, hospital odour), but instead learn the specific odour of the disease (VOC pattern) and successfully complete the generalization process.

Dogs are not only trained to detect the target scent, but also to have excellent social behaviour since, being MAD, they will accompany their owners to work, school, restaurants, etc. and it is important to educate them in these scenarios.

First, they receive general training in which they are taught not to be startled by sudden sounds, running children, or people trying to interact with them. They learn to lie quietly under the table when they go to the office, school or restaurant, and even to walk within a foot of their keeper without paying attention to other animals or people (Gadbois & Reeve, 2014). During this training they learn 30 commands, ranging from the simplest, such as sitting down to the most complex, such as asking for help or obtaining items like the mobile phone, glucose tablets, insulin syringe, etc. The specific training for DAD, which consists mainly in detecting episodes of hypoglycaemia, is divided into three phases (Hardin *et al*., 2015):

- **Positive Control Phase.** It consists of teaching the dog to recognize signs of hypoglycaemia on the skin and exhalation in a glass jar. He is taught to mark the jar with his nose and to sit in front of it. If the dog alerts correctly, it is rewarded with food or games (positive reinforcement). It is important in this phase that the hypoglycaemia samples (one per session) are from different individuals, to avoid memorizing the individual odour and favour the common odour profile (Elliker *et al.*, 2014). Teaching the dog to respond to odours based on a common classification requires that the dog learn a concept that is usually the disease (``Diabetes´´).

When the animal has automated the commands to mark and sit before the sample, the second phase of the training begins.

- **Negative Control Phase.** In this second phase, the glass bottle with the hypoglycaemia sample is placed in a one-litre steel can. The dog must smell the sample, mark it with its nose and sit in front of it to consider it correct.

  Once this point has been worked on, we begin with the Negative Controls, also known as interfering (Porritt *et al*., 2015). These consist of non-target odours that are very important in the evaluation of the detection capacity based on calculating the specificity. The use of distractions teaches dogs to discriminate target odour (hypoglycaemia) from non-targets. Distracting odours are odours normally associated with training or its environment (treats or trainer), with sample containers (steel cans), sample preparation (gloves, bag, pipettes), etc.

  In this phase, in addition to the hypoglycaemia sample, four steel cans containing blank samples and two cans with normoglycemic samples are added. These additional samples are from the same person from whom the hypoglycaemia sample was obtained. Non-target samples (blank and normoglycemic) should contain the same distracting odours as found in the target odour. So, for example, they do not associate the smell of gloves with the target sample but learn its olfactory profile.

  Part of this discriminative training also consists of introducing novel smells to avoid possible distractions in the future. Thus, the dog learns that new odours can appear at any time but that they do not add any value (Minhinnick, 2016).

- **Third phase.** In this last phase, the glass vials with the samples are placed on a person. Instead of sitting down, the dog is taught to give the alert signal by pushing the person with its nose or other even more obvious signals (jumping, pawing, barking...).
  The most frequent alert signals made by alert dogs for changes in blood glucose levels are vocalizations (61%), licking (49%), staring at the owner's face (41.3%), muzzling (41 %) or skip (30%) (Lippi *et al*., 2016).

Once the dog has completed all three phases, samples from different patients are introduced, and training is continued until the dog is fully capable of distinguishing between hypo and normoglycemia in multiple people. From this moment on, the dog is considered a DAD and is prepared to accompany a diabetic person. However, once the dog has a designated future owner, the samples used only come from this person. This assures us that the dog works for the person and helps ensure the best service for its individual owner.

To be classified as DADs, the dog must show at least 70% more hits than false positives.

Collection of samples for training.

Hypoglycaemia and normoglycemia samples are from patients with type I diabetes. Subjects must wipe their neck and forehead with sterile gauze and place it in a zip bag. Immediately after placing the sample in the bag, the patient performs an exhalation of breath and proceeds to close the bag. Each bag is labelled with your name, date, collection time, and its corresponding glucose value at that time. They must send the samples to the place of collection within a maximum period of 48 hours after their collection. Once the samples are received, they are stored in the freezer at a temperature of -18º C.

Patients are indicated to take samples in hypo and normoglycemia in conjunction with the following reference ranges (Workgroup on Hypoglycemia, 2005):

- → Hypoglycemia = <70 mg/dl
- → Fasting normoglycemia = <100 mg/dL; preprandial = 80–130 mg/dl; post-prandial = <180 mg/dl

Training environment.

The training is usually carried out in a closed room, preferably without the presence of any person, to avoid possible distractions or provide information (often indirectly) to the dog. Normally, they are awarded with an automatic dispenser (Pet Tutor) that is activated by the trainer from another room.

The cans with the hypoglycaemia, normoglycemia and blank samples are placed on the floor in a semicircle in a randomly determined order (by dice), to reduce possible positional preferences (Jezierski *et al*., 2008). In front of the samples is the Pet Tutor. We can find a scheme of the training method in *Figure 6*.

The dogs are controlled from each other by video cameras and enter the session individually and randomly, which lasts no more than one minute. We can do up to 8 sessions per day, taking a two-hour break after completing the fourth session.

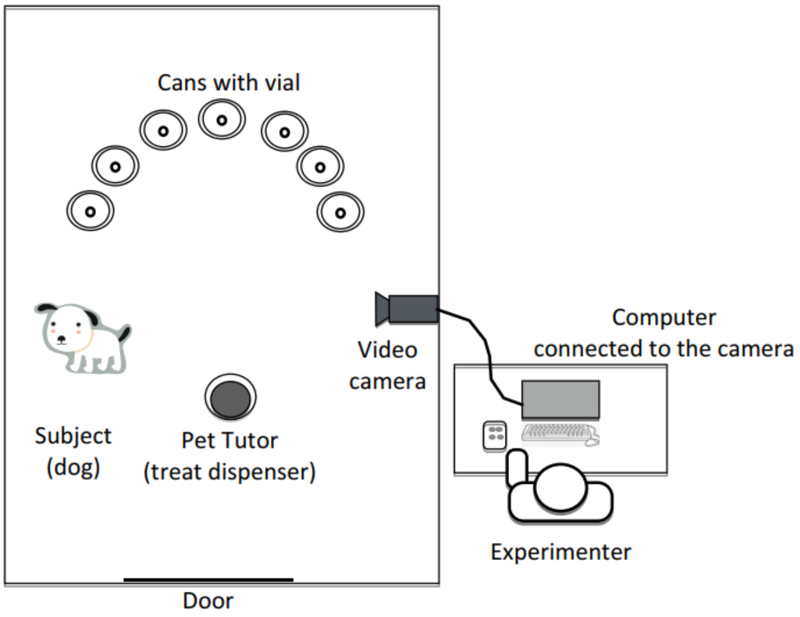

Figure 6. *Room layout for DADs training (Hardin et al., 2015)*

The training record of each dog is important since it is what will measure its sensitivity (proportion of responses to the target odour when that target odour is present) and specificity (proportion of non-target odours that the dog correctly ignores) being able to thus compare the performance between different individuals.

Training conditionals

One obstacle to this type of training is the development of positional preferences. It has been shown that when the hypoglycaemic sample falls in the same position several times in a session, the dog begins to choose those positions because when he does, he is usually rewarded (Lit, 2009). These types of preferences are more common in the initial phases of training when the dog is not yet able to clearly identify the target scent and can be minimized by randomizing positions. Ideally, each position should contain the target (hypoglycaemic sample) the same number of times per session, thus reducing positional biases.

Human influence is another of the training challenges, where the expectations of the observer (trainer) condition the behaviour of the animal. It is common that if the trainer is present, he pays more attention to the areas that contain the targets, which can give clues to the dog, which is very skilled at detecting the subtle and unconscious signals offered by people (DeChant *et al.,* 2020). Also, under real conditions, neither the trainer nor the dog will know where the correct sample is. That is why Double-Blind testing is the gold standard in this type of training or research.

We can minimize these clues by letting the animal work without a leash and/or eliminating the presence of people at the time of training, entering the room only to give the reward.

Another challenge is the subjectivity of the observer. Dogs are trained to alert using a variety of responses (sit, bark, push…) whose interpretation can be subjective. The influence of the observer is found, above all, in cases where the answer is ambiguous and may be conditioned by his assumptions, depending on whether he thinks that the answer was correct or not. This can lead to biases in the rating of the dog. To avoid this, the topography and duration of the alert sign or behavioural reaction are usually estimated.

The ´´set-size effect´´ is a concept that refers to a greater learning due to a greater number of samples. The more samples, the more difficult your individual memorization is, and the learning will be based on identifying the concept that is common to all the tests.

Contamination and maintenance of samples and materials are essential and often interrelated considerations in maintaining the integrity of the training plan. Contamination occurs when odour seeps in and is transferred to other materials or odour sources. Most of the time contamination occurs by introducing odour or saliva from the operators into the target sample. This circumstance can serve as a clue to the dog, which learns to detect the polluting odorants instead of the target odour.

There may also be cases of cross-contamination, in which one of the samples is unintentionally transmitted to another, causing alterations in the training results. In these cases, the animal could mistakenly learn that the target odour is a mixture of polluting odours and that same odour. This usually occurs when we store the samples too close together.

In addition, there may be contamination from the container in which the sample is stored. For example, plastic emits chemicals that can pollute the environment. That is why we use non-corrosive metals (such as steel) or glass to store the samples (Why and How to Control Contamination. Detection Dog Guidance Notes, 2018).

The role of the owner is enormously important in ensuring the success of the program. The owner's adherence to the trainer's instructions, attending to the dog's alerts and having his glucose measured before rewarding him, is crucial. Wilson C. et al observed that owners who met the guidelines had dogs with values of Sensitivity a positive Predictive Positive Value superior to the rest. In contrast, owners who ignored animal alerts owned "worse performing" dogs (Wilson *et al.*, 2019). Over time, inconsistent reinforcement reduces the dog's sensitivity and specificity to hypoglycaemic episodes. Lack of reward, as well as early reward (before measuring glucose values), is totally contraindicated and could lead to detraining of the dog.

Discussion

The emergence of diabetic alert dogs is fairly recent, beginning in 2003, when Armstrong, a Labrador retriever, alerted his type 1 diabetic owner to a hypoglycaemic episode. He was the first (known) dog trained and able to alert an episode of hypoglycaemia (*Figure 7*). (Dogs Diabetics. The Origin of Dogs for Diabetics, 2018).

Figure 7: *Armstrong (Guinness Book of World Records 2003).*

Rooney et al. conducted a study in which the sensitivity of trained dogs to detect changes in blood glucose in people with diabetes was found to be 80% (95% confidence interval [95% CI], 0.44–0.97). Other interesting findings observed in this study after the acquisition of the dog were the reduction in glycosylated haemoglobin (HbA1c) values and the notable decrease in glucose values when it was within the normal range. 75% of patients reported feeling more independent after living with DAD (Rooney *et al*., 2013).

Later, Hardin et al. conducted a study in which they estimated the ability of six dogs trained as DADs (two Labrador retrievers, a flat-coated retriever, a German shepherd, a Siberian husky mix, and a spaniel mix) to detect hypoglycaemia in perspiration samples from different people (Hardin *et al.*, 2015). Dogs were specifically trained to signal hypoglycaemic samples by sitting in front of them or pushing the sample canister. The accuracy, sensitivity, and specificity of the DADs for detecting hypoglycaemia in sweat samples were 93%, 78%, and 96%, respectively.

Putting together, all the articles published on the efficiency of dogs in the event of hypoglycaemic episodes, yield very heterogeneous results, with a specificity and sensitivity with traits between 29-93% and 49-100%, respectively. This very wide range of sensitivity and specificity is characterized by a wide inter-dog variability that has been observed in most of the studies carried out. This variety is due to the diversity of factor that can be observed in the *Figure 8.*

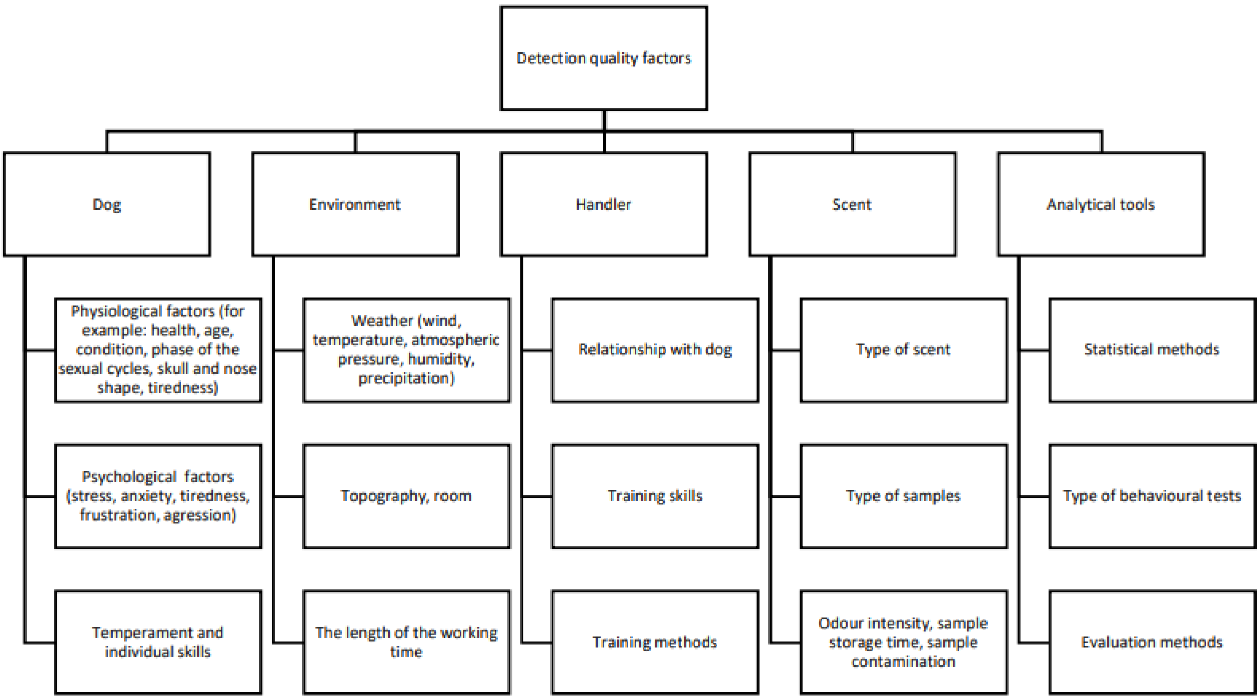

Figure 8. *Factors affecting the efficiency of canine olfactory detection (Kokocińska-Kusiak et al., 2021).*

Studies such as the one by Evan A. et al., which show significantly lower sensitivity and specificity values, have numerous limitations, highlighting the lack of standardization in dog training (Los *et al*., 2017). In longitudinal studies, owner records may not be complete. Thus, some alerts may have been lost and others may have been marked as incorrect, as happens in routine tests, in which when the dog is not there and there is an episode of hypoglycaemia, it is recorded that it has not alerted, when in fact it has not been present.

Sensitivity values in studies conducted with children have also reported lower levels. This is because the dogs are not present in many out-of-range episodes, such as those that occur at school.

Many owners have reported that dogs alert before the glucose values fall below the stipulated range, alerting the dog when the values were still normal, verifying that in the next measurement (20-60 minutes later) they were in hypoglycaemia. Using a Glucose Monitoring System, which provides estimates of the transition time from in to out of range, it has been possible to demonstrate the existence of the ability of dogs to "pre-alert". This pre-alert behaviour would imply that some dogs could respond more quickly than current glucose measurement systems (Rooney *et al*., 2019). DADs can alert owners to hypoglycaemia 62% of the time before even showing symptoms.

In studies that only attempt to estimate the ability of dogs to detect hypoglycaemia, this response would be categorized as erroneous, since only alerts below 70 mg/dl glucose are considered "correct.". It has been shown that dogs trained to detect hypoglycaemic episodes are also able to detect hyperglycaemic events spontaneously. Dogs that are family pets, and that have not been trained to detect changes in blood glucose levels, have been found to have greater sensitivity for detecting hyperglycaemia according to the study by Rooney N. This study suggests that detection of hyperglycaemia could be supported, more than in the response to high glucose levels, in behavioural patterns, a fact that would be facilitated in the case of a close connection with the human.

In order to assess patients' perception of DADs, Petry et al. conducted a study that included 135 subjects (72 diabetics and 63 parents of children with diabetes) to assess the ability of these dogs to detect compromising glucose levels (Petry *et al*., 2015). More than half of the responses revealed that the dog was able to frequently (more than three times per week) identify low glucose values before the owner himself noticed it, with 80% of the participants stating that the dog had saved their lives. lives at least once. Most of the participants stated that the dog was extremely helpful in managing diabetes.

According to various studies, DADs also have a substantially favourable impact on the quality of life of patients, reducing the frequency of hospitalizations, the need for assistance from other people, as well as reducing the feared risk of accidents while driving (Rooney *et al*., 2013).

Lundqvist et al. carried out another longitudinal study including fifty-five owners with their consequent dogs, twenty of them being DADs (Lundqvist *et al*., 2018). A specific questionnaire collecting data on health, quality of life and activity was administered to the owners before adopting the dog and three months after its adoption. Diabetic owners with DADs reported a significantly greater increase in health and quality of life compared to the rest of the population. Analysis of the entire study population revealed an increase in the level of physical activity and well-being after adopting the dog.

Recent studies have shown a strong association between having a DAD and improved glycaemic control related to responsible pet ownership, especially in children and adolescents, where it is more difficult to achieve this control (Maranda & Gupta, 2016). In the study by Rooney et al. we can see a graph that shows the lowest percentage of glucose values below the range before and after the acquisition of the dog. The values were collected overnight, the time when diabetics fear hypoglycaemia the most (*Figure 9*).

The study by Reeve et al. revealed that DADs could additionally alert to other pathological situations and even alert people other than their owners. Dogs trained to detect episodes of hypoglycaemia began to alert their owner and/or other people to other situations. Participants reported a total of thirty-three different conditions that the dog alerted to. The most common additional conditions that dogs alerted to were hyperglycaemia, migraines, epilepsy, and POTS (Postural Orthostatic Tachycardia Syndrome). 84% of animals alerted to multiple conditions (in the same person), 54% to multiple people, and 46% to multiple people and conditions.

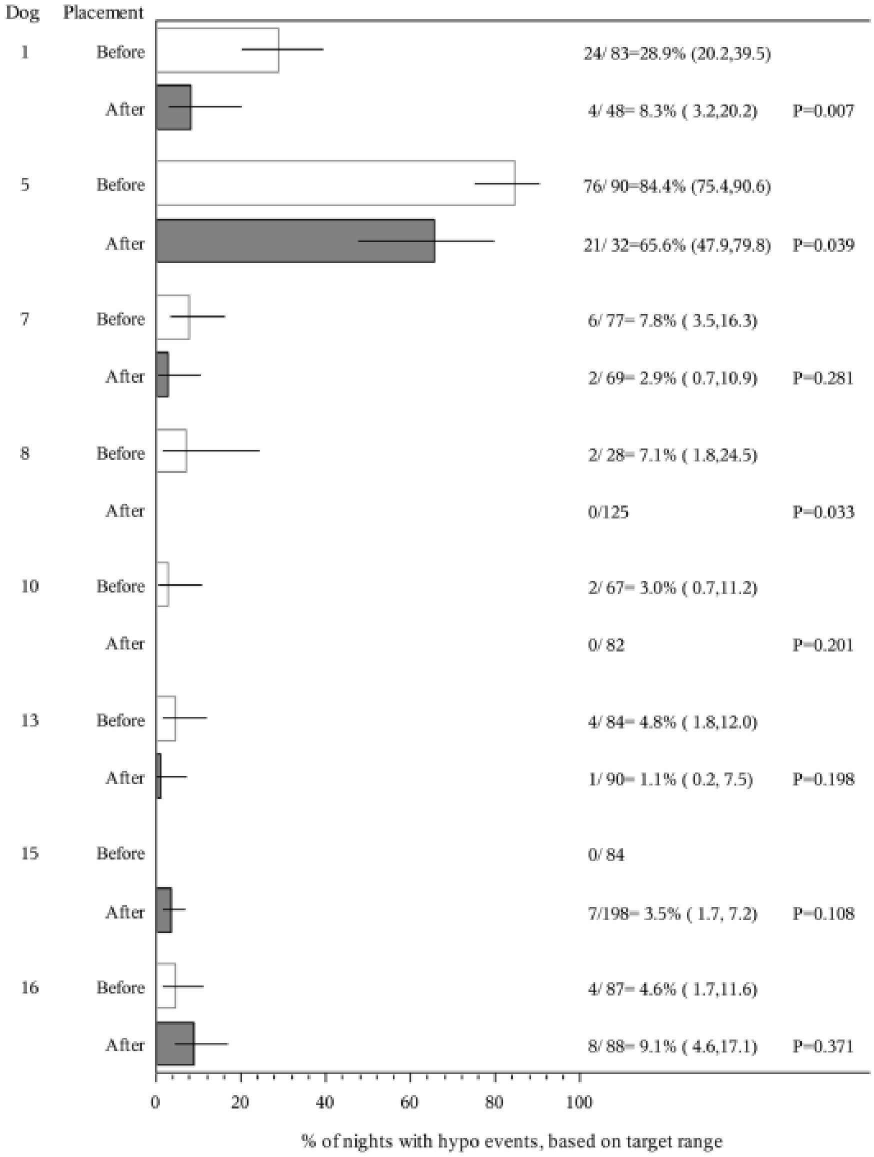

Figure 9*. Percentage of nights in which blood glucose readings were recorded to be below clients' target range before and after dog acquisition (Rooney et al., 2013)*

To explain the higher frequency in these diseases, there is an association between anxiety and epilepsy, as well as between anxiety and migraines (Chu *et al*., 2018). Individuals may experience anxiety or stress prior to a seizure, migraines, or other physiological changes, and the dog may detect this stress preceding the conditions. Although there is some evidence that stress produces detectable VOCs and that dogs can detect odorants related to fear and stress (Zamkah *et al*., 2020) this is a topic that still needs a lot of research.

The dog's reinforcing story could play an important role in the dog's attitude to alert to multiple conditions or people. If the alert occurs when someone other than the owner is undergoing some physiological change, and if the alert is correctly rewarded (positive reinforcement), the dog can learn to respond to new conditions and/or new people (Reeve *et al*., 2021).

In Spain there are several non-profit associations where you can get DADs. On the one hand, there is the Bocalán Foundation, pioneers in this type of dog training, with offices in Madrid, Cantabria, Galicia and Catalonia. On the other hand, in Zaragoza there is the CANEM Foundation where this type of dog is also trained. The biggest disadvantage regarding the acquisition of these dogs is that the procedure is very slow and expensive (in the case of for-profit companies). It is estimated that the cost associated with the breeding, maintenance and training of these dogs is 3,000 euros, with a market value of around 5,000 euros. These organizations are not regulated, each having different training methods and, therefore, different results.

It is important that owners follow the guidelines and use continuous glucose monitoring devices to confirm alerts by measuring blood glucose levels before giving any reward. In this way we can evaluate and maintain adequate performance.

Conclusion

The use of these dogs is an innovative and non-intrusive system that can be of great help in assisting in the recognition of hypoglycaemia and in reducing the emotional burden suffered by people with DM1, especially those who live alone or with asymptomatic hypoglycaemia.

Some studies have shown that "man's best friend" has saved the lives of several diabetics, especially when the glucose monitor has not been available (Lippi *et al*., 2016). Therefore, although this method is not intended to be a substitute for blood glucose meters or continuous glucose monitors (due to the high false positive rate), it is undeniable that the presence of these dogs improves the overall quality of life of people with diabetes, offering extra security, a friendly presence and greater peace of mind.

Organizations should consider developing standards for training DADs and official certifications, as well as criteria for appropriate selection of individuals prior to training, and subsequently to assess their performance after placement.

Finally, closer work between these organizations and the scientific community is necessary to study in depth the VOCs detected by DADs in order to determine their real effectiveness against hypoglycaemia and in order to improve current technologies for the control of diabetes.

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