Interactions of human chorionic gonadotropin with genotype and parity on fertility responses of lactating dairy cows

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ABSTRACT

Fertility-promoting effects of treatment of lactating dairy cattle with human chorionic gonadotropin (hCG) after artificial insemination (AI) have been variable. Here, we tested whether fertility response to hCG in lactating Holstein cows interacts with genotype and parity. Primiparous (n = 538) and multiparous (n = 613) cows were treated with hCG (3,300 IU) or vehicle 5 d after AI. Pregnancy was diagnosed on d 32 and 60 after AI. A subset of cows (n = 593–701) was genotyped for 4 single nucleotide polymorphisms (SNP) previously associated with fertility. Treatment with hCG increased progesterone concentration on d 12 after AI regardless of genotype or parity. Pregnancy per AI was improved by hCG in primiparous cows but not in multiparous cows. Moreover, hCG treatment interacted with a SNP in coenzyme Q9 (COQ9) to affect fertility. Fertility of cows treated with vehicle was greatest for the AA allele, whereas fertility was lowest for the same genotype among cows treated with hCG. Pregnancy per AI was improved by hCG in primiparous cows but not in multiparous cows. Overall, results show that variation in response to hCG treatment on fertility depends on parity and interacts with a SNP in COQ9. Key words: human chorionic gonadotropin, genotype, parity, SNP, fertility

INTRODUCTION

Personalized medicine is a form of individualized medical therapy in which knowledge from genetics is combined with clinical data to guide clinical treatment. An example of personalized treatment is exclusion of women with breast cancer from tamoxifen treatment based on screening for a SNP in CYP2D6 associated with reduced function of the enzyme (Schroth et al., 2009; Zeng et al., 2013; Elia et al., 2018). Personalized treatments could conceivably be important in livestock production. Use of genetic information to identify animals for which treatments are likely to be efficacious could reduce the number of animals receiving treatment and thereby achieving cost savings from reduced drug use, labor costs, and withdrawal of milk and meat from the human food chain. Moreover, effectiveness of treatment would increase because only those animals with predisposition for positive response would receive treatment.

In cattle, one type of therapy that could potentially be made more effective through use of personalized medicine is hormonal administration to improve fertility. There is a great deal of between-study variation in effectiveness of some hormonal treatments administered after AI for improving fertility including GnRH (Peters et al., 2000), human chorionic gonadotropin (hCG; Nascimento et al., 2013), and progesterone (Yan et al., 2016). Some of this variation is likely due to small sample size, but environmental or genetic differences may also be present between herds, affecting efficacy of treatment.

Here, the objective was to test whether effects of one of these hormonal treatments on the fertility of lactating cows depend on genotype as well as parity. The hormonal therapy tested was hCG treatment at d 5 after AI. Administration of hCG during the early luteal phase can be used to induce ovulation of the first-wave dominant follicle, formation of a functional accessory corpus luteum, and an increase in circulating progesterone concentrations (Schmitt et al., 1996; Santos et al.,
HUMAN CHORIONIC GONADOTROPIN RESPONSE

Experimental Design and Treatments

Animals and Management

The experiment was conducted on a commercial dairy farm in northern Florida (Alliance Dairy, Trenton, FL, 29°36′54″N, 82°49′4″W) with lactating Holstein cows housed in freestall barns equipped with fans and sprinklers. Cows were milked 3 times daily and fed a TMR. Animal procedures were approved by the University of Florida Institutional Animal Care and Use Committee. All procedures, including injections, pregnancy diagnosis, blood collection, and timed AI (TAI), were performed while cows were restrained in self-locking head gates at the feed line.

Cows were randomly assigned to receive intramuscular injection of either 3,300 IU of hCG (Chorulon, Merck Animal Health, Millsboro, DE) or an equivalent volume (3.3 mL) of vehicle (diluent) on d 5 after the first insemination following calving. The study was conducted over 2 yr. The first year involved 283 primiparous cows and 489 multiparous cows that received first insemination after calving from June to September 2016, corresponding to hot months of the year. For the second year, the experiment was replicated for 297 primiparous cows receiving first insemination after calving in July and August of 2017.

Twice weekly, a group of 20 to 50 cows was enrolled in the Double-Ovsynch protocol (Souza et al., 2008) to allow TAI at 75 to 80 d after calving. Multiparous cows were inseminated with conventional semen and primiparous cows were inseminated with either conventional (n = 343) or sexed-sorted (n = 237) semen. At the time of hCG or vehicle injection (d 5 after TAI), BCS was estimated as described by Ferguson et al. (1994).

For cows in yr 1 only, a blood sample (7 mL) was collected on d 12 after TAI by puncture of the coccygeal blood vessels using vacutainer tubes containing sodium EDTA (Becton Dickinson and Company, Rutherford, NJ). The samples were placed immediately on ice and brought to the laboratory within 6 h of collection. At the laboratory, 2 mL of blood was collected and stored...

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For a C → G mutation in the third intronic region of pro-
at −20°C for later genotype analyses. The remaining sample was centrifuged at 2,000 × g at −4°C for 20 min for separation of plasma. Plasma samples were frozen at −20°C for later progesterone analyses.

Diagnosis of pregnancy was performed by transrectal ultrasonography on d 32 ± 3 and 60 ± 3 after AI. A cow was determined pregnant when an embryonic vesicle with a viable embryo (presence of heartbeat) was detected.

Genotyping

Cows from the first year of the experiment were genotyped for SNP present in COQ9 (rs109301586), HSPA1L (HSP70C895D), PARM1 (rs111027720), and PGR (rs109506766). Details of these SNP are presented in supplemental material in Cochran et al. (2013b). Frozen samples of whole blood were thawed and DNA extraction was performed using the Puregene Blood Core Kit (Qiagen, Germantown, MD) according to the manufacturer’s instructions. Genotyping was performed by PCR-based KASP assay (LGC Genomics, Middlesex, UK). The assay is a PCR-based technique involving a common reverse primer and 2 allele-specific forward primers, where one allele-specific primer is labeled with fluorescein amidite and the second with hexachlorofluorescein. Primers used for genotyping are described in Table 1. Depending on the SNP, the total number of animals genotyped varied between 593 and 701 because of no-calls.

Each PCR reaction included 5 µL of extracted DNA (20 ng/mL), 5 µL of 2x supermix with low ROX (LGC Genomics), and 0.14 µL of KASP by design primer mix. Amplification and analysis were performed using a CFX96 Real-Time PCR detection System (Bio-Rad, Hercules, CA). Thermal cycling conditions were 94°C for 15 min, followed by 10 cycles of 94°C for 20 s and 61°C for 60 s, where the second temperature was decreased 0.6°C per cycle to achieve a final annealing temperature of 55°C at the end of the 10th cycle. The reaction proceeded for an additional 26 cycles of 94°C for 20 s and 55°C for 60 s, and a read step of 37°C for 60 s. To improve genotype cluster visibility, 3 additional cycles of 94°C for 20 s and 55°C for 60 s and a final read step at 37°C for 60 s were performed. In each run, DNA samples from whole blood of cows of known genotypes were analyzed as controls. Determination of the genotype was performed using the allelic discrimination feature of the CFX96 machine.

Determination of Progesterone Concentrations

Plasma concentrations of progesterone were determined in duplicate using a commercial solid-phase, no-extraction RIA (ImmuChem Coated Tube, MP Biomedicals, Costa Mesa, CA). In the 5 assays performed, quality control samples with concentrations of progesterone representative of the diestrous phase of the estrous cycle (3.7 ng/mL) were included twice (at the beginning and end) in each assay to determine variation within and across assays. The sensitivity of the assay was 0.1 ng/mL and the average intraassay and interassay coefficients of variation for the quality control sample were 6.1 and 6.6%, respectively.

Statistical Analysis

Data on pregnancy per AI were analyzed with the GLIMMIX procedure of SAS v 9.4 (SAS Institute Inc., Cary, NC) with the independent variable, pregnancy outcome (pregnant, nonpregnant), modeled as having a binomial distribution.

Data from yr 1 of the experiment were analyzed using 2 types of models. The first analysis included effects of hCG treatment, parity, month of insemination, and 2-way interactions between hCG treatment, month, and parity. Body condition score was included in the model as a covariate. The second model was used to analyze

| Table 1. Nucleotide sequence of the forward and reverse primers used in the PCR-based KASP assay (LGC Genomics, Middlesex, UK) genotype analysis |
|--------|--------|---------------------------------------------------------------|
| Gene   | Allele | Primer sequence                                             |
| COQ9   | A      | 5'-AAGGTCTTTTGATCAGCAGAAGA-3'                                |
|        | G      | 5'-AAGGTCTTTTGATCAGCAGGAGG-3'                                |
|        | Common reverse primer | 5'-AAAGAAGAGGCGGCGGTTGAT-3'                                |
| HSPA1L | Deletion | 5'-CAAGTCCTGCCCTGCGC-3'                                     |
|        | C      | 5'-CTCAAGTCCTGCCCTGCGC-3'                                    |
|        | Common reverse primer | 5'-GCATCCAGGGCGCTGATTGTT-3'                                 |
| PARM1  | G      | 5'-AAAGGGCGATGAGGCTGGC-3'                                    |
|        | C      | 5'-AAAGGGCGATGAGGCTGGC-3'                                    |
|        | Common reverse primer | 5'-TCCCAACACTCACCTCCCTCAA-3'                               |
| PGR    | G      | 5'-ACCTAATCTTGAAATAATGGTGATCTAAAG-3'                        |
|        | C      | 5'-ACCTAATCTTGAAATAATGGTGATCTAAAG-3'                        |
|        | Common reverse primer | 5'-CTTATTAAATGGTGTCAGCAGATCACCA-3'                        |
the effect of each SNP in 4 separate analyses. For each analysis, the model included hCG treatment, parity, month of insemination, genotype, 2-way interactions of hCG treatment with month, parity, and genotype, and with BCS as a covariate.

An additional analysis of pregnancy data from primiparous cows in yr 1 and 2 was performed. The statistical model included effects of hCG treatment, type of semen (sexed vs. conventional), month-year of insemination, BCS, and 2-way interactions between hCG treatment, month-year, and parity.

Effects on progesterone concentrations were first analyzed by GLIMMIX procedure of SAS to estimate the effect of parity and treatment on the proportion of cows considered to have a functional corpus luteum (progesterone concentration >1 ng/mL) at d 12 after TAI. For this analysis the independent variable, presence of a functional corpus luteum, was modeled as having a binomial distribution. The second set of analyses was performed by the GLM procedure of SAS and only included data from cows classified as having a functional corpus luteum. The model included hCG treatment, parity, month of insemination, genotype, 2-way interactions of hCG treatment with month, parity, and genotype, with BCS as a covariate.

For all analyses, a mean separation test using the DIFF option of Proc GLIMMIX was performed to compare differences between individual means for those effects with \( P < 0.100 \) and that involved multiple degrees of freedom. Differences identified by the DIFF option with a \( P = 0.05 \) or less are reported.

**RESULTS**

*Interaction Between hCG Treatment and Parity on Pregnancy Per AI*

In yr 1, there was no overall effect of hCG on pregnancy per AI at either d 32 or 60 after insemination. Treatment with hCG tended to increase pregnancy per AI in primiparous cows but not in multiparous cows (Figure 1A and B). The probability value for the treatment \( \times \) parity interaction was 0.098 for pregnancy per AI at d 32 and 0.063 for pregnancy per AI at d 60. Inclusion of genotype for *HSPA1L* in the model resulted in the parity \( \times \) treatment interaction being significant for pregnancy per AI at d 32 of gestation \( (P = 0.038) \) and close to significant at d 60 of gestation \( (P = 0.059) \). Similar results were found for inclusion of genotype for *COQ9* and *PGR* (results not shown).

Analysis of all data from primiparous cows in yr 1 and 2 (Figure 1C and D) also indicated that hCG treatment tended to increase pregnancy per AI in primiparous cows. The effect of hCG was \( P = 0.159 \) for pregnancy per AI at d 32 of gestation and \( P = 0.063 \) for pregnancy per AI at d 60 of gestation. There was no effect \( (P > 0.200) \) of semen type (conventional vs. sexed).

![Figure 1](image_url)

**Figure 1.** Effect of human chorionic gonadotropin (hCG) treatment on pregnancy per AI. Values are LSM ± SEM. Numbers of animals per group are indicated above each bar. (A and B) Effect of hCG treatment on pregnancy per AI of primiparous (A) and multiparous cows (B) in yr 1. A tendency was observed for a treatment \( \times \) parity interaction on d 32 (A; \( P = 0.098 \)) and d 60 (B; \( P = 0.063 \)) of gestation, with hCG increasing pregnancy per AI in primiparous cows but not in multiparous cows. (C and D) Effect of hCG treatment on pregnancy per AI of primiparous cows (yr 1 and 2). The probability value for effect of treatment was \( P = 0.159 \) at d 32 of gestation and \( P = 0.063 \) at d 60 of gestation.
or interaction with hCG treatment for pregnancy per AI at d 32 or 60.

**Interactions of hCG Treatment and Genotype on Pregnancy Per AI (Yr 1)**

The effect of the SNP in *COQ9* and its interaction with hCG treatment are presented in Figure 2. A hCG × genotype interaction was observed for pregnancy per AI at d 32 (P = 0.016) and 60 (P = 0.012) of gestation. When treated with vehicle, AA cows had a higher pregnancy per AI at d 32 and 60 of gestation than AG cows, with GG cows being intermediate. When treated with hCG, AA cows had lower pregnancy per AI at d 32 than AG cows. Stated differently, hCG decreased pregnancy per AI for AA cows, increased pregnancy per AI for AG cows, and had no effect on pregnancy per AI for GG cows.

Results for the effect of the SNP in *HSPA1L* and its interaction with hCG treatment on pregnancy per TAI and response to hCG is presented in Figure 3. Pregnancy per AI was affected by genotype for *HSPA1L* at d 32 (P = 0.009) and 60 (P = 0.001) after TAI. However, no interactions were observed between hCG treatment and *HSPA1L* genotype for pregnancy per AI at either day. For pregnancy per AI at d 32, mean separation tests indicated that CD differed from CC (P = 0.005) and DD (P = 0.041) but that CC and DD were similar (P > 0.10). For pregnancy per AI at d 60, mean separation tests indicated that CD differed from CC (P = 0.001) and DD (P = 0.052) and CC and DD were similar (P > 0.100).

The genotype for the SNP in *PARM1* had no effect (P > 0.100) on pregnancy per AI at d 32 or 60, nor were any interactions present between genotype and hCG (Figure 4).

Pregnancy per AI was affected by genotype for *PGR* at d 32 (P = 0.041) after TAI and tended to be affected (P = 0.080) at d 60 after TAI (Figure 5). No interaction was observed between the SNP and hCG treatment.

![Figure 2](image-url)  
*Figure 2.* Effect of *COQ9* genotype on the response to human chorionic gonadotropin (hCG) treatment on pregnancy per AI. Values are LSM ± SEM. Numbers of animals per group are indicated above each bar. A significant interaction was observed between hCG treatment and genotype for *COQ9* on d 32 (P = 0.016; A) and d 60 (P = 0.012; B) of gestation. Bars with different letters differ (P < 0.05). When treated with vehicle, AA cows had a higher pregnancy per AI at d 32 and 60 of gestation than AG cows, with GG cows being intermediate. When treated with hCG, AA cows had lower pregnancy per AI at d 32 than AG cows. Stated differently, hCG decreased pregnancy per AI for AA cows, increased pregnancy per AI for AG cows, and had no effect on pregnancy per AI for GG cows.
For pregnancy per AI at d 32, mean separation tests indicated that CC differed from CG (P = 0.034), but other means were not different from each other (P > 0.100). For pregnancy per AI at d 60, mean separation tests indicated that CC differed from CG (P = 0.025) but other means were not different from each other.

**Progesterone Concentrations**

Presence of a functional corpus luteum was defined as a progesterone concentration at d 12 after insemination >1 ng/mL in 90.6% of the primiparous cows and 93.2% of the multiparous cows. Presence of a functional corpus luteum was not affected by parity, hCG treatment, or genotype (P > 0.100).

Progesterone concentration at d 12 after insemination for animals with a functional corpus luteum was higher (P = 0.0001) for cows treated with hCG than for cows treated with vehicle (4.6 ± 0.09 vs. 3.2 ± 0.08 ng/mL). The difference in progesterone concentration was greater (interaction, P = 0.051) for primiparous cows (5.1 ± 0.13 vs. 3.4 ± 0.13 ng/mL) than for multiparous cows (4.3 ± 0.10 vs. 3.1 ± 0.10 ng/mL). Progesterone concentration was not affected by genotype (P > 0.100), and no interactions (P > 0.100) were observed between genotype and hCG treatment (Figure 6).

**DISCUSSION**

Here we confirm previous results that fertility response to hCG is greater for primiparous cows than multiparous cows (Nascimento et al., 2013). We also show, for the first time, an interaction between genotype and hCG affecting fertility. In particular, the association between a SNP in COQ9 previously shown to be related to fertility in cattle (Coirhan et al., 2013b; Ortega et al., 2017b) and pregnancy per TAI depended on whether cows were treated with hCG. Associations of SNP in HSPA1L and PGR with pregnancy per AI were also found.

The SNP in COQ9 is a missense mutation in which the A allele has been related to higher fertility in dairy cattle (Cochran et al., 2013b; Ortega et al., 2017b). In the present study, pregnancy per AI among animals...
treated with vehicle was highest for cows with the AA genotype. Differences among genotypes were altered for cows treated with hCG with AA cows being the least fertile. Indeed, hCG increased pregnancy per AI in AG cows but decreased pregnancy per AI in AA cows. Thus, results are indicative that one can use genotyping for \textit{COQ9} to identify animals that would respond positively to hCG as compared with those that will experience, on average, decreased fertility or will not respond to treatment.

The mechanism by which hCG decreased pregnancy per AI in cows with the AA genotype while increasing pregnancy per AI in AG cows is not known. \textit{COQ9}, along with other COQ proteins (COQ2–COQ8), is involved in the biosynthesis of COQ10 (Tran and Clarke, 2007; Ben-Meir et al., 2015), which is a component of the mitochondrial electron transport system that is required for mitochondrial adenosine triphosphate synthesis. The A allele, which has a frequency of 49 to 51\% in Holsteins (Ortega et al., 2016a), is associated with enhanced mitochondrial function (Ortega et al., 2017c). One possibility is that actions of hCG on reproductive tissues such as the endometrium (Shemesh et al., 2001) and corpus luteum (Niswender et al., 2000) have negative consequences when mitochondrial function is enhanced. In the endometrium, activation of the LH receptor induces expression of cyclooxygenase, which is associated with increased PGF₂α production that can trigger luteolysis (Shemesh et al., 2001). Because expression of endometrial LH receptor is low during the luteal phase (Shemesh et al., 2001), treatment with hCG 5 d after TAI may not be able to stimulate enough PGF₂α synthesis to trigger luteolysis. However, in cows carrying the AA allele, higher mitochondrial function could conceivably increase cytoplasmic ATP concentration and intensify cellular response to hCG in a way that leads to luteolytic release of PGF₂α.

The \textit{COQ9} genotype had no effect on progesterone concentration at d 12 after AI, indicating either no effect of genotype on luteolysis or that luteolysis induced by the interaction between treatment and the SNP for \textit{COQ9} occurs after d 12. Results regarding progesterone response to hCG are also indicative that the relationship between the SNP in \textit{COQ9} and response to hCG was not caused by a differential effect on ability of hCG to induce accessory corpus luteum because progesterone concentrations were similar between groups.

Figure 4. Effect of \textit{PARM1} genotype on the response to human chorionic gonadotropin (hCG) treatment on pregnancy per AI. Values are LSM ± SEM. Numbers of animals per group are indicated above each bar. No effect was observed of \textit{PARM1} on pregnancy per AI and no interaction between hCG treatment and genotype for \textit{PARM1} at d 32 (A) and d 60 (B) of gestation.
It is also possible that the SNP in COQ9 is acting through additional mechanisms [note that the SNP is related to follicle number on the ovary (Ortega et al., 2017c)] or that the SNP is in linkage disequilibrium with a causative mutation located elsewhere in COQ9 or in other nearby genes.

Although SNP in HSPA1L and PGR did not affect response to hCG treatment, pregnancy per AI was associated with allelic variation in both genes. For HSPA1L, cows that were heterozygous for the locus in HSPA1L had a higher pregnancy per AI than cows homozygous for either allele. For PGR, CC cows were the least fertile. These results were surprising. There was a lack of association between the mutation in HSPA1L and PGR for genetic or phenotypic measurements of fertility in Holsteins (Cochran et al., 2013a; Ortega et al., 2016b, 2017a) and the deletion mutation was associated with a reduced proportion of females pregnant during the breeding season for Brahman cows (Rosenkrans et al., 2010). Small sample size could explain some of these discrepancies. In addition, the present experiment was performed during the hot months of the year and it is possible that the increase in pregnancy rate for cows heterozygous for the deletion mutation in HSPA1L is related to embryonic resistance to the deleterious effect of heat stress on embryonic survival (Hansen, 2014). The deletion mutation in HSPA1L, which encodes for a member of the heat shock protein 70 family, results in increased expression of the protein in response to heat shock (Basiricò et al., 2011) and is associated with cellular resistance to elevated temperature (Basiricò et al., 2011), and increased competence of embryos to develop to the blastocyst stage in culture (Cochran et al., 2013a) and survive to heat shock (Ortega et al., 2016b). Heat stress may also be a factor in the inconsistency in results for PGR. The G variant in this gene has been related to maintenance of low body temperature in Holsteins cattle during heat stress (Dikmen et al., 2015).

Although the G allele in PARM1 was described to be associated with higher DPR, heifer conception rate, and cow conception rate (Cochran et al., 2013b), the mutation in PARM1 was not associated with pregnancy rate in the present study. Ortega et al. (2016b) also did not find an association between the SNP in PARM1

![Figure 5](image-url)
Figure 6. Effects of allelic variation for COQ9 (A), HSPA1L (B), PARM1 (C), and PGR (D) on progesterone concentration at d 12 after insemination for cows with a functional corpus luteum (progesterone concentrations >1 ng/mL). Values are LSM ± SEM. Numbers of animals per group are indicated above each bar. Genotype had no effect on progesterone concentration, and no interaction was present between human chorionic gonadotropin (hCG) treatment and genotype for any locus on progesterone concentration.
and fertility traits. Inconsistency between studies casts doubt on the importance of the SNP in \textit{PARM1} being an important predictor of fertility in Holsteins.

It was also found that hCG treatment was more effective for primiparous cows than multiparous cows. One possible reason for a higher fertility response to hCG in primiparous cows is that the increase in progesterone concentration at d 12 after insemination caused by hCG was greater for primiparous cows than multiparous cows. High progesterone concentration during the early pregnancy is associated with higher embryonic elongation and IFN-tau (IFNT) production (Clemente et al., 2009; Lonergan et al., 2013). In addition, one possible reason for the difference in responses to hCG may be that induction of a second ovulation on the side contralateral of the first ovulation reduces pregnancy per AI in multiparous but not primiparous cows (Baez et al., 2017). These authors proposed that the pregnancy response of multiparous cows to formation of a corpus luteum contralateral to the original corpus luteum results in early regression of both structures. Due to the large uterine size in multiparous cows, the physical diffusion of IFNT from the developing conceptus to the contralateral horn may be compromised as well as the maintenance of the accessory corpus luteum (Baez et al., 2017).

At least 2 explanations are possible for why hCG increased progesterone concentrations to a greater degree for primiparous cows than for multiparous cows. First, it is possible that primiparous cows are more likely to form an accessory corpus luteum than multiparous cows. Also, primiparous cows tend to produce less milk than multiparous cows (Nebel and McGilliard, 1993). Progesterone clearance is inversely related to milk yield (Sangsritavong et al., 2002) and differences in rate of metabolism due to parity could affect circulating concentrations of progesterone. Note that progesterone concentrations in blood may be underestimated because of prolonged storage before processing (Owens et al., 1980), but such a phenomenon is unlikely to compromise comparisons between groups.

In conclusion, treatment with hCG at d 5 after TAI can increase fertility in primiparous cows exposed to heat stress while not being effective for multiparous cows. In addition, fertility is affected by genotype for \textit{COQ9}, \textit{HSPA1L}, and \textit{PGR} and an interaction is present between hCG treatment and genotype at the \textit{COQ9} locus. This last result is the first evidence in cattle that consideration of genetic information could be informative regarding effectiveness of a fertility treatment. Under current genetic screening systems in cattle, cattle are genotyped at thousands of individual loci. It is likely that use of all this genetic information can improve the precision of predicting which animals are most likely to respond to therapeutic treatments. Targeting treatment to those animals most likely to respond could increase efficacy of therapeutics and avoid waste caused by treating animals that will not respond to the therapy.

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